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Effect of Anisotonic Media on Volume, Ion and Amino-Acid Content and Membrane Potential of Kidney Cells (MDCK) in Culture

G. Roy and R. Sauvé
Groupe de Recherche en Transport Membranaire, Départments de Physique et de Physiologie, Université de Montréal, Québec, Canada

Summary. Effects of anisotonic media on a monolayer of confluent kidney cells in culture (MDCK) were studied by measuring: cell thickness and cross-section changes, ion and amino-acid content and membrane potential. The volume was also determined with cells in suspension. When cells in a monolayer were incubated in hypotonic media, the lateral and the apical membranes were rapidly stretched. Afterwards the lateral membranes returned to their initial state while the apical membranes remained stretched. This partial regulatory volume decrease (RVD) was verified with cells in suspension. RVD was accompanied by a loss of K^+, Cl^- and amino acids, but there was no loss of inorganic phosphate. Also a transient hyperpolarization of the membrane potential was observed, suggesting an increase of the K^+ conductance during RVD. Upon restoring the isotonic medium, a regulatory volume increase (RVI) was observed accompanied by a rapid Na^+ and Cl^- increase and followed by a slow recovery of the initial K^+ and Na^+ content while amino acids remained at their reduced content. A transient depolarization of the membrane potential was measured during this RVI, suggesting that Na^+ and Cl^- conductance could have increased. In hypertonic media, only a small and slow RVI was observed accompanied by an increase in K^+ and Cl^- content but without any change of membrane potential. Quinine partly inhibited RVD in hypotonic media with cells in a monolayer while inhibiting RVD completely with cells in suspension. Incubation during four hours in a Ca^{2+} free medium had no effect on RVD. Furosemide and amiloride had no effect on RVD and RVI. Volume regulation, RVD or RVI, was not affected by replacing Cl^- by nitrate. When cells in a monolayer were incubated in a hypotonic K_2SO_4 medium, no RVD was observed. From these results, it seems that MDCK cells in a confluent monolayer regulate their volume by activating specific ion and amino-acid transport pathways. Selective K^+ and Na^+ conductances are activated during RVD and RVI, while the activated anion conductance has a low selectivity. The controlling mechanism might not be the free intracellular Ca^{2+} concentration.

Key Words volume regulation · membrane potential · membrane transport · kidney cells

Introduction

When cells are incubated into anisotonic media, they usually change their volume rapidly because of their large permeability to water. But this initial volume change is not always maintained; many types of cells can modify their internal solute content to return to a near normal volume. This volume regulation in the presence of anisotonic media has been studied in cell suspensions such as red blood cells (reviewed by Kregenow, 1981; Cala, 1983), lymphocytes (reviewed by Grinstein et al., 1984) and Ehrlich ascites tumor cells (reviewed by Hoffmann, 1985) and also in epithelial cells such as frog skin (Ussing, 1985), frog urinary bladder (Davis & Finn, 1985), *Necturus* gallbladder (Larson & Spring, 1984), *Necturus* intestine (Lau, Hudson & Schultz, 1984), mammalian kidney (Macknight, 1983; Strange & Spring, 1987) and *Amphiuma* kidney (Guggino, Oberleithner & Giebisch, 1985). The common denominator of these regulatory volume mechanisms is a loss of KCl in hypotonic media and a gain of NaCl in hypertonic media. But different membrane transport pathways seem to be involved depending on the cell types studied (reviewed by Eveloff and Warnock, 1987). The regulatory volume decrease (RVD) in hypotonic media activates either a neutral K-Cl cotransport or separate K^+ and Cl^- conductances. The regulatory volume increase (RVI) in hypertonic media activates either a neutral Na-K-2Cl cotransport or a neutral Na-H and Cl/HCO_3 exchange.

From these results, it would seem that volume regulation in anisotonic media is only related to a loss of KCl or a gain of NaCl. But it is possible that other cellular osmolytes are also involved. For example, it was shown that hypotonic volume regulation stimulated an important increase in amino-acid transport in Ehrlich ascites tumor cells (Hoffmann & Lambert, 1983). In that case it was found that amino acids, particularly taurine, contributed importantly to the cellular osmolality. In marine species, amino acids are the most important osmolytes and variations of their content play a fundamental

role in their response to anisotonic media (Gilles, 1979).

Kidney cells in culture (MDCK) were used recently by Simmons (1984) to study volume regulation. Epithelial cells in culture are now widely used to study transport mechanisms in epithelia (Taub, 1985) and the MDCK line in particular was found to have many of the transport systems of kidney tissues. They have a furosemide-sensitive Na-K-2Cl cotransport (Aiton et al., 1982), a Na^+/H^+ antiporter (Rindler & Saier, 1981) and a quinine-sensitive Ca^{2+} dependent K^+ conductance (Brown & Simmons, 1982). The volume regulation experiments performed by Simmons (1984) have shown that these cells in subconfluent cultures lost K^+ in hypotonic media through an increase of their K^+ permeability. However, this increase of the K^+ permeability was not inhibited by either furosemide or quinine. These results could mean that the permeability changes were produced by some other K^+-specific membrane pathway or by an unspecific ion leakage opened by the volume increase. These experiments did not demonstrate if the K^+ loss was conductive or neutral and they did not measure the possible losses of other osmolytes like amino acids. Also the time dependence of volume changes with cells in suspension was much more rapid than the loss of K^+ in a cell monolayer.

The purpose of our study was to perform volume regulation experiments with monolayers of MDCK cells in anisotonic media and measure the time dependence of the cross section and the height changes, the ion and amino-acid content and the membrane potential. It will be shown that a confluent monolayer of cells partly regulated their volume in hypotonic media mostly through a change of their lateral dimension accompanied by a loss of K^+, Cl^- and amino acids. There was no loss of inorganic phosphates. Membrane potential measurements established the conductive nature and the selectivity of the ionic permeability changes. The time dependence of volume, cell content and membrane potential were all closely correlated.

Materials and Methods

Cultures

The MDCK line was obtained from the American Type Culture Collection at 58 serial passages. Cells were seeded at medium density and grown until confluency in MEM (Gibco 410–1100) in plastic bottles (Falcon 3023) or petri dishes (Falcon 3002). Experiments were performed with confluent monolayers (3.5 to 4.0 × 10^5 cells/cm^2). The medium contained gentamycin (10 mg/liter), 10% fetal bovine serum (Gibco) and it was changed every two days. Cultures were maintained at 37°C in a humid air atmosphere and the Falcon bottles were maintained closed. Subculture was performed by detaching the cells from the bottles using a trypsin-EDTA solution containing 0.05% trypsin and 0.02% EDTA.

Solutions

MEM was buffered at pH 7.3 with HEPES (25 mM), NaOH (10 mM) and $NaHCO_3$ (6 mM). Earle's medium contained in mM: 121 NaCl, 5.4 KCl, 25 HEPES, 10 NaOH, 6 $NaHCO_3$, 5.6 glucose, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 1.0 NaH_2PO_4. Hypo- and hypertonic media were obtained by either increasing or decreasing the amount of water normally added to prepare isotonic MEM or Earle's media. For example, the quantities of solutes for one liter of isotonic MEM were dissolved in 1.6 liters to give a 125-mosmol solution or in 0.6 liter to give a 485-mosmol solution. The osmolality of each medium was measured with an osmometer (Advanced Instruments). The drugs used were quinine and DPC (diphenylamine-2-carboxylate or N-phenylanthranilic acid) from Aldrich Chemical Co. and furosemide, amiloride and A23187 from Sigma Chemical Co.

Ion and Amino-Acid Content

To measure K^+, Na^+, Cl^- and amino-acid content, 75 cm^2 bottles were washed three times with a cold Tris-SO_4 solution for 45 sec; this solution contained, in mM: 243 Tris, 111 H_2SO_4, 1.8 $CaSO_4$ and 0.8 $MgSO_4$ at pH 7.3 and 290 mosmol. This method is similar to the one used by Simmons (1981) to determine the Na^+ and K^+ concentrations of MDCK cells in monolayer. Our washing medium used Tris-SO_4 instead of choline-Cl and our washing time of 45 sec gave Na^+ and K^+ concentrations similar to the ones measured by Simmons (1981), indicating that our washing method is satisfactory. After washing, each bottle is incubated in 5.5 ml of distilled water for 1 hr. Tests were performed with 4-hr incubations and gave the same results as those of 1 hr. Afterwards 3 ml were used for measuring Cl^-, 2 ml for K^+, Na^+ and 0.5 ml for amino acids. Cl^- was measured with a chloridometer (Buchler) and K^+, Na^+ was measured with a flame photometer (Instrumentation Laboratory Inc.). Amino acids were measured with the nynhidrin reaction (Sigma N1135). In order to eliminate contamination by proteins, the samples were heated at 100°C and centrifuged (100,000 × g) to eliminate the proteins before the nynhidrin treatment. To measure the inorganic phosphate content, separate experiments were performed. The cells were incubated in 3 ml of distilled water during 1 hr; afterwards, 0.5 ml was taken and added to a molybdate reagent (Data Medical Ass. Texas, No. 1600–080) to provide a blue-colored molybdenum complex.

Membrane Potential

Glass microelectrodes were made from 1-mm capillaries (Clark) and filled with 3 M KCl; only those having at least 60 MΩ and less than 8 mV tip potential were used. The equipment for these measurements has been described previously (Roy & Sauvé, 1983). Only the potentials that were stable for 2 min or more were accepted and all the stable potentials larger than 10 mV were considered valid. Initial transient hyperpolarizations were frequently observed upon electrode penetration as in HeLa cells (Roy & Sauvé, 1983) and required a longer time to reach a stable

potential. When the potential was stable, isotonic MEM was flowed through the dish to verify the stability of the potential during a solution exchange. This exchange was always completed within 1 min.

Cell Height

Using the same equipment as for potential measurements, it was possible to measure cell height. When a microelectrode touched the cell surface, a small white dot appeared under phase-contrast microscopy and simultaneously a small potential change was observed. After noting the vertical position of the micromanipulator, the microelectrode was lowered through the cell until it reached the dish surface. This position is detected when the electrode tip appears to advance horizontally on the surface. Recording the vertical reading again and subtracting gave the cell height. Each measurement took 1 min. If the electrode position on the top of a cell was recorded after lowering and raising the electrode repetitively on the same cell, it was found to be reproducible within ± 0.5 μm. A similar variation was found for the bottom position. The height measurements were therefore reproducible within ± 1 μm. Also cell heights were variable from cell to cell. An average cell height was obtained from at least 15 values. From microscope photographs of a confluent monolayer, the average number of cells N per cm^2 is obtained. The value of $1/N$ gives the average cell surface plus the extracellular surface. With the average cell height, the average cell volume plus extracellular volume is obtained.

Volume Measurements

A Coulter counter was used to measure the volume of cells in suspension. The cells were detached with the trypsin solution described above and the suspension was shaken on a vortex to separate the cells. A visual control of cell separation was performed under the microscope. The cell size distribution and their average volume was measured with a pulse-height analyzer (Nucleus, Oak Ridge, Tenn.) connected to the output of the Coulter counter. The pulse-height analyzer was a circuit board connected into an Apple® computer which displayed the distribution. A program was provided by Nucleus to operate the circuit and determine the median of the distribution. This median was compared to the one obtained from a distribution of calibrated spheres and the average cell volume was calculated. Under the microscope cells in suspension appeared as spherical and their diameter could also be measured. An average volume was calculated and the same value as the one obtained with the Coulter counter was obtained.

Results

Cellular Volume

The effect of a hypotonic medium on cells in a monolayer can be observed by phase-contrast microscopy. In a dense confluent culture with an isotonic medium, cells appeared as shown in Fig. 1*a* with intercellular spaces more or less clearly visible. After perfusion with a hypotonic (125 mosmol) medium, the intercellular spaces rapidly and completely disappeared, as shown in Fig. 1*b*. After 5 min they reappeared (Fig. 1*c*) and 2 to 3 min later they were clearly apparent over the whole cell layer (Fig. 1*d*). The cell monolayer remained as shown in Fig. 1*d* as long as the hypotonic medium was maintained. It seems therefore that the intercellular spaces decreased rapidly when the monolayer was in contact with the hypotonic medium and afterwards they increased in about 5 to 6 mim to a stable size similar to those observed in the isotonic medium. When the isotonic medium was restored, there was no apparent increase of these spaces (Fig. 1*e*) and approximately 7 to 8 min later the initial appearance of the monolayer was recovered (Fig. 1*f*).

Since cells in a confluent monolayer have very little space for lateral expansion, it is expected to observe a height increase in a hypotonic medium. Using a microelectrode as described in Materials and Methods, it was possible to measure cell height in isotonic and hypotonic media. The measured values in an isotonic medium were variable from cell to cell, giving an average of 7.2 ± 1.8 μm in a typical confluent culture. The variations of cell heights were partly due to inaccuracies of the measurements but also to variations of cell shapes. During incubation in a hypotonic medium (125 mosmol), cell height measurements were taken every minute during a 20-min period. A typical time course is shown in Fig. 2. There was a rapid height increase and although there was much variation among individual values, there was no apparent decrease during 20 min. It is possible that there was a small recovery from the initial height increase during that period but it was certainly not complete. After restoring the isotonic medium, cell heights were measured for another 20-min period. After the first minute, the cell height returned to values within the range it had initially in the isotonic medium and remained in that range. These experiments were repeated ten times and a similar pattern was observed. The average stable height increase in a hypotonic medium can be obtained by calculating the average of the measurements during a single 20-min experiment. In 125 mosmol MEM, an average height ratio of 1.55 ± 0.11 was obtained. The height ratio could also be measured with a 180-mosmol MEM, giving a value of 1.26 ± 0.9. From these results, it appears that MDCK cells in a monolayer swelled rapidly when incubated in a hypotonic medium by expanding through the intercellular space and by increasing their height. Afterwards they recovered from their lateral expansion but their height remained larger. Cell volume was only partly regulated and this regulation seemed to have occurred

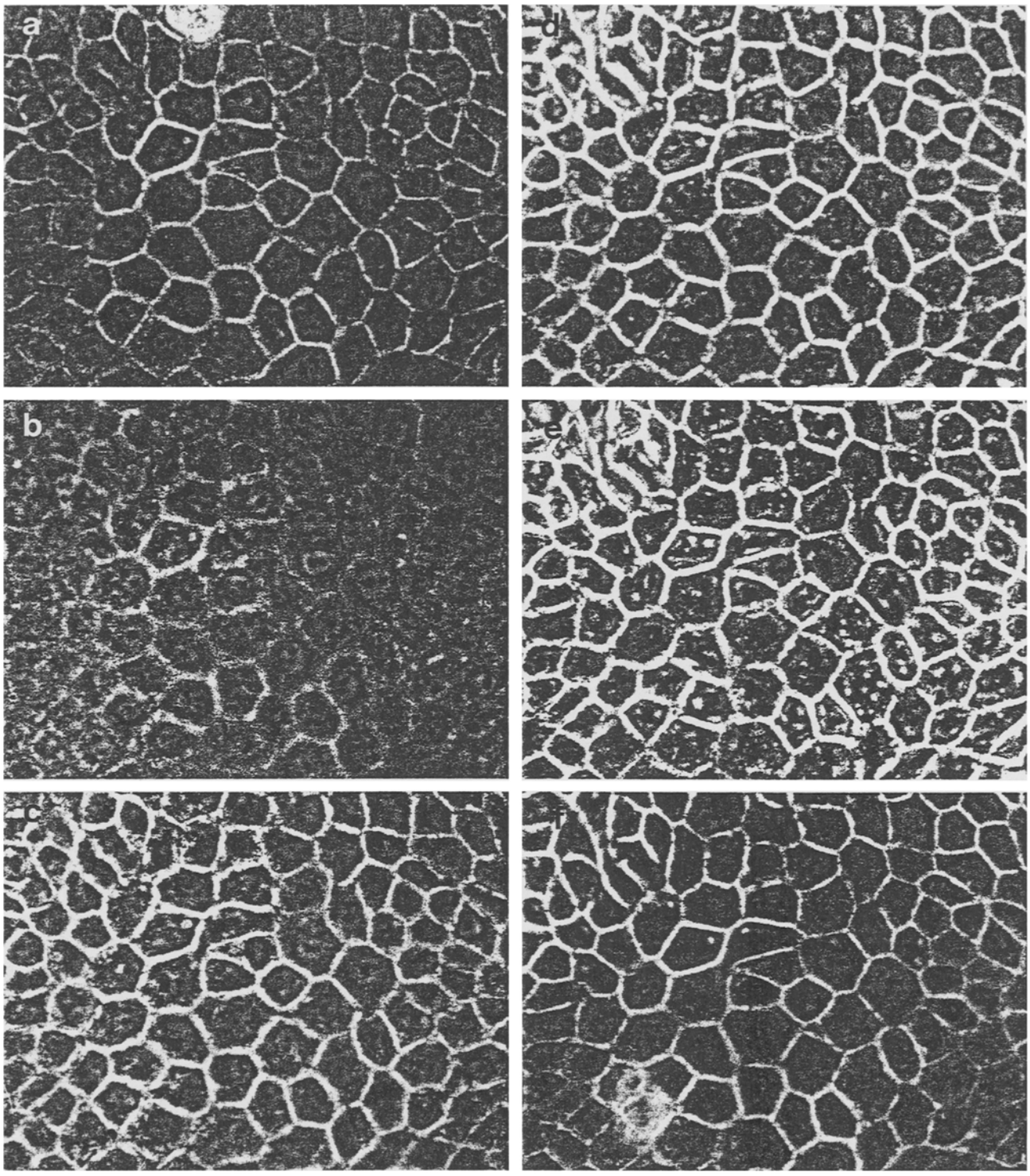

Fig. 1. MDCK cells in phase-contrast microscopy. (*a*) Isotonic MEM. (*b*) After 1 min in 125 mosmol MEM. (*c*) After 5 min. (*d*) After 7 min. (*e*) One min after restoring isotonic MEM. (*f*) after 7 min in MEM. Horizontal line is 20 μm

mostly in the lateral spaces. The time required to complete this volume regulation was at most 7 min. If it is assumed that the cell cross section has recovered to its original value, the stable volume ratio in hypotopic media would be given by the height ratio. In a confluent monolayer with an average cell density of $3.6 \pm 0.4 \times 10^5/\text{cm}^2$, an average cross section of 277 ± 35 μm^2 including cell and intercellular

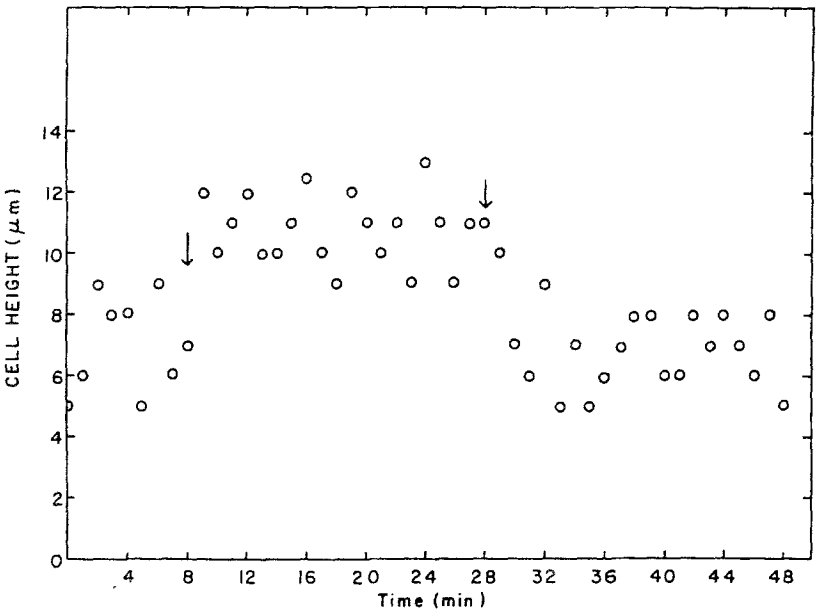

Fig. 2. Time course of cell height in a monolayer during incubation in a 125-mosmol MEM for 20 min followed by restoration of isotonic MEM. Results from a single typical experiment. The total number of height measurements before introducing the hypotonic medium was 20. The average heights before and after incubation in hypotonic MEM were 7.1 ± 1.6 and 6.9 ± 1.4 μm, respectively. The average height in hypotonic MEM was 10.8 ± 1.2 μm

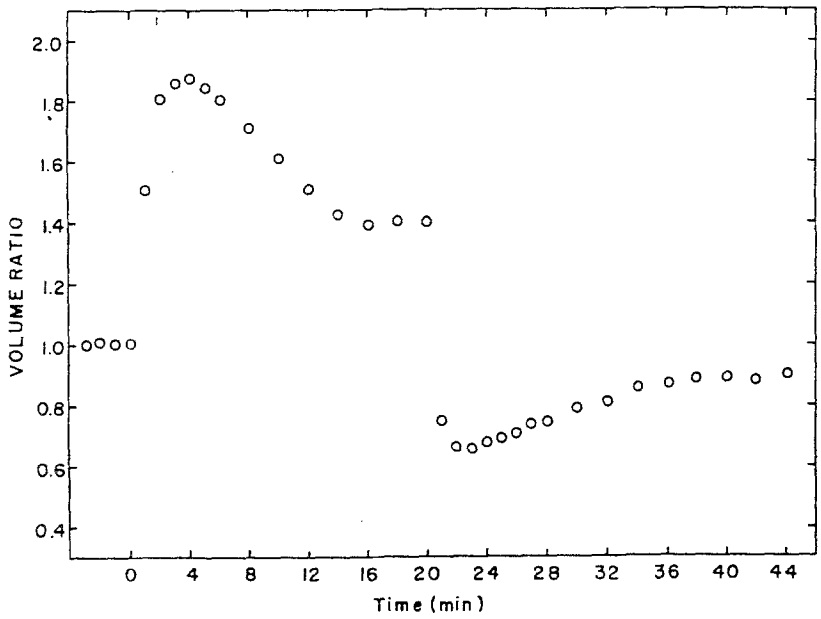

Fig. 3. Volume ratio of confluent MDCK cells in suspension during incubation in a 125-mosmol MEM for 20 min followed by centrifugation and restoration of isotonic MEM. Initial volume was 1.75 pl (picoliter). Results from a single typical experiment

space was obtained. With a cell height of 7.2 ± 1.8 μm, an average cell and intercellular volume of 2.0 ± 0.4 pl (picoliter) was obtained. Since a confluent monolayer has very small intercellular spaces (Richardson, Scalera & Simmons, 1981), this value should be close to the cell volume.

It was also possible to perform volume measurements using a Coulter counter and cells in suspension. That method was easier to use and more accurate, but it was necessary to detach the cells with trypsin. The average volume of confluent cells in normal MEM was found to be 1.8 ± 0.2 pl. This volume was a little smaller than the volume of cells in a monolayer, but the latter included the intercellular space. Therefore, trypsinization did not seem to affect the normal cell volume. This method has been verified by Simmons (1984) on MDCK cells and similar results were obtained. The time course of the volume ratio observed during incubation in a 125-mosmol solution is shown in Fig. 3. It increased rapidly to a maximum of 1.88 and slowly decreased to reach a stable level of 1.40 after 15 min. Incubation times lasting 60 min did not show a further decrease. Confluent cells in suspension demonstrated regulatory volume decrease (RVD) but did not recover completely. This was similar to the ob-

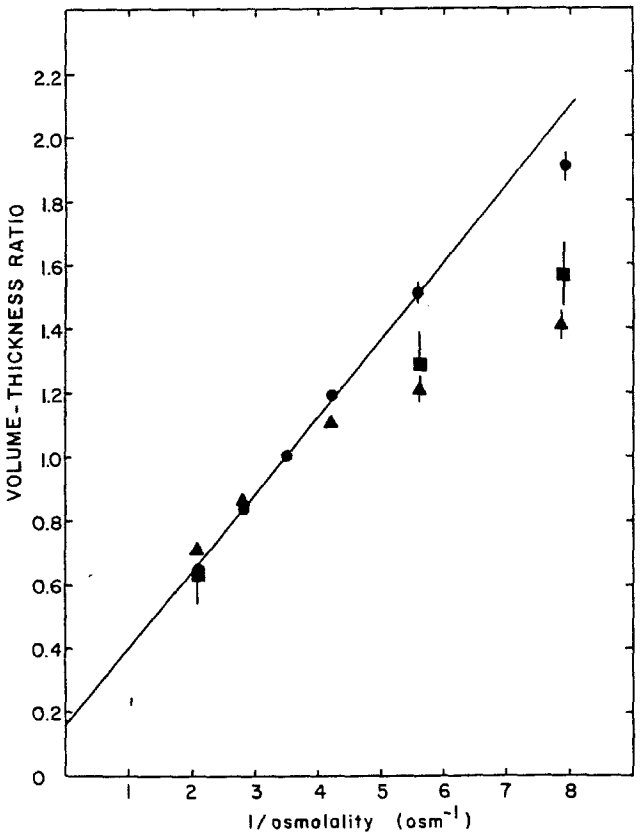

Fig. 4. Maximum (●) and stable (▲) volume ratio of MDCK cells in suspension and (■) cell-height ratio in a monolayer incubated in hypo- and hypertonic MEM plotted against the inverse of osmolality. Average initial volume was 1.8 ± 0.2 pl (picoliter) and average initial thickness was 7.2 ± 1.8 μm

servation of cells in a monolayer which partly regulated their volume, their height remaining larger than its normal value, giving a height ratio of 1.55 ± 0.11. Since the cell cross section in a monolayer appeared to return to its original value, the stable volumes in both preparations were similar. The time taken by cells in suspension to reach their stable volume was about 15 min. Cells in a monolayer required at most 7 min to return to their initial cross section and cell heights remained at their increased level. It would seem that cells in suspension regulated their volume more slowly than cells in a monolayer. That was probably due to the trypsin treatment. Replacing the cell suspension in the isotonic medium following a 20-min preincubation in a 125-mosmol medium gave a rapid decrease of the volume ratio followed by a slow volume regulatory increase (RVI) to about 90% of its normal value as shown in Fig. 3.

Volumes of cells in suspension were also measured with media of 180 and 235 mosmol. The initial (maximum) and the stable volume ratios were plotted against the inverse of osmolalities as shown in Fig. 4. The height ratios for cells in a monolayer are also shown in Fig. 4; their values are comparable to the stable volume ratios of cells in suspension. The results for cells in suspension are similar to those obtained by Simmons (1984) with subconfluent MDCK cells, except that they were obtained on confluent cells. In our cultures of MDCK cells, the same results were observed at different cell densities and different number of days in culture.

Similar experiments were performed with hypertonic media. Observing a cell monolayer with visible intercellular spaces and introducing a 475-mosmol medium, no change was observed; this was surprising since we expected a widening of the spaces. When the cell height was measured in the isotonic and the 475-mosmol media, it was found to decrease rapidly and remained stable at an average height ratio of 0.65 ± 0.08. Using cells in suspension and the same 475-mosmol medium, the volume ratio decreased rapidly to 0.63 ± 0.03 and then increased slightly to reach a stable value of 0.71 ± 0.03 after 1 hr, meaning that there was very little volume regulatory increase in hypertonic conditions. This stable volume ratio is comparable to the height ratio in a monolayer. It is possible that a small change of cell cross section has occurred in the monolayer but it was too small to be observed. The initial (minimum) and the stable volume ratios for cells in suspension were plotted for two hypertonic media in Fig. 4. The initial volume ratios for hyper- and hypotonic solutions follow a line corresponding to that of an ideal osmometer, except for the value at 125 mosmol (8 osmol^{-1}) which is a little lower than expected. The line crosses the vertical axis at a ratio of 0.16 which according to the van't Hoff equation corresponds to an osmotically inactive volume of 16%, a value similar to the one obtained by Simmons (1984), Kirk et al. (1984) and Hoffmann (1977).

Ion and Amino-Acid Content

The cell content of K^+, Na^+, Cl^- and amino acids was measured using isotonic and hypotonic media. The time course of these components during incubation in a 125-mosmol medium is shown in Fig. 5. It can be observed that they all decreased rapidly to reach a stable level, except Na^+ which remained constant. The time to reach stable values was approximately 6 min which would correspond to the time taken by the monolayer cell cross sections to reach a steady state and much shorter than the volume regulation shown in Fig. 3 for cells in suspension. In order to verify that the decrease of amino-acid content was related to a membrane transport into the external medium, a cell monolayer was in-

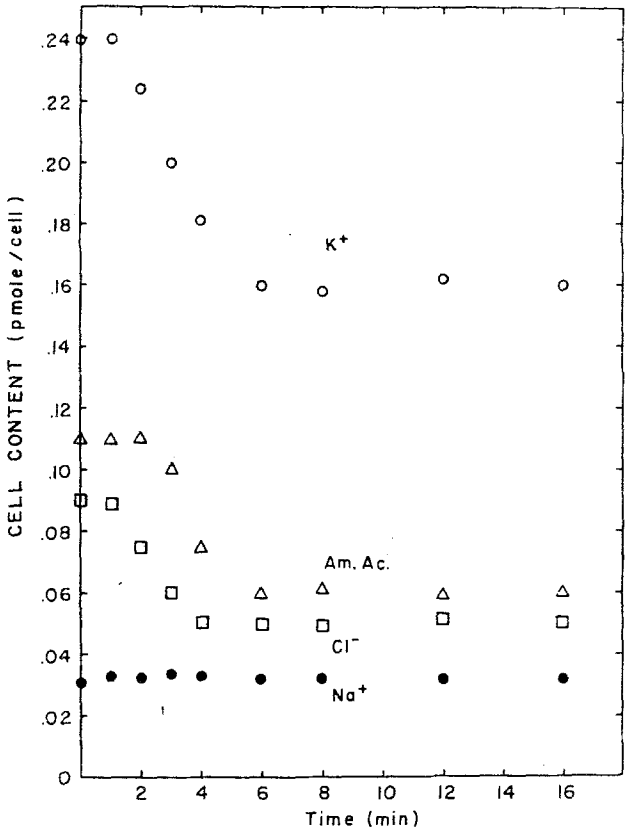

Fig. 5. Time course of MDCK cell content from a confluent monolayer during incubation in a 125-mosmol MEM. (○) K^+, (□) Cl^-, (●) Na^+, (△) amino acids

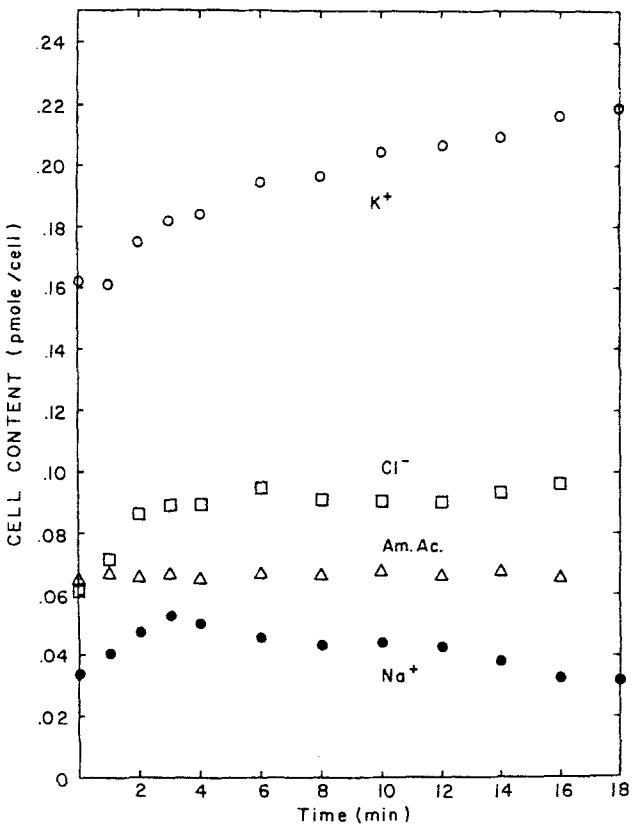

Fig. 6. Time course of MDCK cell content from a confluent monolayer during incubation in isotonic MEM after a preincubation in the 125-mosmol MEM for 15 min. (○) K^+, (□) Cl^-, (●) Na^+, (△) amino acids

cubated for 15 min in 125 mosmol Earle's medium and the amino-acid content of that medium was measured. It was found that 0.051 ± 0.005 pmol/cell of amino acids appeared in the 125-mosmol Earle's medium, a value corresponding to the reduction of the cell content as shown in Fig. 5.

Ion and amino-acid content measurements were performed with cells preincubated for 20 min in the 125-mosmol MEM and restored to their isotonic MEM. The time-dependent changes of K^+, Na^+, Cl^- and amino acids are shown in Fig. 6. The amount of Na^+ and Cl^- increased rapidly while K^+ increased more slowly. While Na^+ subsequently decreased, K^+ slowly increased such that after 30 min both reached the values they had in isotonic MEM. These results demonstrate that the entry of NaCl was occurring rapidly when the normal medium was restored and that Na^+ was replaced by K^+ afterwards. The time course of this NaCl increase corresponds to that of the RVI. The Cl^- content was found on the average to reach a value 20% larger than its initial value in isotonic MEM. This could mean that some of the anion losses in hypotonic media were replaced by Cl^- upon recovery. The amino-acid content did not return to its initial value in isotonic MEM even after 1 hr, thereby explaining the incomplete volume recovery (90%) observed with a suspension of cells. Volume recovery was apparently complete in a cell monolayer after restoring the isotonic medium, but these measurements were not accurate enough to detect 10% differences. In hypertonic media a small accumulation of K^+ and Cl^- was measured, but it was very slow taking about 1 hr to reach a steady state. No change of Na^+ and amino-acid content was measured. It is possible that Na^+ slowly entered the cells but was immediately exchanged for K^+. The Na^+ influx in hypertonic media was probably too slow to produce some accumulation. Since our MEM-HEPES-buffered media are low in bicarbonate and that MDCK cells are known to have a bicarbonate-dependent Na influx (Rindler, Taub & Saier, 1979), a hypertonic MEM (475 mosmol) with 25 mM bicarbonate was used to verify if RVI could depend on the presence of bicarbonate. The results with this MEM-bicarbonate have shown the same contents as those observed with hypertonic MEM-HEPES. An absence of hypertonic RVI was also observed by

Table 1. MDCK cell contents after volume regulation in anisotonic MEM-HEPES media

Osmolality (mosmol)	K^+	Cl^-	Amino acids (pmol/cell)	Na^-
125 ± 2	0.155 ± 0.005	0.045 ± 0.004	0.058 ± 0.006	0.027 ± 0.004
180 ± 2	0.185 ± 0.004	0.060 ± 0.005	0.079 ± 0.006	0.029 ± 0.003
235 ± 2	0.218 ± 0.005	0.072 ± 0.005	0.098 ± 0.005	0.028 ± 0.003
290 ± 2	0.240 ± 0.004	0.091 ± 0.004	0.101 ± 0.004	0.029 ± 0.004
355 ± 2	0.249 ± 0.006	0.099 ± 0.005	0.102 ± 0.005	0.030 ± 0.003
475 ± 2	0.271 ± 0.004	0.118 ± 0.004	0.101 ± 0.005	0.029 ± 0.004

Gagnon et al. (1982) on the rabbit proximal tubule using a 25-mM bicarbonate medium.

Stable values of ion and amino-acid contents were measured with the same hyper- and hypotonic media as in volume measurements and the results are given as a function of osmolalities in Table 1. For the 235-mosmol medium, the loss of K^+ was equal to that of Cl^- and there was no amino-acid loss. For the 180 and 125 mosmol media the loss of K^+ was about twice that of Cl^- and there was an amino-acid loss. This could indicate that part of the amino-acid loss is negatively charged. In hypertonic media, K^+ and Cl^- increased equally but Na^+ and amino acids remained constant. The increase of K^+ and Cl^- are rather small, confirming the limited regulatory volume increase (RVI) in those conditions.

Another important cellular osmolyte is the inorganic phosphate. Its content was measured in separate experiments using isotonic and hypotonic (125 mosmol) MEM. In isotonic MEM its value was 0.024 ± 0.002 pmol/cell and the same content was measured in hypotonic MEM meaning that there was no loss of inorganic phosphates during volume regulation. To further verify that there was no loss of inorganic phosphates during incubation in hypotonic MEM, a hypotonic Earle's medium without phosphates was used and its phosphate content was measured after a 15-min incubation in a cell monolayer. No inorganic phosphate could be detected. Since organic phosphates are much larger molecules, it can be presumed that they were not lost during volume regulation. These results indicate that the ionic losses were selective.

From the values in pmol/cell measured in isotonic MEM as shown in Table 1 and cell volume (1.8 pl), the normal concentrations were calculated assuming that the cell volume contained 84% water, 16% of the volume being osmotically inactive. The results are as follows in mM: 159 K^+, 60 Cl^-, 66 amino acids, 20 Na^+ and 16 for HPO_4^{2-} and $H_2PO_4^-$. As cell swelling or shrinking occurred in hypo or hypertonic media, those concentrations were changed rapidly. For example, in 125-mosmol MEM, the value of $[K^+]$ became 76 mM assuming that the volume of water rapidly increased by a factor of 2.1 as predicted by the line of Fig. 4 if the cells behave as osmometers. During volume regulation, the K^+ and water content decreased but the K^+ concentration did not change. Taking its stable content (0.16 pmol/cell) and the stable volume ratio (1.4), its concentration remained near 76 mM in the 125-mosmol MEM. Similarly, the Cl^- and amino acid concentrations changed very little after their rapid initial decrease in hypotonic media.

Membrane Potential

Since the changes of ionic content were probably related to changes of ionic permeabilities during volume regulation, it was important to measure the membrane potential in the same conditions to determine if selective conducting pathways were involved. During a stable potential recording, the hypotonic MEM (125 mosmol) was introduced; as shown in Fig. 7, the potential rapidly hyperpolarized and returned to its initial value in about 4 min. This time course correlated quite well with the time taken by K^+ and Cl^- to reach their stable values in the same conditions as shown in Fig. 5. The average normal potential was −35 mV (±7) and the hyperpolarized potential had an average value of −55 mV (±9). Upon restoring the isotonic medium, the potential depolarized and returned to its normal value in about 6 min. Again, this time course was closely correlated to the time taken by Na^+ and Cl^- to reach their maximum values as shown in Fig. 6. The depolarized potential had an average of −13 mV (±3). It is possible that the potential variations shown in Fig. 7 reflect changes of the ionic conductances involved in volume regulation. In contrast, when the hypertonic medium (475 mosmol) replaced the isotonic medium, there was no change of the membrane potential. To verify that the hyperpolarized potential change measured in the hypotonic medium was due to a volume change and not to a dilution of the medium, an isotonic Earle's medium containing 48 mM NaCl (the same as in the 125-

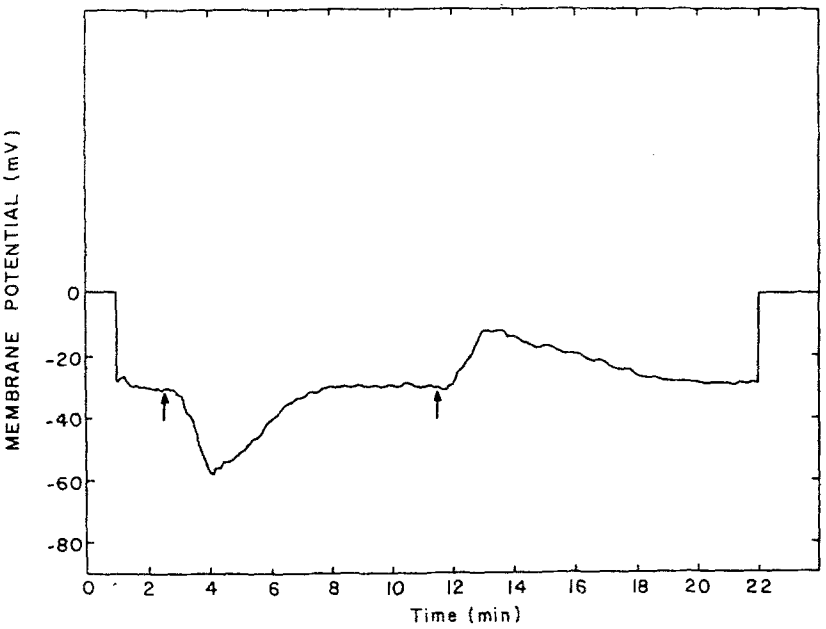

Fig. 7. Membrane potential recording in MDCK cells in a confluent monolayer during incubation in a 125-mosmol MEM (first arrow) and restoration to isotonic MEM (second arrow)

mosmol MEM) and 136 mM sorbitol was used instead. When this medium replaced the isotonic MEM, the membrane potential depolarized instead of hyperpolarizing. It seems therefore that the potential change measured in the hypotonic MEM was due to a volume change.

Effect of Inhibitors

Volume regulation studies in lymphocytes (Grinstein, Dupre & Rothstein, 1982b) and in gallbladder cells (Foskett & Spring, 1985) have shown that quinine, an inhibitor of the K^+ permeability, could block volume regulation. Therefore, MDCK cells in monolayer were incubated in an isotonic medium with 0.1 and 1 mM quinine for 10 minutes, such that quinine could reach the basolateral spaces and then in the 125-mosmol medium with quinine for 15 min. The sequence of events regarding intercellular spaces observed under the microscope was similar to those shown in Fig. 1. Cell heights were measured with the 125-mosmol medium with 1 mM quinine and the height ratio obtained was 1.61 ± 0.12, a value similar to the one obtained in the same medium without quinine. The volume ratio was measured with cells in suspension in the same media. With 0.1 mM quinine, there was no effect on volume regulation, but with 1 mM the volume ratio reached a stable amplitude of 2.0 ± 0.2 after 2 min and remained at this value during 20 min. In a cell suspension quinine seemed to block volume regulation completely and the value of the volume ratio was quite close to that predicted for an osmometer (Fig. 4).

Table 2. MDCK cell contents after volume regulation in anisotonic MEM-HEPES media with 1 mM quinine

Osmolality (mosmol)	K^+	Cl^- (pmol/cell)	Amino acids
125 ± 2	0.197 ± 0.007	0.068 ± 0.005	0.092 ± 0.004
180 ± 2	0.202 ± 0.006	0.070 ± 0.006	0.096 ± 0.005
235 ± 2	0.215 ± 0.005	0.076 ± 0.005	0.098 ± 0.004
290 ± 2	0.230 ± 0.005	0.089 ± 0.004	0.101 ± 0.004

To determine if quinine had some effects on RVD in a monolayer, ion and amino-acid content measurements with the 125-mosmol MEM containing 1 mM quinine were performed. The contents followed a time course similar to those shown in Fig. 5, but the stable values were higher. Those stable values are given in Table 2 for the isotonic and the three hypotonic media. When these results are compared with those shown in Table 1, it can be observed that quinine had no effect on the K^+, and Cl^- losses with a 235-mosmol medium. With the 180- and the 125-mosmol medium the losses of K^+ and Cl^- were reduced by about 50%. Longer incubation periods in a 10-mM quinine isotonic medium lasting up to 2 hr did not show additional inhibition of RVD. In an attempt to observe a more complete inhibition of the K^+ and Cl^- losses, a larger quinine concentration (3 mM) was tried, but it produced toxic effects and destroyed a large number of cells. Another important effect of quinine media (1 mM) was that the loss of amino acids was almost totally blocked. This result was rather surprising and could mean that quinine at such doses has unsuspected

effects on membrane transports other than the K^+ conductance.

Membrane potential measurements with a 1-mM quinine isotonic medium have given an average potential of -20 ± 3 mV, a value much lower than the normal potential. This effect was not observed with 0.1 mM quinine. When the 1 mM quinine medium at 125 mosmol was introduced, a hyperpolarization was still observed but it was smaller reaching a value of -30 ± 5 mV from a stable potential of -20 mV. This result shows that quinine had an effect on the potential of MDCK cells in a monolayer, but that quinine did not completely block the hyperpolarization related to volume regulation.

Other well-known inhibitors of the K^+ conductance are Ba^{2+} and TEA^+. To determine if a Ba^{2+} or a TEA^+-sensitive K^+ conductance was involved during volume regulation, Earle's media containing 15 mM Ba^{2+} or 15 mM TEA^+ were used. Cells in a monolayer were incubated during 10 min in these solutions and then the osmolality was reduced to 125 mosmol during 15 min. Afterwards ion and amino-acid contents were measured and it was found that there was no difference in the K^+, Cl^- and amino-acid losses with either of these media as compared to controls. An incubation period of 2 hr in the Ba^{2+} isotonic medium was also performed before reducing the osmolality to 125 mosmol. There was no inhibition in the ion and amino-acid losses. Therefore, it seems that the volume-activated K^+ conductance in MDCK cell monolayers could not be blocked by external Ba^{2+} or TEA^+ and only partly blocked by 1 mM quinine.

Other inhibitors such as furosemide were found to have an effect on volume regulation of red cells (Kregenow, 1981). Experiments with hypotonic media containing 0.1 or 1 mM furosemide were performed; no effect on volume regulation was observed although a furosemide-dependent K^+ flux has been measured by Aiton et al. (1982) on MDCK cells. Also amiloride has been found (Grinstein, Clarke & Rothstein, 1983) to inhibit the regulatory volume increase (RVI) of lymphocytes in an isotonic medium after treatment with a hypotonic medium. Since MDCK cells also exhibit such a RVI in similar conditions (Fig. 3), amiloride was tested as a possible inhibitor. No inhibition of volume increase was observed using 0.1 or 1 mM amiloride and also 1 mM furosemide did not have any effect. Also DPC, a Cl^- channel blocker in epithelia (DiStefano et al., 1985), was tested to determine if it could have an effect on volume regulation. Cells were incubated in isotonic MEM with either 0.1 or 1 mM DPC for 10 min and transferred to 125 mosmol MEM with the same DPC concentrations. There was no change in the pattern of volume regulation with cells in suspension and also no change in the K^+ and Cl^- losses for cells in a monolayer.

Effects of Ion Removal

In order to determine if a Ca^{2+}-dependent K^+ conductance was involved in K^+ extrusion during volume regulation (RVD), cells in a monolayer were incubated in Earle's medium without Ca^{2+} and containing 1 mM EGTA + 0.01 mM A23187 (a divalent cation ionophore) during periods of 1 to 4 hr. It was shown previously by Brown and Simmons (1982) that A23187 was effective in increasing the K^+ permeability in MDCK cells, presumably by increasing the cytoplasmic-free Ca^{2+} and that in absence of external Ca^{2+} the response was abolished. Therefore, incubation of the cells in a Ca^{2+}-free EGTA medium with A23187 should deplete them of their Ca^{2+}. Afterwards the cells were incubated in this medium diluted to 125 mosmol during 15 min and the cell content in ion and amino acids was measured. The results were the same as in control experiments using a normal Earle's medium with Ca^{2+}. These results would indicate that Ca^{2+} is not a mediator of volume regulation in MDCK cells, but experiments measuring intracellular Ca^{2+} during volume regulation would be required to determine if internal free Ca^{2+} is increased.

Since it was shown in Fig. 6 that there was a transient Na^+ increase during RVI after a hypotonic treatment, Na^+ in the external medium was replaced by $Tris^+$. A complete inhibition of volume increase in a cell suspension was observed. Also measuring the K^+, Cl^- and Na^+ content using the same $Tris^+$ medium in a cell monolayer during RVI, there was no increase such as those shown in Fig. 6. The measured cell contents remained the same as those in the hypotonic media. It seems that the Na^+ influx during RVI cannot be replaced by large cations like $Tris^+$.

Although no inhibitors could completely block RVD in a monolayer, it was nevertheless possible to obtain an RVD inhibition by using a modified Earle's medium in which NaCl was replaced by K_2SO_4 (98 mM, isotonic). Since this medium abolished the outward K^+ gradient, it is possible to prevent RVD in such conditions as shown with lymphocytes (Grinstein et al., 1982*a*). Incubating a cell monolayer in this medium for 30 min, no change of K^+ and amino-acid content was observed while Cl^- was reduced to 30% of its normal content. Cell height measurements have shown no difference compared to those obtained in MEM. When a suspension of cells was incubated in this medium for 30 min, there was no change of volume. After incubat-

ing cells in a monolayer in this medium diluted to 125 mosmol, there was a small increase instead of a decrease of cell K^+ content (0.02 pmol/cell), while the normal amino acid loss (0.04 pmol/cell) was measured. The cell-height ratio was measured in the same conditions and reached a value of 1.56 ± 0.15, a value quite similar to that obtained with the 125 mosmol MEM. Microscope observations made during the height measurements have shown that the cell cross sections did not decrease after their initial increase as observed with the 125-mosmol MEM (Fig. 1*d*), most of them remaining as shown in Fig. 1*b*. It seems that the cells remained expanded through the intercellular space with this hypotonic medium. Incubating a cell suspension in the same medium, no volume regulation was observed; the volume ratio reached a value of 2.0 ± 0.05 and remained at that value. This value is a little higher than the maximum ratio measured in a 125-mosmol MEM and closer to that predicted if cells swelled as osmometers (Fig. 4).

In order to determine if the loss of Cl^- was selective, a modified Earle's medium in which Cl^- was replaced by nitrate was used. After an incubation of 30 min in this medium it was found that the cells had lost 90% of their Cl^-. Afterwards the cells were incubated in the same medium diluted to 125 mosmol for 15 min. The amount of K^+ lost was the same as in a control experiment where a standard 125-mosmol Earle's medium was used. Measuring the volume with a cell suspension using the same nitrate medium, it was found that the isotonic volume was not changed and that volume regulation was not affected. Therefore, it seems that the permeability of MDCK cells for anions is high and poorly selective in isotonic conditions and volume regulation can occur similarly with different anions. These results are similar to those observed on lymphocytes by Grinstein et al. (1982*a*). To determine if the Cl^- influx was selective during RVI after a 15-min preincubation in a 125-mosmol Earle's medium, Cl^- was replaced by nitrate in isotonic Earle's medium and the K^+ and Na^+ contents were measured after 10 min in a cell monolayer. It was found that these contents had reached the same values as in a normal isotonic Earle's medium. It seems that the anion pathways used in both RVD and RVI are not very selective and probably involve conducting channels.

Discussion

The use of epithelial cell cultures in monolayer has provided a very useful approach to the study of volume regulation. The ion and amino-acid content measurements can be performed accurately and can be easily reproduced. Their time course during incubation in anisotonic media were measured and gave the time dependence of volume regulation. The most difficult problem with cell monolayers is volume measurement because the cells are thin and their shape irregular. Microscope observations permitted an evaluation of the time course of cross-section changes during RVD. Cell-height measurements were also possible with a simple method and gave values from which cell plus external volume could be calculated. Volume of cells in suspension can also be measured accurately and the values obtained are the same as those in monolayers, although trypsinization can produce some changes related to the time course of volume regulation. From the increases in cell height measured in hypotonic media, it seems that an important part of the volume changes in a monolayer occurred in that direction. Intercellular spaces could be observed under the microscope and it would seem that following incubation in a hypotonic medium they rapidly decreased because of a cell cross-section increase. Although the cross-section changes could not be measured quantitatively, it is possible to calculate them. For example, a maximum volume ratio of 2.0 was obtained with cells in suspension in 125 mosmol MEM and a height ratio of 1.55 was measured in the same medium for cells in a monolayer. From these values, the cross section would have increased by a factor of 1.29. Such a small cross-section increase would be expected because of the limited external space between cells in a confluent monolayer (Richardson et al., 1981). Since losses of ions and amino acids were measured in hypotonic media, it is expected that a volume decrease should follow them as observed with cells in suspension. The volume ratio in that case decreased to a value of 1.4. It was observed that the cross sections in a monolayer decreased following a time course similar to that of ion and amino-acid losses and apparently recovered their initial value, meaning that the cross-section ratio would decrease from 1.29 to about 1.0. Although the height ratio did not seem to decrease during that period, it is possible that a small decrease occurred and was not detected. If both cell diameter and height have decreased symmetrically by the same factor (f), the cross-section decrease would appear larger (f^2) than the height decrease (f). In other words because of a limited lateral space, cells in a monolayer would increase their volume asymmetrically in hypotonic media but the volume decrease during RVD could occur symmetrically. The lateral membranes would apparently return to their initial position while the apical membranes did not because expansion in that

direction was larger and RVD was not complete. It is possible that the losses of ions, amino acids and water during volume regulation occurred mostly through the lateral spaces. This possibility is supported by the fact that the basolateral membrane is much more permeable to K^+ than the apical membrane in MDCK cells (Aiton et al., 1982). Also Strange and Spring (1987) have shown that the basolateral membrane was much more permeable to water than the apical membrane in rabbit kidney cortical collecting tubule.

In hypertonic media cell shrinking did not seem to reduce the cross section while the height was reduced in the same proportions as the total volume. It should be realized that the basal membrane is attached to a rigid surface and the lateral membranes are attached to each other by tight junctions, leaving only the apical membrane free to move. This appears as the main reason to explaining why the volume changes were mostly reflected in height changes.

Volume regulation either in a monolayer or with cells in suspension is not complete in MDCK cells as also observed in Ehrlich ascites tumor cells (Hoffmann, Simonsen & Lambert, 1984). There was an incomplete volume regulation for the whole range of hypotonic media studied with MDCK cells. This could indicate that the controlling factor is not membrane stretching because in that case the final volume would be independent of osmolality. It is not clear why the cell content is not reduced further such that the isotonic volume is restored. It would seem that the controlling factor is eliminated too rapidly stopping the cell content losses before a complete restoration of isotonic volume.

The total content of amino acids and their loss in hypotonic media are similar to those observed by Hoffmann and Lambert (1983) in Ehrlich ascites tumor cells. These results suggest that amino acids are an important factor in cell osmolality and its regulation in mammalian cells as it is in marine species (Gilles, 1979). Some negatively charged components of the intracellular medium are also lost during volume regulation because the loss of K^+ is twice as large as the loss of Cl^- in the 180- and 125-mosmol media. Among the amino acids an important fraction could be negatively charged and it would contribute to this anion loss. Another anion that could be involved in RVD would be bicarbonate. Since it was not measured in these experiments, it is difficult to estimate its contribution to the anion loss. Because our external media are low in bicarbonate (6 mM), its cell concentration could also be in the same range. It is possible that other small organic anions are extruded, since it was shown that volume regulation was not modified by replacing Cl^- with nitrate. Grinstein et al. (1982*a*) and Hoffmann et al. (1984) have also shown that volume regulation was not influenced by similar small anion replacements. But in MDCK cells these anion losses do not include inorganic phosphates which were not lost during RVD.

The average membrane potential (-35 mV) measured in isotonic MEM was similar to the one measured by Stefani and Cereijido (1983). The membrane potential measurements in a hypotonic medium demonstrated a transient hyperpolarization following approximately the time course of the volume changes and of the ion and amino-acid losses. This hyperpolarization probably reflects an increase in a K^+ conductance activated during cell swelling. A transient increase of the K^+ permeability during volume regulation was measured on MDCK cells by Simmons (1984). A Cl^- conductance increase could also occur in parallel with the K^+ conductance increase. A simultaneous change in both conductances is compatible with a hyperpolarization. It cannot be excluded that the time course of the potential recovery is partly dependent on some transient accumulation of KCl in the small basolateral spaces. It was shown by Paulmichl, Gstraunthaler and Long (1985) with MDCK cells that the membrane potential decreased as external K^+ increased. But since the potential returned to its normal value following a time course similar to the K^+ and Cl^- losses and remained stable for as long as 15 min this possibility seems unlikely. Upon restoring the isotonic medium, the potential was depolarized rapidly (Fig. 7). Since there was a rapid increase of Na^+ and Cl^- content in those conditions (Fig. 6), it seems likely that there was an increase in a Na^+ and a Cl^- conductance. It would mean that the influx of Na^+ and Cl^- were occurring through independent channels and it was not a coupled transport as in lymphocytes (Grinstein et al., 1983), mouse kidney (Hebert, 1986) and rabbit kidney (Eveloff & Calamia, 1986). This is supported by the absence of inhibition of regulatory volume increase when the media contained either amiloride or furosemide. When Na^+ was replaced by $Tris^+$ in the external medium, there was no recovery of K^+ and Cl^- showing that there is a specific Na^+ conductance involved. Since there is no parallel increase of amino acids, it is not mediated by a coupled sodium-amino acid transport. It is possible that an unidentified Na^+ channel was activated by cell shrinking only after a hypotonic treatment, because this effect was not observed with hypertonic media.

The effect of quinine on volume regulation has shown a complete inhibition of RVD in cell suspensions while only a partial inhibition of the K^+ and Cl^- losses was observed in cell monolayers. It was

shown by Brown and Simmons (1982) that MDCK cells had a Ca^{2+}-dependent K^+ conductance activated by the divalent cation ionophore A23187 and requiring 1 mM quinine for its inhibition. Our membrane potential measurements in normal MEM with 1 mM quinine have shown a depolarization which could be explained by a decrease of the K^+ conductance by quinine. During incubation in a hypotonic medium, a hyperpolarization was still observed suggesting that quinine did not inhibit completely the K^+ conductance increase. Therefore, although quinine could inhibit a Ca^{2+}-activated K^+ conductance, it seems that it could not inhibit completely the volume-activated K^+ conductance in cell monolayers. It is possible that the trypsin treatment has increased the quinine sensitivity compared to cells in a monolayer. The effect of quinine on RVD inhibition in other types of cells as lymphocytes (Grinstein et al., 1982*b*) and gallbladder epithelia (Foskett & Spring, 1985) seems to occur at lower concentrations than in MDCK cells. It is possible that the K^+ conductance of cell monolayers activated by the hypotonic media is such that 1 mM quinine applied externally is not sufficient to block it completely. Quinine blocking of K^+ channels is not complete in rabbit kidney cells (Guggino et al., 1987) and also the K^+ channels are more sensitive to quinine when it is applied intracellularly.

The membrane potential of MDCK cells was shown previously to be depolarized in the presence of 1 mM Ba^{2+} (Paulmichl et al., 1985), presumably by blocking a K^+ conductance. The absence of effect on RVD by external Ba^{2+} could mean that this ion is not an efficient blocker of the K^+ conductance in RVD when applied externally. Again Ba^{2+} was found to be much more efficient intracellularly (Guggino et al., 1987) and only a partial inhibition of RVD by Ba^{2+} was observed on rabbit kidney tubules (Welling, Linshaw & Sullivan, 1985). We thought that longer incubations in a Ba^{2+} medium could have increased internal Ba^{2+}, but the results have shown no RVD inhibition. TEA^+ was tested because it is known to be more efficient externally (Guggino et al., 1987) and also because it was shown to be an effective inhibitor of the K^+ efflux (Brown & Simmons, 1982). Since TEA^+ did not inhibit RVD either, it would appear that the K^+ conductance activated by a hypotonic medium in MDCK cells is only sensitive to quinine at large concentrations. Although long incubation times did not increase blocking, it is still possible that some portions of the basal membrane in contact with the plastic bottle could not be reached by the inhibitors leaving many channels unblocked. Experiments made with cells grown on porous support would settle this point.

In conclusion, the results we obtained with confluent MDCK cells indicate that volume regulation involves the activation of a specific K^+ conductance during RVD and a specific Na^+ conductance during RVI with a rather unspecific anion conductance for both RVD and RVI. The transport pathways activated during RVD appear to have some similarity with those observed on other cell types while those activated during RVI seem to be different. An increased transport of amino acids was observed during RVD and there was no loss of inorganic phosphate. Although it was demonstrated (Grinstein et al., 1982*b*; Hoffman et al., 1984; Davis & Finn, 1985; Wong & Chase, 1986) that an increase of internal Ca^{2+} could be a trigger for ionic permeability increase during RVD, there is no convincing evidence for such a mechanism in MDCK cells since depletion of internal Ca^{2+} did not have a notable effect on volume regulation. Membrane transport pathways are controlled by extracellular as well as intracellular factors and the latter could be released when cell volume is increased to protect the cell. These controlling factors could be sequestered in organelles or in the plasma membrane which would release them upon swelling. MDCK cells appear to be a useful model epithelia to study these controlling mechanisms.

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