Simultaneous Recording of Cell Volume Changes and Intracellular pH or Ca\textsuperscript{2+} Concentration in Single Osteosarcoma Cells UMR-106-01*

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We present a new technique for the simultaneous measurement of cell volume changes and intracellular ionic activities in single cells. The technique uses measurement of changes in the concentration of intracellularly trapped fluorescent dyes to report relative cell volume. By using pH- or Ca\textsuperscript{2+}-sensitive dyes and recording at the ion-sensitive and -insensitive (isosbestic) wavelengths, the method can measure both cell volume changes and intracellular ionic activities. The technique was used to study the mechanisms of regulatory volume decrease (RVD) in the osteosarcoma cell line UMR-106-01 grown on cover slips. Swelling cells in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered hypotonic medium was followed by stable cytosolic acidification and a decrease in cell volume back toward normal. The recovery of cell volume could be blocked by depolarization, treatment with ouabain, or depletion of cell Cl\textsuperscript{-}. These suggest the conductive efflux of K\textsuperscript{+} and Cl\textsuperscript{-} during RVD. The cytosolic acidification that accompanied cell swelling was not blocked by amiloride, baflomycin A, or removal of Cl\textsuperscript{-} and could not be reproduced by depletion of cellular ATP. These findings exclude Na\textsuperscript{+}/H\textsuperscript{+} and Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange, intracellularly generated acid, or increased metabolism, respectively, as the cause of the acidification. The cell swelling-induced acidification was inhibited by depolarization, suggesting the involvement of an electrogenic pathway. The acidification, as well as RVD, was inhibited by short incubation with deoxyglucose, and these effects could not be reversed by valinomycin. Thus, the anionic pathway(s) participating in RVD and the acidification are sensitive to the cellular level of ATP. Together, these studies indicate that RVD in UMR-106-01 cells in HEPES-buffered medium is mediated by the conductive efflux of K\textsuperscript{+}, Cl\textsuperscript{-}, and OH\textsuperscript{-}.

Regulation of cell volume is a constant feature of cells in the face of water entry due to the presence of impermanent osmoles in the cytosol. In addition, regulation of cell volume can play an important role in the physiological function of cells. For example, in ion-absorbing or -secreting epithelia, cell volume regulation is necessary for uninterrupted vectorial transport of the ions (1). Osteoblasts stimulated with calcitropic hormones undergo a marked shape change (2), which may be accompanied by changes in cell volume. These changes are thought to be required for bone turnover (3).

Cells use a variety of mechanisms to regulate their volume after swelling or shrinkage (4). The most common mechanism for regulatory volume decrease (RVD) is the K\textsuperscript{+} and Cl\textsuperscript{-} efflux through a parallel conductive pathway (5). In many (6), although not in all (7) cells, RVD is regulated by the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i). [Ca\textsuperscript{2+}]i is believed to activate both the K\textsuperscript{+} (8,9) and the Cl\textsuperscript{-} (10) channels. In the case of the osteoblastic cells, UMR-106-01, we reported that RVD is affected by [Ca\textsuperscript{2+}]i (11) and that a [Ca\textsuperscript{2+}]i increase causes a reduction in intracellular K\textsuperscript{+} (12).

[Ca\textsuperscript{2+}]i, an important regulator of RVD, has been shown to oscillate when recorded from single cells (13); yet we know very little about volume regulation by single cells. Two methods have been reported for measurement of volume in single cells. The first involves measurements of changes in impermeant ion concentration with microelectrodes (14) or by optical means (15). The second is based on direct optical measurements of cell shape or cross-sectional area and relating these changes to changes in cell volume (16, 17). Previous methods to simultaneously record cell volume and intracellular ionic activities were successfully performed in several cell types (18-20) but required specialized optical arrangements that include two light sources and two separate data acquisition setups (18, 19) or the use of several ion-selective microelectrodes (20).

Here, we report a simple noninvasive technique for the simultaneous recording of cell volume changes and intracellular ionic activities in single cells, which allows the study of the relationship between the dynamic changes in these cellular activities. The technique is based on measurements of changes in the concentration of intracellularly trapped dyes such as BCECF and Fura 2 while also recording changes in pH, or [Ca\textsuperscript{2+}]i. Using the technique, we show that in HEPES-buffered medium, single UMR-106-01 cells regulate their volume after swelling (RVD) by the conductive efflux of K\textsuperscript{+} and Cl\textsuperscript{-}. Cell swelling is also followed by stable cytosolic acidification that appears to be due to the conductive efflux of OH\textsuperscript{-} together with a maintained inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange in hypotonic medium. The efflux of OH\textsuperscript{-} mediates part of the RVD.

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The abbreviations used are: RVD, regulatory volume decrease; RVI, regulatory volume increase; [Ca\textsuperscript{2+}]i, intracellular free Ca\textsuperscript{2+} concentration; BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid]; NMG, N-methyl-D-glucamine; DOG, deoxyglucose.
MATERIALS AND METHODS

Reagents and Solutions—Amiloride, ouabain, deoxyglucose, N-methyl-D-glucamine, nigericin, and valinomycin were from Sigma. The dimethyl ester form of 9,10-dimethyl-11-hexyl-9H-carbazoylfluorescein (BCECF) and Fura 2 were obtained from Molecular Probes, Inc. (Eugene, OR). Basilomycin A was a generous gift from Drs. D. Stone (University of Texas Southwestern Medical Center) and M. Sato (Merck Sharp and Dohme). The UMR-106-01 cells were cultured exactly as described before (11, 12), either in 75-cm² flasks for maintenance or in thin glass coverslips for single cell recording. The cells were used between 5 and 8 days in culture. Since RVD in UMR-106-01 cells is dependent on the subpassage at early subpassages (21), cells between subpassages 8 and 16 were used for the present studies.

The following solutions were used during the experiments. The standard solution A (SA) contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES (pH 7.4 with NaOH), 10 mM glucose, 1 mg/ml bovine serum albumin, and either 1 mM CaCl₂ or 0.2 mM EGTA (Ca⁡²⁺-free solution A). The Na⁺-free solution B (SB) was similar to solution A except that NaCl was replaced isosmotically with N-methyl-D-glucamine (NMG⁺) Cl⁻, and the pH was adjusted with Tris to pH 7.4. The osmolarity of all solutions was adjusted with the major salt to 310 mOsm.

Fluorescence Measurements—UMR-106-01 cells grown on thin glass coverslips were washed twice with serum-free tissue culture medium (Ham's F-12, Dulbecco's modified Eagle's medium) and incubated in the same solution containing either 1 µM BCECF/AM or 2.5 µM Fura 2/AM. For BCECF loading, the cells were incubated for 15-20 min at 37 °C, whereas for Fura 2 loading they were incubated for 45-60 min at 37 °C. Then the cells were washed once and kept in the same medium at room temperature until use.

For fluorescence recording, the coverslips were mounted in a perfusion chamber and continuously perfused. The perfusate volume in the chamber was adjusted to 0.3 ml, and the cells were perfused at 10-12 ml/min. The perfusate was delivered through an eight-way valve to a heat exchanger and then to the chamber and maintained at 37 °C. The recording system included a Nikon Diaphot with a Nikon Diaphot inverted microscope equipped with a high numerical aperture Neofluor ×100/1.3 numerical aperture (Zeiss) oil immersion objective. The microscope was attached to a Photon Technology International Delta Scan 1 spectrofluorometer, which provided the dual wavelength excitation light of 450 and 500 nm (BCECF) or 360 and 380 nm (Fura 2). The excitation light was selected by a spinning chopper mirror and directed to the cell by a dichroic mirror. The emitted light of 510 nm wavelength was monitored by a photomultiplier tube at a resolution of 3 kHz, and the signal was stored in a NEC Power Mate 1 computer for further analysis. The changes in cell volume were read from the fluorescence intensity recorded at the isosbestic wavelengths of 450 (BCECF) or 360 (Fura 2) nm after correction for cell volume-independent change in fluorescence intensity (see below). pH, was obtained from the uncorrected ratios of 500/450 after appropriate calibration (22). Briefly, the calibration procedure involved perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, 5 mM nigericin at pH between 6.3 and 7.8 (adjusted with NaOH). This procedure allows for calibration while the dye is in the cells. The fluorescence ratio values (500/450 nm) were linear, with pH, between 6.3 and 7.6.

Analysis of the Results—All experimental protocols presented were repeated at least three times with cells from different subpassages with results similar to those presented in the figures. Where appropriate, results are reported as mean ± S.E.

RESULTS

Measurement of Cell Volume Changes in Single Cells—The technique used to measure changes in cell volume is based on determination of changes in the concentration of intracellularly trapped fluorescent dyes. This is demonstrated in Fig. 1 for BCECF and Fura 2. Fig. 1a (top panel) shows a recording of BCECF fluorescence intensity at its isosbestic point. The decline in fluorescence represents a combination of photo-bleaching and dye leakage. Similar to findings in proximal tubule cells (15), this decline followed a single exponential course. The rate constant of these curves, however, varied between experiments, and therefore it was determined for every experiment from 3-5 min of fluorescence intensity recording at the isosbestic point prior to a change in medium osmolarity. Due to the exponential decline of the fluorescence intensity, it was advantageous to perfuse the cells continuously for 15-20 min prior to the osmolarity change and to use the last 3-5 min of recording for calculation of the rate constant and thus the quench curve for the correction of the data. On average, with Fura 2, the cell volume-independent change in fluorescence intensity was about 5 times slower than that recorded with BCECF (not shown).

Fig. 1b (top panel) shows the changes in BCECF fluorescence intensity upon step alterations in medium osmolarity. The solutions used were Ca²⁺-free and buffered with HEPES to minimize volume regulation by these cells (11). The fluorescence intensity gradually decreased as medium osmolarity was reduced stepwise from 310 to 155 mosM and then increased nearly to the original level when the osmolarity was returned to 310. Fig. 1, bottom panel, shows the relationship between the fluorescent signal and external osmolarity in cells loaded with BCECF or Fura 2. A linear relationship was observed, as expected for changes in dye concentration in cells exhibiting osmometric behavior.

Fig. 1 demonstrates that measurement of fluorescence intensity by recording at the isosbestic point can be used to follow the volume changes of single cells. To study RVD, it was necessary to swell the cells by exposure to hypotonic medium. Preliminary experiments revealed the following observations. When medium osmolarity was decreased from 310 to 190-150 mosM, some cells became permeable and lost all of their dye. There was no obvious way to predict which cell
Volume Changes in Single Cells

A major advantage in using BCECF or Fura 2 is that cell volume changes and pH, or [Ca\(^{2+}\)], can be simultaneously followed in the same cell. This is illustrated in Fig. 2, where cell volume and the accompanying pH changes were measured in cells perfused with HEPES-buffered, Ca\(^{2+}\)-containing solutions. Fig. 2a shows the fluorescence signal recorded from a BCECF-loaded cell that was alternately exposed to media of different osmolarities. This raw signal represents the changes in intracellular BCECF concentrations and dye leakage/photobleaching. The volume-independent changes in fluorescence intensity for the entire experiment was determined from a 3.9 min of fluorescence recording prior to the first osmolality change and assuming a single exponential course for the remaining part of the experiment. The resulting curve is illustrated in Fig. 2b (broken line), which is the calculated, cell volume-independent change in fluorescence intensity. The rate constant describing this curve is 0.0718 min\(^{-1}\). Although the rate constant for the cell volume-independent reduction in fluorescence varied between experiments, it remained nearly constant in a given experiment despite the changes in medium osmolality and cell volume. This can be seen in Fig. 2c for step reduction in medium osmolality. Similar findings were made when the osmolality was reduced from 310 to 190 mM while RVD was prevented (not shown). Thus, the cell volume-independent curve for an entire experiment can be calculated from the 3–5 min of recording prior to osmolality changes. Using the curve in Fig. 2b, the fluorescence recorded in Fig. 2a can be corrected to obtain the changes in cell volume as illustrated in Fig. 2c.

Fig. 2 shows that reducing medium osmolality from 310 to 190 mM resulted in cell swelling, which was followed by RVD. RVD was completed in 8 min of incubation at 37 °C (range, 6–10 min in 78 out of 97 cells studied). Cell swelling also induced a stable decrease in pH, from a resting level of 7.26 ± 0.02 to 6.94 ± 0.02 (n = 72). Increasing medium osmolality back to 310 caused cell shrinkage with subsequent regulatory volume increase (RVI) and an increase in pH, back to resting values.

Cell Volume and Na\(^{+}/H\(^{+}\) Exchange—In the present studies, we investigated the mechanisms responsible for RVD, the initial pH, decrease, and the relationship between pH, changes and RVD in single UMR-106-01 cells attached to substratum rather than in suspension. The mechanisms underlying RVI and the pH, increase in HEPES-buffered medium will be described in detail elsewhere.

Since reduction of extracellular Na\(^{+}\) from 140 to 80 mM was required for cell swelling, we first tested the possible contribution of the Na\(^{+}/H\(^{+}\) exchanger present in these cells (23) to the initial acidification. Fig. 3, a and b, shows that including amiloride in the hypotonic medium did not prevent the sustained acidification or normal RVD. Furthermore, reducing Na\(^{+}\) from 140 to 80 mM under isotonic conditions (NMG replacing Na\(^{+}\)) caused only small acidification, and subsequent exposure of the cells to hypotonic medium by removal of NMG resulted in the typical sustained acidification and RVD (Fig. 3, c and d). The pH, was reduced to 6.89 ± 0.03 (n = 6), similar to that measured under control conditions. Hence, the reduction of extracellular Na\(^{+}\) to 80 mM did not induce sufficient Na\(^{+}/H\(^{+}\) reverse) exchange to account for the acidification. Therefore, the large acidification is caused by cell swelling and not a simple reduction in extracellular Na\(^{+}\) concentration.

In this (23) and many other cell types (5), when the cytosol is acidified to pH of 6.9 in HEPES-buffered medium, the Na\(^{+}/H\(^{+}\) exchanger is activated and restores pH, to normal. Yet cell swelling caused acidification, which was maintained constant as long as the cells were exposed to hypotonic medium. A possible explanation for the observed sustained

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**Fig. 2. Simultaneous recording of cell volume and pH.** A BCECF-loaded cell was perfused with solution A containing 1 mM CaCl\(_2\) (310 mM). Where indicated, the osmolality was reduced to 190 mM and then increased back to 310 mM. The raw signal recorded at the isosbestic wavelength of 450 nm is shown in trace a. Trace b (broken line) is the calculated, volume-independent change in fluorescence intensity. Trace c is the corrected signal depicting the volume-dependent change in BCECF fluorescence. The signal recorded at the pH-sensitive excitation wavelength of 500 nm together with the signal in trace a was used to obtain the uncorrected ratio of the fluorescence, and the ratio was converted to pH, after calibration (trace d), as described under “Materials and Methods.”

**Fig. 3. Effect of amiloride and low external Na\(^{+}\) on pH, and cell volume.** For traces a and b, the cell was perfused with solution A and then solution A containing 0.5 mM amiloride. Where indicated, the osmolality was reduced to 190 mM by reducing the NaCl concentration in solution A while including 0.5 mM amiloride in the perfusate. Finally, the cell was perfused with solution A. For the experiment in traces c and d, the cell was perfused with solution A (310 mM) and then with solution A in which 60 mM NaCl was replaced with 60 mM NMG-Cl (310 mM) before reducing the osmolality to 190 mM by removing 60 mM NaCl from solution A. Traces a and c show the corrected fluorescence signals at 450 nm excitation wavelength, and traces b and d show the corresponding changes in pH.
Acidification is that the Na\(^+\)/H\(^+\) exchanger is inhibited by cell swelling and remains inhibited despite the following RVD. Na\(^+\)/H\(^+\) exchange is reactivated or stimulated during the subsequent cell shrinkage. That this is the case is demonstrated in Fig. 4. Fig. 4, a and b, shows that shrinking the cells by return to isotonic medium while maintaining external Na\(^+\) at 80 mM resulted in recovery of pH\(_r\) and RVI. Increasing Na\(^+\) to 140 mM had only a small further effect on pH\(_r\) and cell volume. Fig. 4, c and d, shows that these changes in pH\(_r\) can be blocked by amiloride, and the removal of amiloride was followed by an increase in pH\(_r\).

Role of Cl\(^-\) in RVD and pH\(_r\).—Two additional potential mechanisms mediating the acidification can be the efflux of acid stored in internal organelles (24) to the cytosol or the activation of Cl\(^-\)/HCO\(_3\)(OH\(^-\)) exchange (25) and H\(^+\) pumping by vacuolar-type H\(^+\) pumps (26). Fig. 5, a and b, shows that cell swelling was followed by the typical acidification in the absence of Cl\(^-\). On the other hand, RVD occurred only during the first 46 s, coinciding with the completion of the decrease in pH\(_r\), but then abruptly stopped. In the absence of Cl\(^-\), the cells recovered 26 ± 4.3% (\(\bar{x} = 6\)) of their volume, as compared with cells incubated in the presence of Cl\(^-\). Another finding shown in Fig. 5, a and b, is that cell shrinkage by return to isotonic Cl\(^-\)-free medium restored pH\(_r\) and cell volume back to normal without a significant overshot in cell volume.

**Fig. 4.** Inhibition of Na\(^+\)/H\(^+\) exchange by cell swelling. A cell perfused with solution A (310 mosm) was exposed to a solution of 190 mosm for approximately 10 min. Then the osmolality was increased to 310 while maintaining Na\(^+\) at 80 mM by replacing 80 mM NaCl with NMG-Cl in solution A. Finally, the cell was perfused with solution A (traces a and b). Traces c and d show the results of a similar experiment except that 0.5 mM amiloride was included in the solutions of 310 mosm used to shrink the cell after RVD.

**Fig. 5.** Effect of bafilomycin and Cl\(^-\) on pH\(_r\) and cell volume. Traces a and b, BCECF-loaded cells were washed once and then incubated in solution C (Cl\(^-\)-free)(SCl-CI) for 30 min before mounting in the perfusion chamber. After perfusion with solution C (310 mosm), where indicated, the osmolality was reduced to 190 mosm by reducing the Na\(^+\) gluconate concentration in solution C to 80 mM. Then the osmolality was increased back to 310 mosmol by perfusion with solution C. Traces c and d, BCECF-loaded cells were incubated in solution A (SA) containing 2.5 \(\mu\)M bafilomycin for 15 min and then, where indicated, they were exposed to a solution of 190 mosmol also containing 2.5 \(\mu\)M bafilomycin.

In the second protocol, cells were treated for 15 min with 2.5 \(\mu\)M of the intracellular H\(^+\) pumps blocker, bafilomycin (27). In separate experiments, we found that such treatment prevented the accumulation of acridine orange by the cells, and 2.5 \(\mu\)M bafilomycin caused the complete efflux of acridine orange from cells preequilibrated with the dye (not shown). The use of acridine orange accumulation as an index of intracellular acid spaces in intact cells was described before (28). Fig. 5, c and d, shows that dissipation of acid gradients in intracellular organelles and inhibition of vacuolar-type proton pumps by bafilomycin did not prevent the acidification due to cell swelling or RVD.

Ion Gradients and Membrane Potential in RVD and pH\(_r\).—Fig. 6, a and b, shows the effect of treatment for 2 h with ouabain on RVD and the swelling-dependent cytosolic acidification. Treatment with ouabain dissipates the K\(^+\) and Na\(^+\) gradients and depolarizes the cells without depleting the cells from ATP. As expected, exposing ouabain-treated cells to a hypertonic solution resulted in cell swelling and almost complete inhibition of RVD. Return to isotonic medium resulted in cell shrinkage back to near the control level. The treatment with ouabain almost completely prevented the swelling-induced cytosolic acidification. The remaining acidification seen (0.16 ± 0.03 pH units, \(n = 3\)) is probably mediated by reversal of the Na\(^+\)/H\(^+\) exchanger, since the ouabain treatment is expected to increase intracellular Na\(^+\) and, during the swelling, external Na\(^+\) is reduced from 140 to 80 mM.

To test directly the role of membrane potential in RVD and the acidification, the effect of elevating external K\(^+\) on these activities was measured. Fig. 6, c and d, shows that increasing external K\(^+\) from 5 to 40 mM during cell swelling prevented RVD and the swelling-induced cytosolic acidification in a manner similar to that observed in ouabain-treated cells. Thus, depolarizing the cells at the time of cell swelling prevented the acidification and RVD. Under these conditions, the partial initial RVD observed under Cl\(^-\)-free conditions (Fig. 5, a and b) was also blocked.

Regulation by ATP.—While studying the effect of membrane potential on RVD and the acidification we noticed that these activities are sensitive to the level of ATP in the cells. The concentration of ATP was reduced by incubating the cells with 10 mM deoxyglucose (DOG) for 10 min at 37 °C. Fig. 7, a and b, shows that such incubation had no effect on cell volume, whereas pH\(_r\) was transiently reduced by about 0.11 ± 0.03 (\(n = 8\)) pH units. When these cells were exposed to hypotonic medium containing 10 mM DOG, they swelled but did not recover their volume. After DOG treatment, the swelling-induced acidification was completely inhibited.

**Fig. 6.** Effect of Na\(^+\) and K\(^+\) gradients on pH\(_r\) and cell volume. Traces a and b, cells were incubated for 2 h in solution A containing 1 mM ouabain. After mounting in the perfusion chamber, the cells were perfused with solutions of the indicated osmolalities that contained 0.5 mM ouabain. For the experiment in traces c and d, cells in solution A (310 mosm) were exposed to a solution of 190 mosmol in which 35 mM KCl replaced 35 mM NaCl (final KCl concentration was 40 mM) and then solution A in which 35 mM KCl replaced 35 mM NaCl.
Fig. 7. The role of ATP in pH and volume responses after cell swelling. For the experiments in traces a and b, the cell was incubated for 10 min in solution A in which 10 mM DOG replaced the glucose before exposure to the hypotonic and then isotonic solutions containing DOG. For the experiment in trace c, the cell was incubated in solution A (SA/310 mosM) containing 10 mM DOG for 10 min. Where indicated, the cell was exposed to 20 mM NH4Cl, when osmolarity was maintained at 310 mosM by reducing NaCl concentration. NH4Cl was removed by perfusion with the glucose-free solution A containing 10 mM DOG.

Rather, pH, increased by about 0.05 ± 0.02 (n = 8) pH units. Shrinking these cells with isotonic medium was followed by return to normal volume with small reduction in pH. Hence, treatment with DOG blocked pH changes and was even more effective than Cl−-free conditions in inhibiting cell volume regulation.

ATP level in the cells can affect volume and pH, regulation directly through regulation of a key, common pathway or indirectly by dissipation of the Na+ and K+ gradients due to inhibition of the Na+ pump. We have performed two types of experiments to distinguish between these possibilities. In the first protocol, we found that incubating the cells for 10 min with ouabain did not prevent the initial swelling-mediated acidification (not shown). Ouabain is expected to have the same effect as DOG on Na+ and K+ gradients but without reducing cellular ATP levels. In the second protocol, we tested the effect of DOG treatment on Na+/H+ exchange activity. An increase in intracellular Na+ is expected to inhibit the Na+/H+ exchange due to reduction in the Na+ gradient and interaction of Na+ with the internal regulatory site (23, 29, 30). Fig. 7c shows that DOG treatment did not prevent normal alkalization in cells treated with NH4Cl to impose cytosolic acidification. The recovery of pH, after NH4Cl treatment is dependent on external Na+ and blocked by amiloride (not shown), indicating normal activity of Na+/H+ exchange. Hence, the inhibition of RVD and the acidification by DOG treatment was not due to inhibition of the Na+ pump and dissipation of the Na+ and K+ gradients.

Since the swelling-dependent acidification and RVD can be blocked by membrane depolarization, partial depletion of ATP could prevent RVD and the acidification either by interfering with K+ or Cl− efflux. To obtain some evidence as to the pathway affected by DOG treatment, we tested whether the effect of DOG can be reversed by the K+-conductive ionophore valinomycin. Fig. 8, a and b, shows that including 2 μM valinomycin in the hypotonic medium did not reverse the inhibitory effect of DOG, suggesting that the effect of DOG was not mediated by inhibition of K+ channels under the conditions of our experiments.

DISCUSSION

In the present studies we introduced a simple technique for the simultaneous recording of changes in cell volume and pH, or [Ca2+] in single cells. We reasoned that a high numerical aperture objective should allow measurements of dye fluorescence intensity from a restricted plane of the cells and thus be a function of the dye concentration in this area. Since dye concentration is inversely proportional to cell volume, measurement of changes in fluorescence intensity at the isosbestic point should report the dynamic changes in cell volume. That this was indeed the case is shown in the curves of Fig. 1, which display a linear relationship between medium osmolarity and fluorescence intensity. The relationship is linear because dye concentration is inversely proportional to cell volume, and cell volume is inversely proportional to external osmolarity for cells that exhibit osmometric behavior. This finding also indicates that most of the BCECF and Fura 2 were in osmotically-sensitive space(s) in UMR-106-01 cells.

A major advantage in using trapped ion-sensitive fluorescence dyes is that cell volume and intracellular ionic activities can be simultaneously followed in the same cell. Recording at the ion-sensitive wavelength reports changes in cell volume and intracellular ionic activities, whereas recording at the isosbestic point reports only cell volume. The ratio of the fluorescence recorded at both wavelengths and appropriate calibration then yields the changes in intracellular ionic activities independent of changes in cell volume.

The measurement of changes in impermeable ion concentration as a measure of changes in single cell volume is similar to that described before (14, 15). However, our technique avoids the necessity of using image acquisition and analysis or reversibly permeabilizing the cells to small ions and impelling the cells with microelectrodes. In addition, the tetraethylammonium technique does not report changes in other ionic activities such as pHi, or [Ca2+], unless additional microelectrodes are used. Optical techniques based on measurement of changes in cell shape have also been successfully used before to measure cell volume (16-19). These techniques are based on tracing the cross-sectional area of an optically thin section(s) through the cells with an image analysis system. Cell volume is then determined by three-dimensional reconstruction of cell shape (18) or by using appropriate geometric assumptions (17-19). The need to manually (16, 18, 19) or semiautomatically (17) analyze the cell shape is time-consuming and requires expensive equipment. This may compromise the time resolution possible; thus, rapid changes in cell volume may be lost.

We used the simple technique introduced here in single UMR-106-01 cells to characterize the mechanisms governing
RVD and the swelling-dependent acidification in HEPES medium. Our results suggest that RVD is largely mediated by the conductive efflux of K+ and Cl⁻ with the obligatory efflux of H₂O. The acidification appears to involve conductive efflux of OH⁻ which contributes to but does not dominate RVD under these experimental conditions. Below, we present the arguments in support of these conclusions.

The mechanisms of RVD have been studied in several cell types (1, 4-6). In most cells, swelling results in activation of K⁺ and Cl⁻ channels to allow the efflux of KCl and H₂O (4, 5, 10). In red blood cells, KCl efflux appears to be mediated by a coupled KCl cotransporter (31). Activation of K⁺ and HCO₃⁻ channels with the consequent efflux of K⁺/HCO₃⁻ and H₂O has been reported in cells of the inner medullary connecting duct (32). In these cells, RVD is independent of the Cl⁻ content of the cells (32). Single UMR-106-01 cells undergo RVD when swollen in hypotonic medium, which is similar to findings in cell suspension reported before (21). The rate of RVD measured in single cells at 37 °C is similar to that measured with cells in suspension at room temperature. These findings are compatible with RVD being mediated by the conductive efflux of K⁺ and Cl⁻, since ion transport through the channel is relatively temperature-independent.

The activation of K⁺ and Cl⁻ channels by cells swelling in UMR-106-01 cells is concluded from the findings that RVD can be blocked by membrane depolarization brought about either by increasing external K⁺ or by treatment with ouabain (Fig. 6). In the following paper, we show that in HCO₃⁻ medium, RVD is also blocked by the K⁺ channel blockers quinidine and Ba²⁺ (33). Similar findings were made in HEPES-buffered medium. The blockers did not prevent the [Ca²⁺], increase due to cell swelling (11) as measured with Fura 2 (not shown), and therefore, the inhibition of RVD was likely due to the inhibition of K⁺ channels. In suspension of UMR-106-01 cells, the K⁺ ionophore valinomycin accelerates RVD when added to cells from early subpassages (21). In addition, depletion of intracellular and extracellular Cl⁻ largely prevented RVD occurring between 2 and 10 min after cell swelling (Fig. 5). Taken together, these findings are compatible with the conductive efflux of K⁺ and Cl⁻ during RVD.

Interestingly, some RVD occurred even when the cells were incubated for 2 h in Cl⁻-free medium. It is likely that this partial RVD is mediated by K⁺ and OH⁻ efflux and is related to the acidification, since the only conditions identified as effective in blocking the partial RVD are also effective in blocking the acidification observed on cell swelling. Hence, it appears that in HEPES medium, RVD in UMR-106-01 cells is mediated by K⁺, Cl⁻, and OH⁻ efflux. We showed previously that swelling of UMR-106-01 in suspension or attached to coverslips also increases [Ca²⁺] (11, 21) and that the presence of external Ca²⁺ is required for RVD (11). Therefore, it is likely that one or more of the conductive pathways participating in RVD are activated by a [Ca²⁺] increase.

In the present studies, we also provided evidence that the cytosolic acidification due to cell swelling is not mediated by a Cl⁻/HCO₃⁻ exchange or intracellularly generated acid. Hence, inhibition of Cl⁻/HCO₃⁻ exchange by depletion of Cl⁻ (12, 34, 35) did not prevent the acidification. Dissipation of acidic gradients in intracellular organelles by incubation with bafilomycin A (24, 27) was equally ineffective in preventing the acidification or RVD. Hydrolysis of intracellular ATP initiated by incubation with DOG did not reproduce the acidification observed upon cell swelling, suggesting that an increase in metabolism cannot account for the acidification.

In the following paper, we show that in the presence of HCO₃⁻, swelling of UMR-106-01 cells reduces pH to a level much lower than that observed in HEPES medium (33). HCO₃⁻ increases the buffering power of the cytosol (36, 37) and thus should have attenuated any acidification due to increased metabolism. These findings indicate that the acidification due to cell swelling is not mediated by Cl⁻/HCO₃⁻ exchange, acid efflux from internal organelles to the cytosol, or increased metabolism.

Another mechanism not participating in the acidification is the Na⁺/H⁺ exchanger. Not only did reducing Na⁺ from 140 to 80 mM under isotonic conditions fail to reproduce the cell swelling-triggered acidification, but including amiloride in the hypotonic medium did not prevent the acidification. Furthermore, our studies show that the Na⁺/H⁺ exchange is inhibited by cell swelling, which accounts for the sustained acidification under these conditions. The Na⁺/H⁺ exchanger remains inhibited despite the recovery of normal cell volume within 10 min of incubation in hypotonic medium. Indeed, shrinkage of cells exposed to hypotonic medium for 10 min, without changing external Na⁺, resulted in activation of the exchanger and recovery of the pH (Fig. 4). These findings are in agreement with previous observations (30) showing a reduction in V_max of the exchanger by cell swelling. Since cell shrinkage under hypertonic conditions activates the Na⁺/H⁺ exchanger in this (30) and other cell types (5, 38), it is not clear at present whether isotonic shrinkage (after hypotonic RVD) only relieves the inhibition observed in hypotonic medium or actually further activates the exchanger. It is worthy to note that isotonic shrinkage at 140 mM external Na⁺ resulted in an overshoot of pH, (for example Figs. 2 and 3), suggesting possible activation of the exchanger, beyond relief of the inhibition, to occur during isotonic shrinkage.

The only condition found to inhibit the cell swelling-dependent cytosolic acidification is depolarization prior or during cell swelling. These findings strongly suggest that the acidification was mediated by the conductive influx of H⁺ or efflux of OH⁻ in the HCO₃⁻-free medium. It is, however, likely that OH⁻ efflux rather than H⁺ influx was responsible for the acidification. Although either of these electrogenic pathways will facilitate the efflux of K⁺ required for RVD, the augmentation of the acidification by HCO₃⁻ (33) supports the movement of OH⁻ under HEPES conditions. In the case of H⁺ influx, HCO₃⁻ should have attenuated the acidification due to the increased cytosolic buffer capacity. It is not surprising that the cell swelling-dependent acidification was largely mediated by OH⁻ efflux since activation of Na⁺/H⁺ or Cl⁻/HCO₃⁻ exchange is expected to inhibit RVD, whereas conductive efflux of OH⁻ will contribute to the net efflux of K⁺, which will otherwise be limited due to the limited availability of intracellular Cl⁻.

Partial depletion of cellular ATP by treatment with DOG inhibited RVD. This effect was not reversed by valinomycin, which was shown previously to hyperpolarize these cells (11) and increase RVD (21). We, therefore, conclude that inhibition of RVD or DOG is not due to inhibition of K⁺ channels, but more likely due to inhibition of Cl⁻ or OH⁻ efflux. It is not clear at present whether OH⁻ and Cl⁻ efflux occur through separate or common pathways causing RVD. Limited evidence tends to support a common pathway. The complete inhibition of RVD in the presence of valinomycin must result from inhibition of both OH⁻ and Cl⁻ efflux, i.e. preventing the acidification. In case depletion of ATP with DOG inhibited only OH⁻ efflux, activation of K⁺ channels with valinomycin together with Cl⁻ efflux (Fig. 5) should have resulted at least with partial and slow RVD. Of course, it is possible that
depletion of ATP inhibited two separate Cl\(^-\) and OH\(^-\) channels to account for the complete inhibition of RVD. Further studies are required to obtain direct evidence to distinguish between one or two separate channels mediating the efflux of OH\(^-\) or Cl\(^-\) during RVD in UMR-106-01 cells.

In summary, in the present studies, we utilized simultaneous recording of cell volume and pH in single cells to show that following hypotonic swelling of the osteosarcoma cells UMR-106-01, cation- and anion-specific channels are activated to allow the efflux of K\(^+\), Cl\(^-\), and OH\(^-\) and the obligatory H\(_2\)O for RVD. At the same time, the Na\(^+\)/H\(^+\) exchanger is inhibited, probably to prevent further cell swelling. At least the activity of the anion permeating channels is regulated by the level of cytosolic ATP. In HEPES medium, the efflux of OH\(^-\) mediates only a small part (about 25%) of RVD. In the following paper, we show that in the presence of HCO\(_3\)\(^-\), HCO\(_3\) flux through these channels dominates RVD.

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