Measuring volume perturbation of proximal tubular cells in primary culture with three different techniques

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RAAT, N. J. H., P. DE SMET, W. VAN DRIESSCHE, R. J. M. BINDELS, and C. H. VAN OS. Measuring volume perturbation of proximal tubular cells in primary culture with three different techniques. Am. J. Physiol. 271 (Cell Physiol. 40): C235–C241, 1996.—Osmotic cell volume perturbations of rabbit proximal tubule (PT) in primary culture were measured using three independent techniques. Automatic cell thickness monitoring of PT monolayers revealed that cell volume rapidly increased by 39 ± 2% in hypotonic medium (150 mosM), which was followed by partial regulatory volume decrease (RVD). Subsequent incubation in hypertonic medium (500 mosM) rapidly decreased cell volume by 54 ± 2% not followed by regulatory volume increase (RVI). When cell volume in PT monolayers was derived from concentration changes in the trapped fluorescent dyes, fura 2 or 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, osmotically induced cell volume changes appeared much smaller (17 ± 1 and 22 ± 2% for similar hypo- and hypertonicity, respectively). However, changes in fluorescence intensity were most often not in agreement with anticipated cell volume changes. With the Coulter counter, a much larger shift in cell volume was observed in PT cell suspensions. In this situation, cell swelling in hypotonic medium amounted to 74 ± 2% but was still followed by partial RVD. Hypertonicity resulted in a decrease in cell volume of 42 ± 3% not followed by RVI. In conclusion, our study indicates that automatic cell thickness monitoring of an epithelial cell layer cultured on a permeable support provides more reliable data than monitoring changes in fluorescence intensity of trapped dyes.

cell volume; proximal tubule; fura 2; regulatory volume decrease; regulatory volume increase

MAINTAINING A CONSTANT CELL VOLUME is important for proper cell function, and it is therefore not surprising that most mammalian cells are capable of regulating cell volume, not only in isotonic environments but also as a reaction to osmotic perturbation. After cell swelling most cells restore their volume by a process called regulatory volume decrease (RVD), and in analogy hypertotonically shrunken cells recover volume by regulatory volume increase (RVI; Refs. 6, 7, 9).

In recent years it has become clear that cell volume regulation is more complex than originally anticipated. A wide variety of transport systems has been shown to be involved in cell volume regulation, and most systems are probably regulated by volume-sensitive signal transduction pathways (16, 20). Despite the fact that most mammalian cells are normally not exposed to large differences in extracellular osmolality, cell volume can also be challenged at isotonic conditions, for example, by hormonal activation of transporters or during cell division (7). Regulation of cell volume and of the transport mechanisms involved varies widely among different cell types (13). To study the role of signal transduction pathways in cell volume regulation both parameters should therefore be measured at the single cell level. The techniques to measure concentration changes in intracellular ions at the single cell level have improved enormously in recent years. However, accurate measurement of cell volume changes remained difficult and depends largely on the cell type under study.

Until now, most techniques measure cell volume in a suspension of cells, like the Coulter counter, which is most often used with blood cells (11) but has also been used in cell suspensions obtained by trypsinization of attached cells (5, 19). Measuring cell volume in confluent monolayers on a substrate is technically more demanding. Previously, morphometrical methods such as the change in tubule diameter have been used (2); however, this method does not allow one to measure changes in cell volume at the single cell level. Recently, an automatic cell thickness measuring system was developed by van Driessche et al. (4) in which the thickness of a single cell within an epithelial monolayer was monitored by alternatively focusing on the apical and basal membranes of the cell, which were marked with fluorescent microbeads. Another method to monitor cell volume changes has been described by Tawe et al. (22) and by Muallim et al. (14) and consists in measuring the intensity of a trapped fluorescent dye. In theory, the last method provides an opportunity to measure simultaneously changes in intracellular Ca2+ concentration ([Ca2+]i) or intracellular pH (pHi) and cell volume in a single cell.

In the present study we have compared three methods, i.e., 1) automatic cell thickness monitoring, 2) measuring trapped dye concentrations, and 3) the Coulter counter to evaluate their reliability and usefulness in studying cell volume regulation in rabbit proximal tubule (PT) cells in primary culture.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise specified. Bumetanide was obtained from Leo Pharmaceutical Products (Ballerus, Denmark). Fetal calf serum (FCS) was purchased from Sera-Lab (Sussex, UK). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and tris(hydroxymethyl)aminomethane (Tris) were from Research Organics (Cleveland, OH). Gentamicin was obtained from Schering (Kenilworth, NJ). Fura 2-acetoxyethyl ester (AM), 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM), and fluorescent microbeads were from Molecular Probes (Eugene, OR).

Solutions and media. The isotonic (300 mosM) medium was a Krebs-Henseleit buffer (KHIB), which contained (in mM) 110 NaCl, 5 KCl, 2 NaH2PO4, 1.2 MgSO4, 10 Na-acetate, 4

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L-lactate, 10 n-glucose, 1 t-alanine, and 1 CaCl₂, 20 HEPES, calibrated with Tris to pH 7.4. Hypotonic (150 mosM) medium was similar to this solution except that NaCl concentration was reduced to 50 mM. Hypertonic medium (500 mosM) was isotonic buffer to which 200 mM mannitol was added. The osmolarity of the solutions was checked with an osmometer (Osmette A, Precision Systems, Sudbury, MA) and marginally adjusted to the desired value with mannitol. K⁺ medium consisted of a 1:1 mixture of Dulbecco’s modified Eagle’s medium (Imperial, Hampshire, UK) and Ham’s F-12 medium (GIBCO, Paisley, UK), supplemented with gentamicin (10 µg/ml), NaHCO₃ (25 mM), glutamine (14 mM), 0.5% (vol/vol) 100 times nonessential amino acids (GIBCO), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (50 nM), prostaglandin E₁ (70 ng/ml), triiodothyronine (5 pM), and Na₂SeO₃ (50 nM) and having a pH of 7.4.

Primary culture of rabbit kidney PT cells. Rabbit kidney PT cells were isolated and subsequently cultured as described previously (18). Briefly, PT cells were immunodepleted from rabbit kidney with monoclonal antibodies 85C8 and 10IE 12. Subsequently, cells were seeded either on coverslips, on culture flasks, or on transparent filters that were all coated with rat tail collagen (~40 µg/cm²). Cells were seeded at a density of 2.10⁶ cells/cm². In Coulter counter experiments, cells were detached from the culture flasks by 3-min trypsinization with one times trypsin-EDTA solution (GIBCO). Before seeding cells on filters, 1 ml of a 1:2,000 dilution of fluorescent polystyrene latex sulfate microbeads with a diameter of 1 µm (Molecular Probes, ref. no. L-5081) were applied for per fusion; IP, inlet port for perfusion. For calculation of a mean graph of experimental data, the experiment with the smallest time period was chosen as a basis. With use of linear regression the time points for the other experiments were calculated.

Measurement of cell volume using the Coulter counter. A suspension of trypsinized PT cells was kept on ice in a stock of 4.5 x 10⁶ cells/ml; 200 µl of this stock were allowed to warm to 37°C before mixing with 10 ml of hypo- or hypertonic buffer at 37°C. After 1, 5, 9, 14, and 20 min of incubation cell volume was determined. Cells measured in K₁ medium containing HCO₃⁻ were kept at 37°C in a 5% CO₂-95% air atmosphere. Cell volume was measured in a Coulter counter model ZF.

Fig. 1. Scheme of the closed Ussing chamber used in automatic cell thickness monitoring, consisting of an upper and bottom part. T, tissue; proximal tubule cell grown on transparent filter; OP, outlet port for perfusion; IP, inlet port for perfusion.
(Coulter Electronics, Harpenden, UK). Each sample was measured for 30 s, and the number of cells counted ranged between 20,000 and 30,000. Output of the Coulter counter was directed to a Coulter channelizer (Coulter Electronics, Dunstable, UK) in which a size distribution of 100 channels was sampled. The output was connected to an analog-to-digital converter (Maclab, World Precision Instruments, Sarasota, FL), and the output was written to file. Data were smoothed by a binomial smoothing procedure using the software program Igor (Wavemetrics, Lake Oswego, OR), and the channel with the maximum number of cells was used as a measure for the mean volume. Besides 300 mosM, calibration was also performed in a 150 and 500 mosM medium by using polymer latex particles with a diameter of 17.9 μm and a volume of 3 μl (Coulter, Luton, UK). This calibration indicated that during incubation in hypotonic medium cell volume will be overestimated by ~10% because of the lower Na⁺ concentration and smaller conductance of the hypotonic medium. This difference was corrected for by multiplying the outcome of the hypotonic measurement by the ratio of the isotonic and hypotonic values. Assuming that a linear relationship between channel number and cell volume does exist, changes in cell volume were expressed as changes in percentage of channel number.

**Statistics.** Measurements were performed on cells derived from at least three different preparations. Statistical significance was determined by one-way analysis of variance or a paired t-test. Data are presented as the means ± SE.

**RESULTS**

**Measuring cell volume by automatic cell thickness monitoring.** Cell thickness of primary cultured rabbit PT cells in a confluent monolayer was measured by an automatic monitoring system developed by van Driessche et al. (4) as described in MATERIAL and METHODS. Because the water permeability of the apical membrane of A6 cells, used by van Driessche et al. (4), is extremely low it was sufficient to change only the osmolality at the basolateral side to induce cell swelling. In the present study, PT cells were used of which the apical and basolateral sides are very water permeable. Changing the osmolality only on one side resulted in a transcellular water flow without change in cell volume. Therefore, osmolality of the solutions had to be changed simultaneously at both sides. Because the monolayer is viewed from the apical side with a water immersion objective we encountered the problem that when the apical solution was changed the image of the fluorescence beads was distorted and the software could no longer keep track of the beads. The Ussing type perfusion chamber as used by van Driessche et al. (4) was therefore adapted as displayed in Fig. 1, allowing a fast replacement at both sides of the objective. In contrast to A6 cells, PT cells in culture have a low transepithelial resistance, reflecting the leaky paracellular pathway of the proximal tubule, and this has the disadvantage that transepithelial resistance during osmotic cell volume perturbation does not provide cell membrane conductance changes.

As shown in Fig. 2 and in Table 1, PT cell thickness rapidly increased when the monolayer was incubated in hypotonic KHB (150 mosM). In most cells this swelling was followed by a RVD, which partly restored cell volume. Mean initial cell thickness was 10.5 ± 0.5 μm, and cell thickness increased by 39 ± 2% to 14.5 ± 0.7 μm. Because of RVD, cell thickness decreased by 23 ± 2% to 12.1 ± 0.6 μm after 10 min. When cells were subsequently incubated in hypertonic KHB (500 mosM) cell volume decreased rapidly by 54 ± 2% to a cell thickness of 5.9 ± 0.4 μm (Fig. 2, Table 1). No significant increase (6 ± 3%) in volume because of RVD was observed during 10-min incubation in hypertonic medium or in isotonic medium after incubation in hypotonic medium. A remarkable heterogeneity was observed among different cells during swelling. On the same filter, one cell increased in volume by 18%, whereas another exhibited a 88% increase. In addition, a heterogeneity in initial cell thickness was observed, and the smallest cell thickness measured was 4 μm, whereas the tallest cell amounted to 15 μm.

**Table 1. Relative change in cell volume after antisolmmotic incubation of rabbit proximal tubule cells in primary culture**

<table>
<thead>
<tr>
<th>Osmolarity Change, mosM:</th>
<th>Hypotonic</th>
<th>Hypertonic</th>
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<tbody>
<tr>
<td></td>
<td>300 - 150</td>
<td>150 - 500</td>
</tr>
<tr>
<td>Automatic cell height monitoring</td>
<td>39 ± 2</td>
<td>-54 ± 2</td>
</tr>
<tr>
<td>Fluorescent trapped fun 2</td>
<td>17 ± 1</td>
<td>-22 ± 2</td>
</tr>
<tr>
<td>Fluorescent trapped BCEOIV</td>
<td>13 ± 1</td>
<td>-15 ± 2</td>
</tr>
<tr>
<td>Coulter counter</td>
<td>74 ± 2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE. Initial changes in relative cell volume after 10 min of incubation in hypotonic medium (150 mosM) and subsequent incubation in hypertonic (500 mosM) medium or after direct superfusion with 500 mosM medium. With Coulter counting cell volume was measured directly and with the other 2 methods cell volume was based on changes in cell thickness or changes in ion-insensitive fluorescence intensity of fun 2 or 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCEOIV). Change in fluorescence intensity was presumed to be linear with changes in cell volume. ND, not determined.
Monitoring cell volume by measuring trapped dye concentrations. Alterations in total, Ca\(^{2+}\)-independent, fluorescence of fura 2 and the pH-insensitive BCECF fluorescence excited at 440 nm were used to measure cell volume changes. To determine ion-insensitive fura 2 fluorescence the isobestic wavelength at 360 nm has been used in previous studies (3, 14). We used both ratio probes simultaneously to measure, in addition to cell volume, changes in \([Ca^{2+}]_i\) and \(pH_i\), but only four excitation wavelength filters could be placed in the filter wheel. Therefore, ion-insensitive fura 2 fluorescence was obtained from summation of the 340 and 380 nm fluorescence signals, instead of the 360 nm signal. Figure 3 indicates that the percentage change in total fluorescence intensity (340 + 380 nm) and in the isobestic fluorescence at 360 nm of fura 2 is equal. Also the shape of the two curves is similar, and this is in agreement with the findings of Alonso et al. (1).

During the experiment the intensity of the fluorescence declines because of photo bleaching and/or dye leakage. This decline in fluorescence could be fitted to a single exponential curve (14), but the time constant varied among individual cells and experiments. Therefore, for each individual cell its own decay rate constant was calculated from the first 5 min in isotonic medium and was used to correct the succeeding signal, as described previously by Muallem et al. (14). An example of the changes in the uncorrected and corrected fura 2 fluorescence after osmotic perturbation is shown in Fig. 3.

Cell volume in an individual PT cell after osmotic perturbation was determined by measuring the changes in ion-insensitive fluorescence in a restricted area within the cell. However, the changes in ion-insensitive fluorescence of fura 2 and BCECF were not always as anticipated. In addition, cell volume changes derived from the 440 nm signal of BCECF are sometimes opposite of those derived from the fura 2 signal. This variation in results is shown in Fig. 4, which displays fluorescence signals from two different cells on the same coverslip containing fura 2 and BCECF. Only 38% of the measured cells (n = 45) showed a response that could unambiguously be interpreted as cell volume changes, and these cells were further analyzed. After hypotonic (150 mosM) superfusion these cells showed a rapid drop in total fura 2 fluorescence of 17 ± 1% (Table 1), which was followed by a partial recovery in the following 10-min incubation to 5 ± 2% below the initial fluorescence level. Subsequent hypertonic (500 mosM) incubation increased fluorescence intensity by 22 ± 2% (Table 1) with a partial recovery in the following 10 min to 7 ± 1% above the initial level. Superfusion with hypertonic medium after isotonicity increased fluorescence by 12 ± 2% (Table 1) with no significant restoration of volume in the following 10 min.

Deriving volume changes from the pH-insensitive fluorescence of BCECF resulted in a decline in fluorescence of 13 ± 1% (Table 1) after hypotonic superfusion, with no recovery in the following 10 min. Subsequent exposure to hypertonic medium increased fluorescence by 15 ± 2% (Table 1) with a partial recovery in the following 10 min to 7 ± 2%. Superfusion with hypertonic medium preceded by isotonic conditions increased fluorescence intensity by 9 ± 2% (Table 1) followed by a partial restoration of cell volume to 5 ± 2% below the initial volume. The presence of probenecid might theoretically influence the processes of RVD and RVI. Therefore control studies were done with automatic cell height monitoring and the Coulter counter, but no effect of probenecid could be observed on cell swelling and RVD with these techniques.

Monitoring cell volume in the Coulter counter. A suspension of PT cells (4.5 \times 10^6 cells/ml) were either resuspended in hypotonic (150 mosM) or hypertonic (500 mosM) medium and incubation in isotonic medium (300 mosM) was used as a control. During incubation in isotonic medium no change in relative cell volume was measured over a period of 20 min (Fig. 5, A and B). When cells were incubated in 150 mosM medium an initial increase in volume. The presence of probenecid might theoretically influence the processes of RVD and RVI, but no effect of probenecid could be observed on cell swelling and RVD with these techniques.
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RVI were observed. Incubation of cells in 400 mosM medium resulted in a cell shrinkage of 28 ± 3% (n = 4), but also with this smaller osmotic shock RVI did not occur.

**DISCUSSION**

In the present study cell volume changes in single attached PT cells were determined using automatic cell thickness monitoring (4) and imaging of trapped intracellular fluorescent dye concentration (3, 14, 22). In addition, the Coulter counter was used to investigate whether differences exist between cell volume regulation in attached and suspension of PT cells.

With all three techniques, RVD was observed after swelling of PT cells in a 150 mosM medium, as has been reported in other studies (19, 21). Compared with cell volume changes measured with automatic cell thickness monitoring, a much larger increase in cell volume was seen in the Coulter counter in media of equal osmotic strength. The difference between both methods may be explained by the fact that the lateral surface can expand of cells in suspension but not of cells in monolayers. This is especially valid in cell swelling, but absent in cell shrinking experiments. Indeed, exposure to a hypertonic medium of 500 mosM in the Coulter counter and in automatic cell thickness monitoring yielded similar cell volume changes. In addition, a decrease in the lateral intercellular space during hypertonic cell swelling will go unnoticed when height of cells in monolayers is used as a measure of cell swelling. In our experimental setup we have no means of measuring changes in the size of lateral intercellular spaces, and therefore we are unable to evaluate this possible source of error in automatic cell thickness monitoring.

Bibby and McCulloch (3) measured cell volume in attached fibroblasts with fura 2 and in fibroblasts in suspension using the Coulter counter and found a similar discrepancy between attached cells and cells in suspension. Another difference between PT cells in a monolayer and in suspension is the time needed to restore cell volume. This phenomenon reflects the larger increase in cell volume in PT cells in suspension. Additional factors, however, delay RVD in suspended cells because RVD observed in the Coulter counter is more than twice as slow as RVD in attached PT cells. This may be indicative of damage of cytoskeletal elements in single isolated PT cells where lateral support is absent.

With the Coulter counter and with automatic cell thickness monitoring no RVI was observed in PT cells after cell shrinkage. In contrast, the fluorescent signals suggested a partial recovery after a hypertonic shock. This could, however, be an artifact introduced by the correction for dye leakage because cell shrinkage might...
is dependent on medium osmolarity (17). From the first exposed to a hypotonic shock and underwent RVD. Whereas a hypertonic shock of similar magnitude did not provoke RVL In several cell types it has been shown that transport mechanisms involved in RVI have to be altered the time constant for dye leakage. To exclude that the osmotic shock differed too much from the physiological condition, we also performed a shock of 400 mosM, but again no RVI could be demonstrated with the Coulter counter technique. Also the literature is equivocal with respect to RVI in PT cells. Lohr and Grantham (12) showed that PT cells maintain their volume when medium osmolarity is gradually increased to 360 mosM, whereas a hypertonic shock of similar magnitude did not provoke RVI. In several cell types it has been shown that transport mechanisms involved in RVI have to be activated by a preceding RVD. This so-called pseudo-RVI or post-RVD-RVI has also been demonstrated in PT cells by Linshaw et al. (10). However, with two different techniques to measure cell volume we were unable to demonstrate RVI, even when PT cells were first exposed to a hypotonic shock and underwent RVD. In a previous study, we have reported that cultured PT cells express Na+-K+-2Cl- cotransport, which activity is dependent on medium osmolarity (17). From the present study we conclude, however, that Na+-K+-2Cl- cotransport is not contributing significantly to RVI in PT cells.

Cell volume changes derived from changes in fluorescence intensity of trapped dyes were rather erratic in the present study. The method is based on the assumption that the total fluorescence in a single cell does not change when properly corrected for photo bleaching and dye leakage. By measuring the concentration of fluorescent dye in a restricted area within the cell an estimate of a change in cell volume can be made (14, 22). Tauc et al. (22) were able to convert directly changes in fluorescence intensity into volume changes. In our study, cell volume changes measured with fluorescent probes were much smaller than in the study of Tauc et al. (22). A similar observation was made by Bibby and McCulloch (3) using fura 2; these investigators observed a volume change of 11% after a change from 300 to 150 mosM. Mualem et al. (14) did not convert changes in fluorescent intensity into cell volume changes but used this method in a qualitative way. When compared with automatic cell thickness monitoring, we conclude that changes in fluorescence intensity of trapped dyes underestimate cell volume changes in PT cells in monolayers.

There are several possibilities why cell volume measurements with fura 2 and BCECF fluorescence are less reliable. First, it is important to obtain a homogeneous dye loading. Compartmentalization of dye in intracellular organelles disturbs the reliability, because it is likely that the permeabilities of intracellular membranes and the plasma membrane are different. Second, cell volume changes derived from two simultaneously present probes should yield similar results. However, in many experiments changes in BCECF and fura 2 intensities were different and even opposite, which indicates that additional factors influence the fluorescence intensity of both probes. Third, accurate volume measurements require a precise correction of the decay in fluorescence intensity due to photo bleaching and dye leakage. Despite the presence of probenecid during dye loading and experiments and despite restricted exposure to ultraviolet light, the fura 2 or BCECF fluorescence signals decreased in 25 min to 50% of the starting level. The fluorescence signal was corrected assuming an exponential decrease in fluorescence intensity in the first 5 min of the experiment at isotonicity. Volume perturbation, however, might influence the rate of dye leakage. In support of this notion is the fact that fluorescence intensity decreased again after a hypertonic shock, whereas with the two other techniques no RVI was observed. In addition, in several experiments no correction of the fluorescence signal resulted in an overestimation of the RVD or RVI responses. In certain conditions measuring cell volume with fluorescent probes could be an attractive technique that allows one to measure changes in cell volume simultaneously with changes in intracellular ions as Ca2+ and H+. The present study indicates that this method underestimates cell volume changes. Reliability may vary with the sort or size of the cell under

![Graph](image-url)

**Fig. 5.** Effect of medium osmolarity on relative cell volume of cultured PT cells in suspension measured by the Coulter counter. A: PT cell volume increase after incubation in hypotonic 150 mosM medium (O). Cells were also incubated in nominal Ca2+ free (+0.1 mM La3+) 150 mosM medium (△) and 150 mosM Ca2+ free medium containing 2 mM EGTA (D). Response in isotonic 300 mosM medium (O) is shown as control. Data are presented as the means ± SE of at least 3 different preparations. B: PT cell volume decrease after incubation in hypertonic 500 mosM medium (O). Cells were also incubated in 500 mosM K+ medium with HCO3− to which mannitol (△) or NaCl (D) was added to increase osmolarity. Response in isotonic 300 mosM medium (O) is shown as control. Data are presented as the means ± SE of at least 3 different preparations.
study. Confocal microscopy could well be a more reliable alternative because it precludes disturbance of the signal by fluorescence from other focal planes. Automatic cell thickness monitoring measures cell volume most straightforward, but this method is so far only applicable when confluent monolayers are under study.

We thank J. A. F. van Boxtel and J. De Beir-Simels for technical assistance and R. Huijben for instruction on the Coulter counter.

This study was supported by the Netherlands Organization for Scientific Research, NWO Grant 810-409-004.

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Received 23 February 1995; accepted in final form 29 January 1996.

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