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ORIGINAL ARTICLE

**Stefan W. Schneider · Philipp Pagel · Christian Rotsch
Timm Danker · Hans Oberleithner
Manfred Radmacher · Albrecht Schwab**

Volume dynamics in migrating epithelial cells measured with atomic force microscopy

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Abstract Migration of transformed renal epithelial cells (transformed Madin-Darby canine kidney cells, MDCK-F cells) relies on the activity of a Ca^{2+}-sensitive K^+ channel (IK channel) that is more active at the rear end of these cells. We have postulated that intermittent IK channel activity induces local cell shrinkage at the rear end of migrating MDCK-F cells and thereby supports the cytoskeletal mechanisms of migration. However, due to the complex morphology of MDCK-F cells we have not yet been able to measure volume changes directly. The aim of the present study was to devise a new technique employing atomic force microscopy (AFM) to measure the volume of MDCK-F cells in their physiological environment and to demonstrate its dependence on IK channel activity. The spatial (*x*, *y* and *z*) co-ordinates of each pixel of the three-dimensional image of MDCK-F cells allow calculation of the volume of the column "underneath" a given pixel. Thus, total cell volume is the sum of all pixel-defined columns. The mean volume of 17 MDCK-F cells was 2500±300 fl. Blockade of the IK channel with the specific inhibitor charybdotoxin (CTX) increased cell volume by 17±4%; activation of IK by elevating the intracellular [Ca^{2+}] with the Ca^{2+} ionophore ionomycin decreased cell volume by 19±3%. Subtraction images (experimental minus control) reveal that swelling and shrinkage occur predominantly at the rear end of MDCK-F cells. In summary, our experiments show that AFM allows the measurement not only of total cell volume of living cells in their physiological environment but also the tracing of local effects induced by the polarized distribution of K^+ channel activity.

Key words Atomic force microscope · Elasticity · IK channel · Polarization · Migration

S.W. Schneider · P. Pagel · T. Danker · H. Oberleithner
Physiologisches Institut, Robert-Koch-Strasse 27a,
D-49148 Münster, Germany

C. Rotsch · M. Radmacher
Lehrstuhl für Angewandte Physik, Amalienstrasse 54,
D-80799 München, Germany

A. Schwab (✉)
Physiologisches Institut, Röntgenring 9, D-97070 Würzburg, Germany
e-mail: albrecht.schwab@mail.uni-wuerzburg. de
Tel.: +49-931-312724, Fax: +49-931-312741

Introduction

Cell volume regulation and the cytoskeleton are closely interrelated with each other. Thus, cell volume regulation depends on an intact actin cytoskeleton [5], and perturbations of cell volume themselves change the polymerization of actin filaments [8] (see [14] for review). Based on these considerations it becomes evident that cell migration – a process in which a complex and co-ordinated turnover of actin filaments is of central importance [2, 15, 19] – also relies on cell volume homeostasis. Accordingly, we have shown that migration of transformed renal epithelial cells (transformed Madin-Darby canine kidney cells, MDCK-F cells) is impaired after cell shrinkage or swelling [33].

Previously, we have studied the role of a Ca^{2+}-sensitive K^+ channel of intermediate conductance (IK channel) in migration [31, 33, 35]. IK channels are activated during regulatory volume decrease in MDCK cells [36]. In migrating MDCK-F cells this K^+ channel is more active at the rear end of the cell than at the front [31]. We have shown that the IK channel is responsible for the intermittent efflux of 10–20% of the cellular K^+ content, implying a net volume decrease of the same order of magnitude during bursts of K^+ channel activity [3, 31]. To date, however, we have not been able to measure the inferred volume changes directly or to test whether K^+ channel-mediated changes of cell volume are, in fact, restricted to the rear end of migrating MDCK-F cells.

One of the aims of the present study, therefore, was to develop a new technique for measuring the volume of MDCK-F cells under physiological conditions using atomic force microscopy (AFM). The atomic force microscope is a tactile instrument that scans the surface of

a sample in the *x*-, *y*- and *z*-directions and calculates a three-dimensional (3D) image from these co-ordinates [1]. The great advantage of AFM is that living cells can be imaged with nanometre resolution under physiological conditions so that dynamic vital processes can be visualized [10, 18, 22, 23, 25, 26, 28]. This new technique enabled us to determine directly changes of cell volume and elasticity elicited by modulating K^+ channel activity. Due to the high spatial resolution of AFM we could also show that K^+ channel-dependent volume changes are restricted to the cell body of MDCK-F cells.

Materials and methods

Cell culture

Experiments were carried out on alkali-transformed MDCK-F cells [21]. The transformed MDCK-F cells were kept at 37 °C in humidified air containing 5% CO_2 and grown in bicarbonate-buffered Minimal Essential Medium (MEM; pH 7.4) with Earle's salts (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom). For experiments cells were plated on coverslips coated with poly-L-lysine (0.1 g/l; Serva, Heidelberg, Germany). Experiments were performed 1–2 days after seeding. During the experiments MDCK-F cells were kept in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered MEM supplemented with 10% FCS.

Atomic force microscopy

We used the BioScope (Digital Instruments, Santa Barbara, Calif., USA) in conjunction with an inverted optical microscope (Axiovert, Zeiss, Oberkochen, Germany) working in "contact" and "force-volume" modes [22]. For AFM experiments we used a continuously perfused (5 ml/h), temperature-controlled (37 °C) fluid chamber, the bottom of which was formed by a glass coverslip (thickness 1 mm). The coverslip on which MDCK-F cells were grown (approximately 7 mm in diameter) was fixed with fast glue (cyanoacrylate adhesive) on the bottom of the fluid chamber. Charybdotoxin (CTX, Alomone, Jerusalem, Israel) or ionomycin (Sigma, Deisenhofen, Germany) was added directly to the bath from a 10× stock solution to obtain the desired final concentration. After adding CTX or ionomycin the bath perfusion was stopped. The effect of CTX or ionomycin on volume or elasticity of MDCK-F cells was studied in a paired fashion.

Determination of cell volume

Scanning MDCK-F cells with AFM was in so far challenging as the height of lamellipodium and cell body differ by a factor of 10. The dramatic height change between lamellipodium and cell body of 2–3 µm leads to a small additional deflection of the AFM tip and therefore to an increase in loading force in the transition zone between these two cell poles. This is due to a temporal delay in the feedback regulation of the AFM tip when the position in the *z*-axis changes rapidly to such a great extent. In the contact mode the loading force was less than 0.3 nN over the lamellipodium (thickness: 300–400 nm) and up to 0.5 nN over the cell body (height: 2.5–3 µm). The loading force was adjusted by using the force calibration mode. By scanning MDCK-F cells with pre-set forces over a period of up to 30 min we could assess the degree of mechanical perturbation induced by the interaction between the AFM tip and the cell surface. Mechanical perturbations are kept at a minimum by never exceeding a scanning force of 0.5 nN in the contact mode. Scanning with forces exceeding 2–3 nN results in the retraction of cell body or lamellipodium; this is often followed by the detachment of the cells. The scan area in the *x*-*y*-plane usually was 100×100 µm.

During the experiments force calibrations were made before and after each image to maintain the scanning force constant and at the lowest level possible. The applied force indented the cell body by approximately 300 nm. Since the apparent elasticity of the lamellipodium of some cells is much higher [26] the loading force between AFM tip and cell surface can be adjusted more precisely at this cell pole. The indentation leads to a systematic underestimation of cell volume under control and experimental conditions. In the present study, volume measurements were not corrected for this error. For volume measurements we used silicon nitrite tips with spring constants of 60 (Digital Instruments) or 8 (Park Scientific, Sunnyvale, Calif., USA) mNm^{-1}. AFM images were generated at line frequencies of 1–1.5 Hz with 256 or 512 lines per image. To reduce the risk of detaching cells from their support the scan direction was always parallel to the longitudinal axis of MDCK-F cells, i.e. in the direction of locomotion.

3D images of MDCK-F cells were analysed with software provided by the AFM manufacturer. Determination of cell volume is based on the following principle. Each pixel is defined by its *x*-, y- and z-co-ordinates. The *x*- and *y*-co-ordinates are determined by the size of the scan area and the number of scan lines per image. The *z*-co-ordinate is given by the specific grey level of each pixel. Thus, each pixel represents a column with precisely defined *x*-, *y*- and *z*-dimensions so that the volume (in femtolitres) of such a column can be calculated. The sum of all columns corresponds to total cell volume [6]. Before determining cell volume images are "planefitted" (planefit order 1) whereby the plane of the glass coverslip serves as the plane of reference. Volume measurements were made by using the so-called "bearing analysis" software provided with the instrument. This software provides a method of plotting and analysing the height distribution of a sample surface. Image subtraction was also performed with the manufacturer's software. The grey levels of corresponding pixels of two images are subtracted from each other whereby the plane of the glass coverslip serves as plane of reference. Images can only be subtracted from each other when the identical visual field is scanned consecutively and when cellular displacement is minimal.

Force-volume measurements

Volume measurements are only reliable when the elasticity of the cell does not change during the experiment. Any change of elasticity will alter the indentation of the cell by the AFM tip and thus, depending on the elasticity the AFM will determine different height profiles, thereby falsely implying volume changes. Therefore, we also performed paired force-volume measurements under control and experimental conditions in which we quantified the indentation of the AFM tip to calculate the elasticity [11]. We used silicon nitrite tips with spring constants of 8 mNm^{-1} (Park Scientific) for force-volume measurements. Since the cell body accounts for 90% of the total cell volume, force-volume measurements were made at this cell pole. The time resolution of force-volume measurements in MDCK-F cells is low (more than 20 min for the entire cell surface) so that measurements were limited to an area of 4 µm ×4 µm (32×32 data per measurement) over the cell body. The principle of force-volume measurements is as follows. Due to the mechanical stiffness of the cell the AFM tip is deflected when it is placed on the cell surface and the cell is indented. In the force-volume mode the cell is indented until the AFM tip is deflected by exactly 100 nm, corresponding to a loading force of 800 pN. Depending on its mechanical properties a tip deflection of 100 nm results in a more or less pronounced indentation of the cell. Then the AFM tip is retracted from the cell surface and force curves are generated (loading force plotted as a function of the vertical position of the AFM tip). This manoeuvre is repeated 1024 times per measurement. The indentation of the cell can be determined by comparing force curves on the cell with those measured on a stiff sample (e. g. glass coverslip). Tip deflection and cell indentation allow the calculation of elasticity according to the

Hertz model for each of the 1024 data [4, 26]. Cell elasticity (Young's elastic modulus in kiloPascal) is given as mean±SEM of these 1024 data. Quantification of the indentation of the cell during the course of the experiment also allows calculation of the systematic over- or underestimation of cell volume. These calculations were always referred to an average cell height of MDCK-F cells of 2.5 µm.

Statistics

All data are presented as mean±SEM. Student's t-test for paired or unpaired samples was performed where applicable. Significance was assumed when $P<0.05$.

Results

Resolution of volume measurements with AFM

To confirm that the scanning process itself does not alter the volume of MDCK-F cells we always generated three images at 4-min intervals under control conditions. As depicted in Fig. 1A, cell volume (2500±300 fl; $n=17$) remains virtually constant during the control period. Force-volume measurements revealed that the indentation of the cell does not change during the course of the experiment, either under control or experimental conditions. Accordingly, the error of cell volume measurements is less than 1% (see Fig. 1B). The mechanical interaction between the AFM tip and the cell membrane does not trigger volume regulatory mechanisms that alter the volume of MDCK-F cells. Under control conditions the elasticity of the cell body was 8.5±1.7 kPa ($n=10$). Elasticity did not change significantly upon application of CTX (+19±7%; $n=4$) or ionomycin (–2±10%; $n=6$).

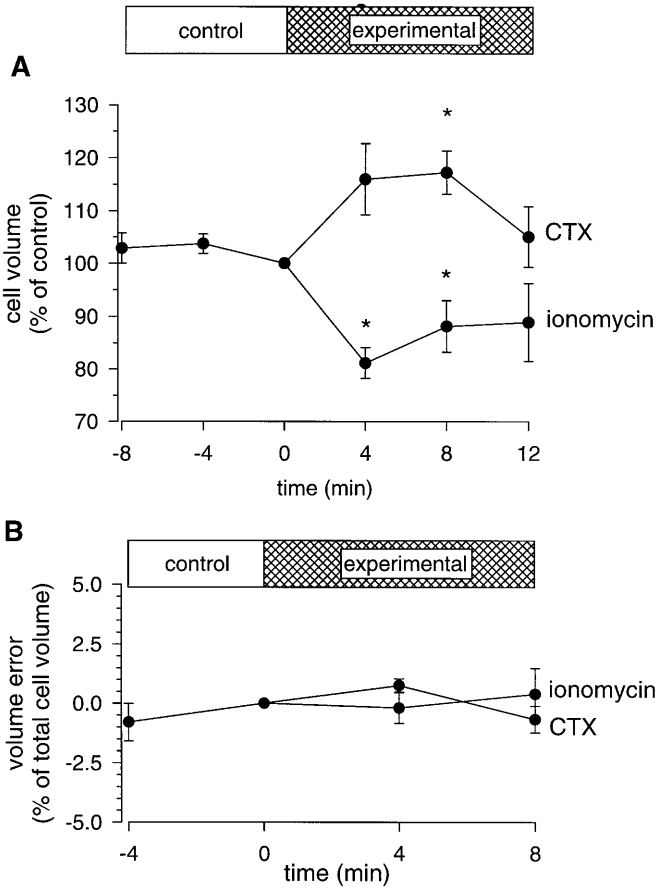

Fig. 1A, B Summary of volume measurements in alkali-transformed Madin Darby canine kidney (MDCK-F) cells with atomic force microscopy. **A** The volume of living MDCK-F cells (normalized with respect to the last control value at time $t=0$ min) rises after the addition of the specific IK channel inhibitor charybdotoxin (*CTX*) and falls after maximally activating the IK channel by elevating the intracellular [Ca^{2+}] with the Ca^{2+} ionophore ionomycin. **B** Time course of the calculated error of volume measurements. Since the indentation does not change after IK channel inhibition or activation the error of volume measurements is minimal.

Effect of CTX

Previously, we have shown that bursts of IK channel activity induce intermittent K^+ loss of the order of 10–20% of cellular K^+ [3, 31] and have suggested that K^+ loss is accompanied by cell shrinkage of the same order of magnitude. By using AFM we now wanted to measure cell volume of MDCK-F cells directly and to show its modulation by K^+ channel activity. Figure 2A and B shows a MDCK-F cell under control conditions and 4 min after the addition of 5 nmol/l CTX. In this example total cell volume increased by 13%. In five further cells volume rose by up to 17±4% (Fig. 1B). Based on the determination of the cell elasticity, the calculated error of volume measurements of MDCK-F cells is less than 1% in the presence of CTX (Fig. 1B). Hence, these experiments provide direct evidence that inhibition of the Ca^{2+}-sensitive K^+ channel in MDCK-F cells is followed by cell swelling. These experiments are summarized in Fig. 1A.

The corresponding section analyses (lower part of Fig. 2A and B) reveal that CTX-induced swelling of MDCK-F cells is primarily due to an increase in height of the cell body. The maximal height of the cell body shown in Fig. 2 increased from 2.6 to 2.9 µm after treatment with CTX. In contrast, the height of the lamellipodium remained constant (–17±13 nm). These data suggest that CTX does not affect the entire cell uniformly but that swelling occurs predominantly in the cell body. This conclusion is confirmed by subtracting the images shown in Fig. 2B and A from each other. The resulting image and section analysis are depicted in Fig. 2C. A decrease of volume is indicated by a dark colour whereas an increase of cell volume is shown in a bright colour. Figure 2C shows that the gain of cell volume after application of CTX is restricted essentially to the cell body.

Effect of ionomycin

If inhibition of the IK channel is followed by cell swelling, activation of the channel should be followed by cell

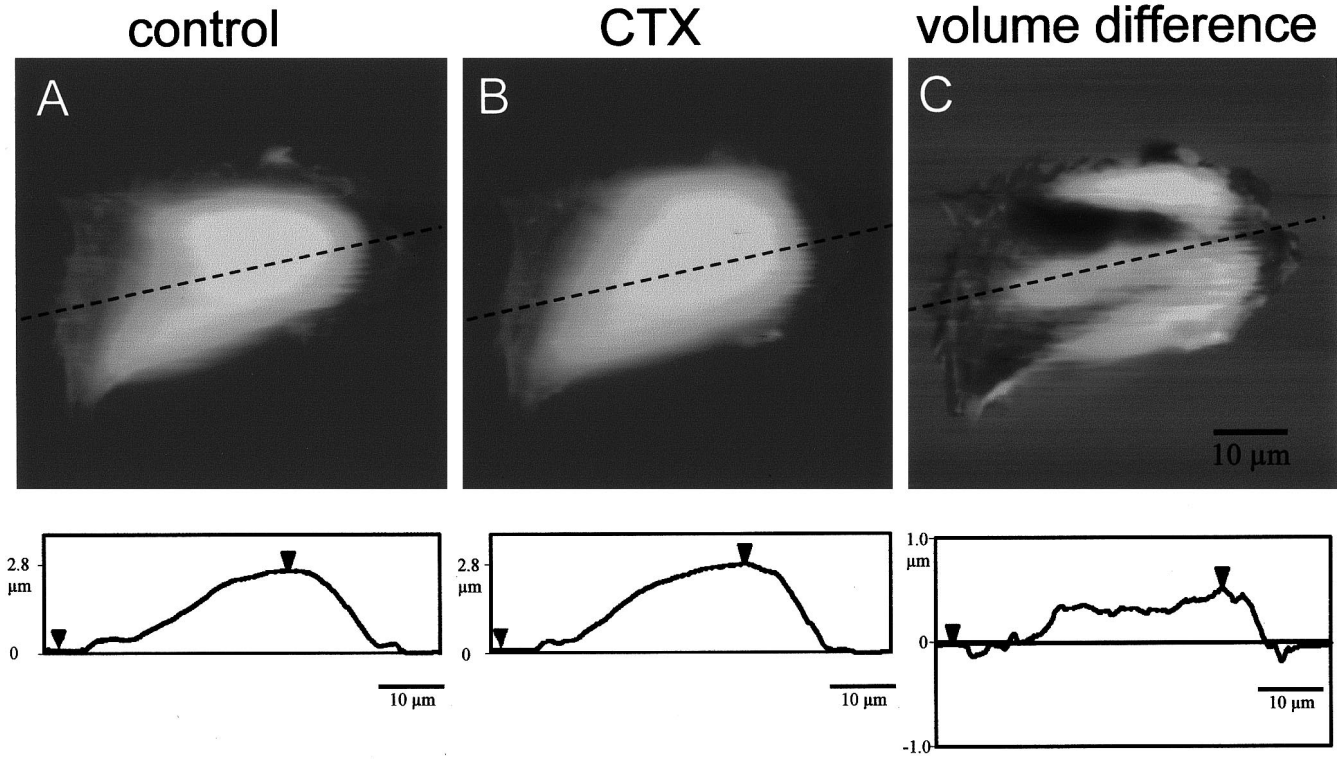

Fig. 2A–C Effect of CTX on the volume of an MDCK-F cell. **A** Control; **B** 4 min after exposure to CTX. **C** Subtraction image (**B** minus **A**). The corresponding section analyses *below* each image show the height profile along the *dotted lines*. The colour coding in **A** and **B** reflects the height of the cell surface (the brighter the hue the higher the cell surface). The colour coding in **C** shows the volume changes. A dark colour indicates volume loss; a bright colour volume gain. The section analysis in **C** shows the change of cell height due to CTX. Note the different *height scale* compared with section analyses in **A** and **B**

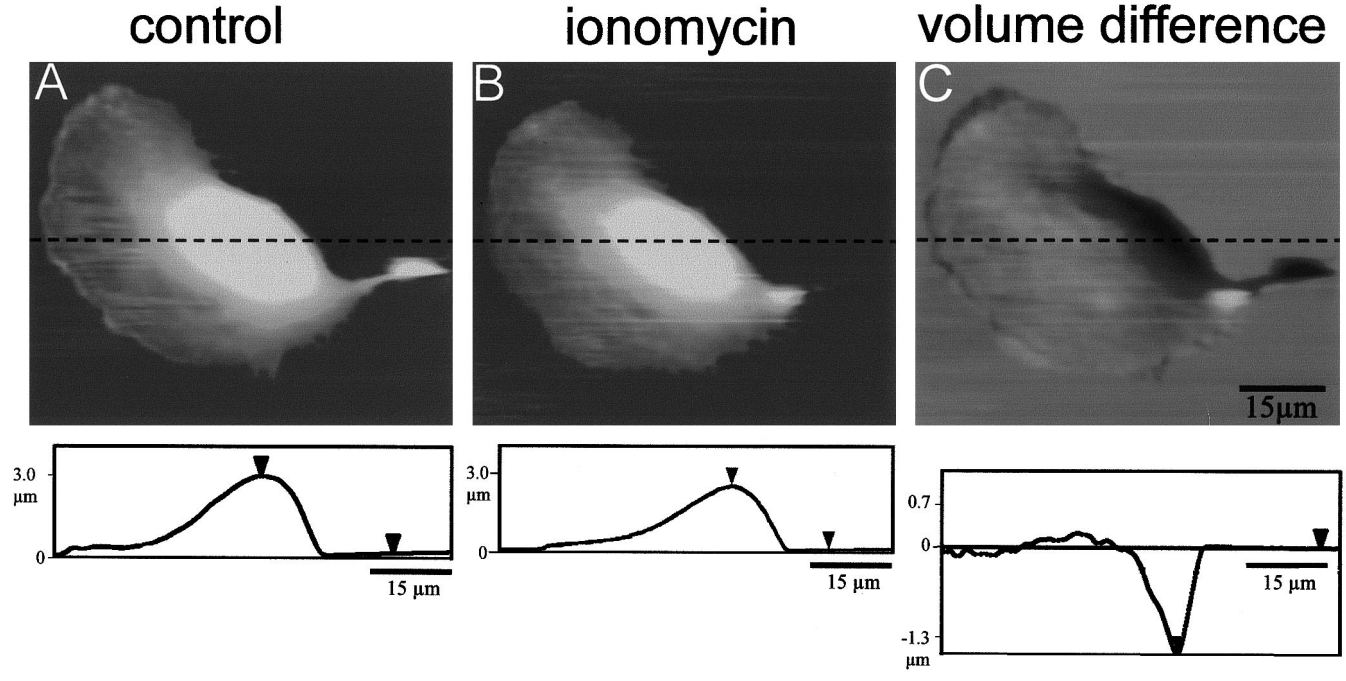

Fig. 3A–C Effect of IK channel activation on the volume of a living MDCK-F cell. **A** Control; **B** 4 min after IK channel activation by elevating the intracellular [Ca^{2+}] with ionomycin. **C** Subtraction image (**B** minus **A**). The corresponding section analyses *below* each image show the height profile along the *dotted lines*, colour coding as in Fig. 2. Note the different *height scale* compared with the section analyses in **A** and **B**

shrinkage. To test this and our previous hypothesis that K^+ loss in MDCK-F cells is followed by volume loss [3, 31] we determined cell volume after maximally activating the Ca^{2+}-sensitive K^+ channel by increasing the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with the Ca^{2+} ionophore ionomycin [32]. Figure 3A and B show images and the corresponding section analyses before and 4 min after the application of 1 µmol/l ionomycin. In this example cell volume decreased from 1700 to 1200 fl. In five further cells ionomycin elicited cell shrinkage by 19±3% within 4 min. These experiments are summarized in Fig. 1A. Based on the determination of the cell elasticity the calculated error of volume measurements of MDCK-F cells is less than 1% in the presence of ionomycin (see Fig. 1B). Thus, activation of the Ca^{2+}-sensitive K^+ channel leads to a decrease of cell volume. Figure 1B also demonstrates that K^+ channel-mediated fluctuations of the volume of MDCK-F cells are of the same order of magnitude as the K^+ efflux from MDCK-F cells [3, 31].

Inspection of the section analyses in Fig. 3A and B reveals that the major effect of ionomycin is a decrease of height of the cell body. In this example the maximal height fell from 2.7 to 2.4 µm (measured between the arrows). In contrast, ionomycin had no effect on the height of the lamellipodium (+17±13 nm). The cell body appears to be more susceptible to K^+ channel-mediated height and thereby volume fluctuations than the lamellipodium. Fig. 3C is a subtraction image of Fig. 3B minus Fig. 3A. It is apparent that IK channel-mediated cell shrinkage is restricted predominantly to the cell body.

Discussion

The aim of this study was to develop a new technique for measuring the volume of migrating MDCK-F cells and its modulation by a Ca^{2+}-sensitive K^+ channel (IK channel) under physiological conditions. This K^+ channel is required for migration and it is more active at the rear end (cell body) than at the front (lamellipodium) of MDCK-F cells [30, 34]. Since the Ca^{2+}-sensitive K^+ channel is responsible for the intermittent efflux of 10–20% of cellular K^+ we have proposed that it supports cytoskeletal mechanisms of migration by inducing localized changes of cell volume at the rear end of MDCK-F cells [3, 31]. To verify this model the new technique for measuring cell volume had to meet three major demands. First, it was absolutely necessary to study *living* MDCK-F cells that maintained their polarized morphology with lamellipodium and cell body. Second, the spatial resolution had to be high enough to be able to detect even minute changes in the height of cell body or lamellipodium. Finally, in addition to measuring cell volume the new technique had to permit accurate monitoring of changes in the morphology of the MDCK-F cells. In our experience these criteria are met only by AFM and not by "conventional" methods of volume measurements such as the Coulter counter [7] or confocal microscopy [24]. By measuring cell elasticity, too, we were able to exclude the possibility that the observed volume changes were caused by differences in mechanical indentation of MDCK-F cells by the AFM tip.

The resolution limit for measuring cell volume with AFM depends largely on the properties of cells under study. We were able to resolve volume changes in MDCK-F cells of the order of 5–10% of total cell volume. The major limitation for volume measurement in MDCK-F cells is the extreme height difference between cell body and lamellipodium. In particular, the loading force between AFM tip and the cell surface cannot be kept constant in the transition zone between lamellipodium and cell body since there is a certain delay in the feedback regulation of the piezo of the *z*-axis. At a scan frequency of 1.5 Hz the *z*-position of the AFM tip has to be changed by 2–3 µm within a few milliseconds. Due to the delay in the feedback regulation the AFM tip senses this height change "too late" so that the loading force automatically rises and the cell is indented. However, we kept this additional indentation at a minimum by never exceeding a scan frequency of 1.5 Hz. Our force-volume measurements indicate that the cell body is indented by 290±24 nm so that cell volume is underestimated by approximately 10%. The pronounced pleomorphism of MDCK-F cells [21] is responsible for a certain variation of the systematic underestimation of cell volume from cell to cell. Since we measured cell volume and cell elasticity in paired experiments before and after K^+ channel modulation and since K^+ channel modulation has no effect on cell elasticity this systematic error does not affect the detection of relative volume changes. The resolution of volume measurements with AFM is considerably higher in cells, such as endothelial cells, with a morphology more uniform than that of MDCK-F cells. When studying these cells the resolution limit is in the order of 3–6 % of total cell volume (S.W. Schneider, unpublished observations).

In addition to accurately determining volume dynamics of individual cells, AFM also provides a detailed, 3D image of the cell under study with nanometre precision. In other words, cell volume and cell morphology can be correlated with each other at any time during the experiment. In our view this is the great advantage of AFM technology which is of particular importance for the present study. We are now able to visualize localized changes of cell volume and show that the volume of cell body and lamellipodium of a migrating cell are modulated independently of each other. In addition, image subtraction allows motion artefacts to be distinguished easily from true volume changes. In our study motion artefacts were negligible for CTX and ionomycin since both drugs completely inhibit MDCK-F cell migration [30, 35]. Thus, AFM is a powerful tool for demonstrating dynamic changes both at the molecular level [20] and at the level of the living cell.

Volume and elasticity measurements with AFM on MDCK-F cells harbour one problem. As explained above MDCK-F cells must be scanned slowly (1–1.5 Hz) due to the dramatic height change between lamellipodium and

cell body and since they are only loosely attached to their substratum. Thus, it usually takes 2–4 min to scan an MDCK-F cell in the contact mode and 20–30 min when measuring elasticity in the force-volume mode. The scanning process can not be accelerated since higher scan frequencies inevitably detach the MDCK-F cell from the glass coverslip. Time resolution of volume measurements with AFM will be considerably higher for smaller cells that require a smaller scan area and for cells that are attached more firmly to their matrix, such as an epithelial monolayer. Time resolution of volume changes can be reduced to less than 1 s if it is not necessary to image the entire cell and only relative volume changes are measured [26]. Under such circumstances a line scan can be performed, i.e. the AFM tip is moved back and forth at the same *y*-position and changes in the *z*-axis along the scan line are recorded as a function of time. Time resolution of elasticity can only be accelerated by reducing the scan area. Therefore, we could determine the elasticity only for an area of 4 μm ×4 μm over the cell body.

The present experiments disclose a functional difference between cell body and lamellipodium in MDCK-F cells. This is in good agreement with our previous findings [31] namely, that IK channel modulation only affects the volume of the cell body. At the moment we can only speculate on the mechanisms that restrict volume changes to the rear end of MDCK-F cells. Actin filament solutions resist osmotic water flow to some extent [13] and they impede osmotic swelling or shrinkage when cross-linked by actin-binding protein [12]. It is thus conceivable that osmotic water flow is impaired in the lamellipodium of a migrating cell, too, since the lamellipodium contains a dense, three-dimensional meshwork of actin filaments [9]. An alternative explanation involves differences in the mechanical properties of the lamellipodium and the cell body. The lamellipodium of MDCK-F cells is much stiffer [23], and is attached more firmly to the substratum, than the cell body. Whenever MDCK-F cells detach from the glass cover slip during the scanning process, the cell body always loses contact first. With this in mind it is feasible that the rigid actin gel and the tight adherence to the substratum prevent the lamellipodium from increasing or decreasing its volume to the same extent as the cell body. We are aware that both explanations are highly speculative and need to be verified experimentally.

Nonetheless, our finding of localized IK channel-induced cell shrinkage adds to the understanding of IK channel function in migrating cells. Recently, we have presented a model in which we postulated that cell volume links IK channel activity to migration of MDCK-F cells [33]. According to this model, periodic bursts of IK channel activity [34] that are triggered by oscillations of $[Ca^{2+}]_i$ lead to fluctuations of cell volume. In the present study we show that volume loss is confined to the rear end of MDCK-F cells. In addition to activating the IK channel the intermittent rise of $[Ca^{2+}]_i$ also loosens cell-matrix connections at the rear end [16, 17] and solates the cortical actomyosin gel, thus allowing a contraction at the rear end [15]. Thus, IK channel-mediated volume loss and cytoskeletal mechanisms act in concert during retraction of the rear end of a migrating cell.

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