Preparation of basal cell membranes for scanning probe microscopy

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Received 17 June 1998; revised version received 31 August 1998

Abstract Scanning probe microscopy has the potential for investigating membranes in a physiological environment. We prepared with a lysis-squirting protocol basal cell membranes, that are suitable for scanning probe microscopy. Investigations using atomic force microscopy under liquid revealed cellular filaments which correlated perfectly with fluorescently stained actin filaments. Globular structures with a diameter as little as 10 nm could be resolved by stripping cytoplasmic components from the membranes. Therefore, cytoplasmic sides of supported basal cell membranes prove useful to gain high resolution with scanning probe microscopy in studies of plasma membrane associated structures and processes under buffer solution.

Key words: Atomic force microscopy; Basal cell membrane; Confocal laser scanning microscopy; Cytoskeleton; Scanning probe microscopy

1. Introduction

The cell membrane is an important mediator of intercellular and intracellular interactions. This function requires the correct assembly of various molecular structures in and around the cell membrane including receptors, transport proteins and specialized membrane domains such as caveolae, clathrin coated pits, or cell-cell and cell-substrate adhesion structures [1]. Atomic force microscopy (AFM) as well as other members of the scanning probe microscopy (SPM) family have been shown to be ideally suited for investigations of cells, cell compartments and membranes under physiological conditions, because many of these instruments can be operated under liquid with high resolution down to individual proteins [2–6]. Using AFM structures on the cell surface and, remarkably, internal cellular structures could be identified. In addition the elastic behavior and dynamic cellular processes could be visualized [7–15].

Identifying structures inside cells, such as stress fibers, by scanning on the cell surface has one major drawback. Indenting the probe tip into the membrane limits the resolution, because no direct interactions between tip and target are established [16–18]. Furthermore, many internal structures cannot be reached with this surface scanning technique. By using isolated cell membranes effects like cell elasticity, motility, and height fluctuations are diminished. In addition, the AFM tip can interact directly with structures on the isolated membrane, which is, at the same time, more rigid due to the firm attachment. This all leads to higher resolution. Currently, the highest resolutions with AFM are obtained on regular protein/lipid complexes [3,4,17,19–24].

For a better understanding of protein structure and function at the inner membrane surface, a lysis-squirting protocol was used for SPM. This technique has been used to cleave intact cells grown on glass or plastic substrates to analyze attached basal cell membranes by electron microscopy [25]. The still attached cytoplasmic components can be further modified by treating basal cell membranes with various protocols [26–30]. These possibilities together with the simple, easy to perform protocol results in a very versatile method. In this work, we show that highly resolved AFM images of the actin cytoskeleton as well as of globular structures can be obtained on attached basal cell membranes after cleaving adherent cells with the lysis-squirting protocol. In contrast to electron microscopy, high resolution can be achieved, while working in a physiological, liquid environment.

2. Materials and methods

2.1. Preparation of membranes

Sodium phosphate transporter type II transfected Madine-Darby canine kidney (MDCK) cells were cultivated overnight to subconfluency according to Quabius et al. [31] on thermanox (Nunc, Roskilde, Denmark) (for electron microscopy) or ethanol washed glass cover slips (for optical microscopy and AFM). The cells were washed with ice cold 20 mM PIPES, 150 mM KCl, pH 6.2, incubated in hypotonic buffer (4 mM PIPES, 30 mM KCl, pH 6.2) for 3 min on ice and subsequently squirted using 5 ml of the same buffer through a 25-gauge needle (Fig. 1a). Incubation in high salt was done in 2 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM Na_2HPO4, pH 7.2 for 30 min at room temperature. Membranes were fixed in 2% formaldehyde or 1% glutaraldehyde, respectively, in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM NaH_2PO_4, 1.5 mM K_2HPO_4, pH 7.2) for 30 min at room temperature.

2.2. Staining of membrane and cytoskeletal components

Actin was stained with a 1:75 dilution of rhodamine or fluorescein-5-isothiocyanate (FITC) labeled phalloidin (Molecular Probe, Eugene, OR, USA) in PBS for 30 min at room temperature. The membrane was fluorescently labeled using a green fluorescent lipophilic stain PKH-2 (Sigma). The sodium phosphate transporter type II was labeled with a polyclonal rabbit antibody (1:500; [31]) followed by a goat anti rabbit Texas Red antibody (1:100; Jackson Immuno Research, West Grove, PA, USA).

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Abbreviations: AFM, atomic force microscopy; FITC, fluorescein-5-isothiocyanate; MDCK, Madine-Darby canine kidney; PBS, phosphate buffered saline; SNOM, scanning near field optical microscopy; SPM, scanning probe microscopy

PII: S0014-5793(98)01118-1

2.3. Transmission electron microscopy

Cells or cell membranes generated by lysis-squirting were post-fixed with 1% glutaraldehyde and 1% OsO_4 in 50 mM sodium cacodylate, pH 7.3, dehydrated in increasing concentrations of ethanol and embedded in epon. Post-staining of 60-nm thick sections was performed using 4% uranyl acetate in 50% ethanol for 20 min at room temperature followed by 133 mM $Pb(NO_3)_2$, 200 mM $Na_3(C_6H_5O_7)$ (sodium citrate) [32] for 15 min at room temperature. This procedure optimizes the contrast of membranes and protein structures [33].

2.4. Optical microscopy

Images of fluorescently labeled membranes were recorded with a confocal laser scanning microscope (LSM 3; Zeiss, Oberkochen, Germany), if no AFM was performed on the basal cell membranes.

2.5. Instrumentation and operation of the AFM

Images were recorded in PBS buffer in tapping mode with a commercial AFM (BioScope; Digital Instruments, Santa Barbara, CA, USA) and oxide sharpened silicon nitride tips (Digital Instruments) on an inverted fluorescence microscope (Axiovert 135; Zeiss). In tapping mode the tip is oscillated with a high frequency in the vertical direction and is only intermittently in contact with the sample [34]. Fluorescence images were acquired using an oil immersion 63× objective with a cooled CCD camera (Hamamatsu, München, Germany) immediately preceding scanning the sample with the AFM. The resolution on AFM images was determined by measuring the distance between two discernible structures using the software provided by Digital Instruments.

3. Results and discussion

For insight into the function and organization of membrane proteins at the inner side of basal cell membranes, a lysis-squirting protocol to cleave adherent cells was developed for SPM. Epithelial cells tend to differentiate after reaching confluency resulting in the formation of an apical and basal membrane compartment [35–39], where some proteins are no longer equally distributed (e.g. the sodium phosphate transporter type II in MDCK cells (not shown)). To avoid differentiation subconfluent MDCK cells were used for investigations. Living cells, attached firmly to a suitable growth substrate, were incubated and, subsequently, squirted with a hypotonic buffer resulting in their lysis and cleavage, but their basal cell membranes were still attached to the support (Fig. 1a). Transmission electron microscopy before lysis-squirting showed cells that were completely stretched and did not form a continuous sheet, but had already established contacts to each other (not shown). Vesicles interacting with the basal, as well as the apical, membrane and some filaments lining the basal cell membrane could be seen clearly (Fig. 1b). The contact of the basal cell membrane to the substrate was very tight. This is indicated by darker stained areas, possibly focal contacts, in direct interaction with the substrate. After squirting, membranes were labeled with the lipophilic stain PKH-2 to confirm that they were attached to the cover slip and not lost by the strong washing procedure (Fig. 1c). Only a minor fraction of the cells was removed. Brighter areas correspond to either lateral or apical parts of the cell or to membranes of internal cell compartments lying on the basal cell membrane.

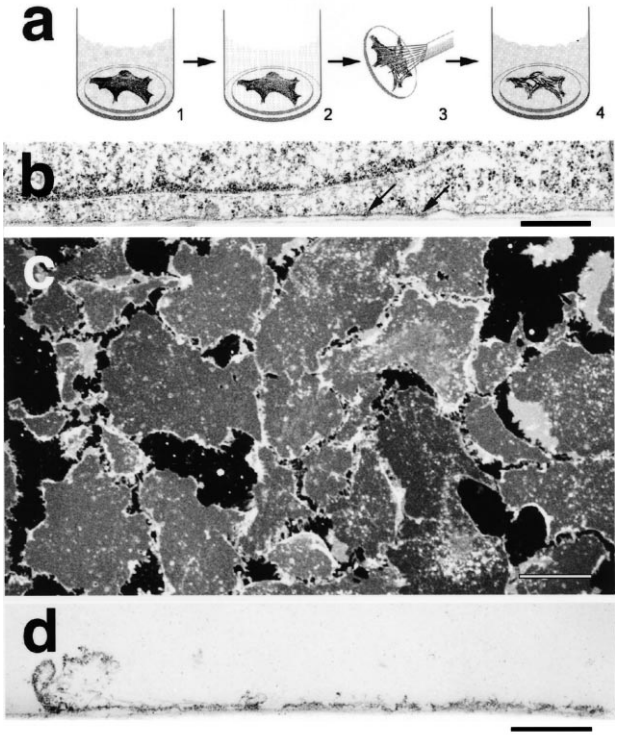

Fig. 1. Preparation of basal cell membranes from MDCK cells by lysis and squirting. a: Cells grown on a solid support were (1) washed with ice cold PIPES buffer, then (2) incubated in hypotonic PIPES buffer, (3) squirted and (4) further processed for atomic force microscopy in buffer solution. b: Ultrastructure of intact MDCK cells before lysis-squirting. Note the focal contacts (arrows) at the basal cell membrane. c: Fluorescence microscopic image of isolated basal cell membranes labeled with the lipophilic stain PKH-2. d: Ultrastructure of a basal cell membrane firmly attached to the support after lysis-squirting. Bars: (b, d) 0.5 μm and (c) 10 μm.

Unstained small discontinuities indicated where the membrane was ruptured.

Supported basal cell membranes can be produced by other methods, such as sandwich-cleaving (pressing a nitrocellulose filter to the surface of cells with the subsequent tearing away of the filter) [25,40]. In our hands, squirting-lysing proved to be a very simple and reliable protocol that results in well attached basal cell membranes suitable for AFM (or for scanning near field microscopy; data not shown) even when using different types of confluent or subconfluent cells (e.g. COS-7 cells, not shown). In addition, it was reported to work with non-adherent cells after appropriate attaching [41–43]. It is important that after squirting the membranes were still in close contact and attached to the substrate as shown by the cross section through a membrane with transmission electron microscopy (Fig. 1d). This is a significant requirement for high resolution imaging with AFM under buffer solutions.

Transmission electron microscopy also indicated that the

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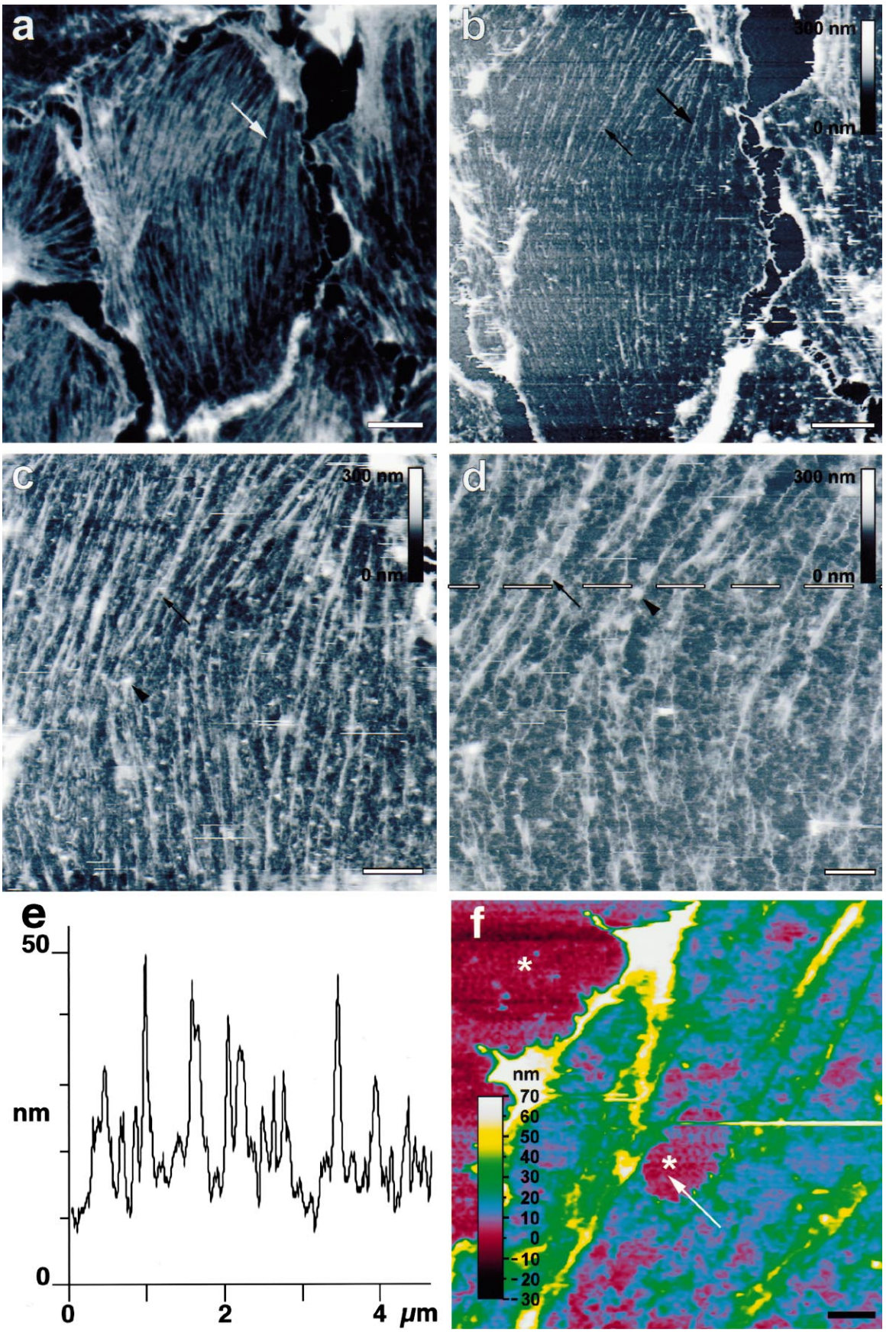
Fig. 2. Fluorescence and atomic force microscopy of basal cell membranes. a: Fluorescence image of rhodamine-phalloidin labeled F-actin. b–d: Sequence of AFM images from the same membrane preparation (a) acquired by tapping mode in solution. The large arrows in a and b indicate a corresponding actin filament. The thin arrows point to the same filament shown in various magnifications in b–d and the arrow head points to globular structures. e: Height profile along the intermittent line in d. f: A false color graphical representation of the height distribution of a membrane. The membrane is practically devoid of cytoplasmic and filamentous structures allowing evaluation of its thickness. Note that the membrane is ruptured (arrow). Glass surfaces are marked with an asterisk. Bars: (a) 6 μm, (b) 5 μm, (c) 2.5 μm and (d) 1 μm, (f) 0.5 μm.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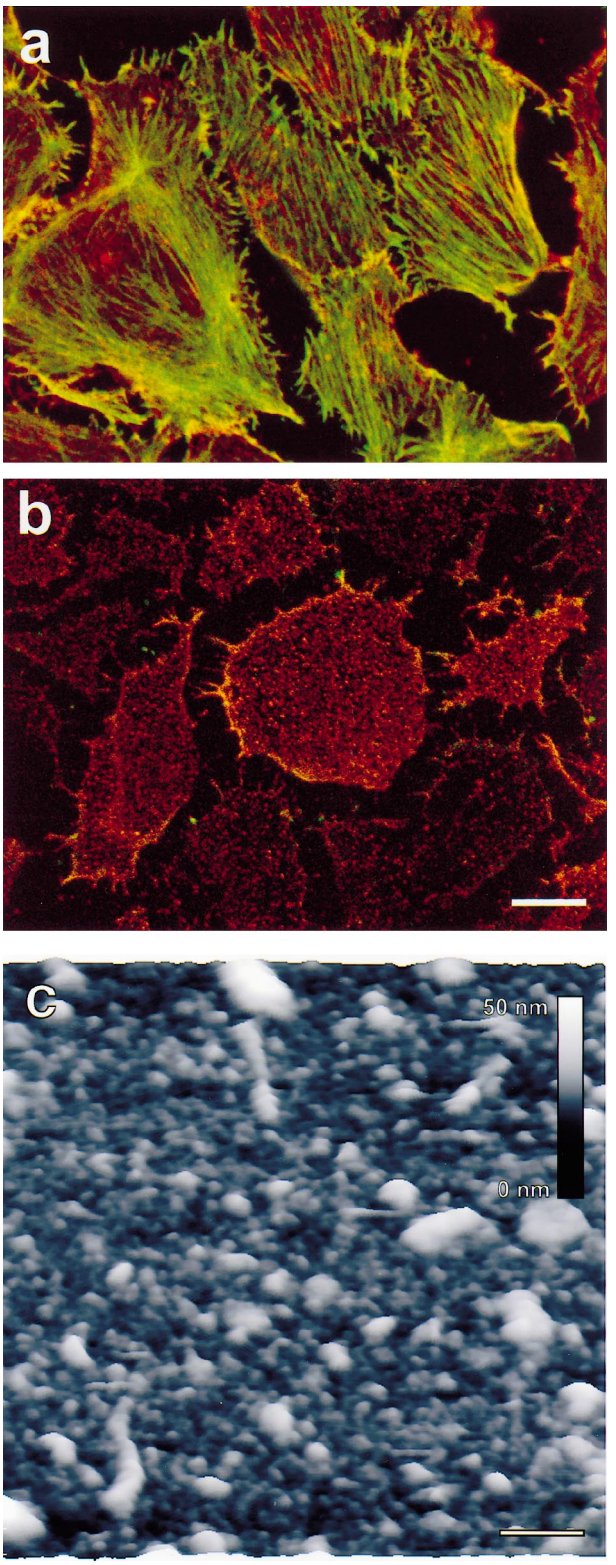

Fig. 3. Basal cell membranes treated with high salt buffer. a, b: Fluorescence microscopy of labeled basal cell membranes before (a) and after (b) high salt buffer incubation: the sodium phosphate transporter type II is colored in red, actin filaments in green. c: AFM image of the cytoplasmic side of a basal cell membrane not labeled with antibodies. Note the almost complete absence of filamentous structures on the membrane. Bar: (a, b) 5 μm and (b) 150 nm.

cytoplasmic side of the membranes was still covered by filaments (Fig. 1d). We could identify actin filaments after fluorochrome conjugated phalloidin labeling of membranes (Fig. 2a, Fig. 3a). These filaments were often very abundant and formed a network covering almost the entire basal cell membrane of MDCK cells. Attached basal cell membranes were tested with AFM to observe actin filaments and integral membrane proteins. Membranes were not processed any further after squirting and fixation except for staining the actin filaments by fluorescently labeled phalloidin. Phalloidin ($MW < 1.5$ kDa) is ideally suited for the simultaneous investigation with light microscopy and AFM, because it increases the diameter of filaments only slightly compared to staining by antibodies (MW 150 kDa). Up to now, structural information of the cytoskeletal filaments lining the cytoplasmic side of cell membranes comes mainly from electron microscopy [25,40,44]. AFM of basal cell membranes under buffer revealed long, filamentous structures which could be perfectly correlated to fluorescently stained actin filaments seen by simultaneous fluoresence microscopy (Fig. 2). Some globular structures were found in areas of the membrane covered with cytoplasmic components. These globular as well as the filamentous structures were generally firmly attached and not removed by subsequently scanning smaller areas to increase the resolution. The topography of the basal cell membrane as seen by AFM is confirmed by Nermut [25] who described a similar morphology of the cytoplasmic side of basal cell membranes. Using tapping mode AFM and not contact mode allowed resolution of these thin filaments. During this study, operating the AFM in contact mode with cantilevers having a nominal force constant of 0.06 N/m regularly resulted in very fuzzy images; filaments and sometimes the whole membrane were swept away and we were never able to see the filamentous network on the membrane. Le Grimellec et al. [45] who were the first to study sandwich-cleaved basal cell membranes with AFM showed low magnification scans of the resulting cell fragments in contact mode under buffer. In contrast to our results, no filaments were observed. Also, the work of Henderson et al. [16] and Chang et al. [46] gives an extra confirmation of our results: they looked at F-actin at the periphery of intact living cells by AFM and found similar filaments such as those we have shown in Fig. 2. Even if structures can be imaged through the apical membrane, their dimensions will be enlarged due to the cell membrane being between the tip and the cytoplasmic structures. In addition, nuclear and cytoplasmic material increase the thickness resulting in a greater softness as well as height fluctuations leading to a reduced resolving power in central regions of the apical membrane. The small filamentous network on the inner surface of the basal cell membranes, organized between the optically identified F-actin (Fig. 2a, d), could only be seen with AFM under buffer after cleavage of intact cells and by operating the AFM in tapping mode to reduce shear forces.

The height of the filaments is very heterogenous due to the presence of filament bundles of varying thickness [44]. Small filaments protruding approx. 7 nm higher than the average height of the membrane could clearly be discerned. The height of small filaments measured with AFM under liquid was 9 nm (9 ± 1.1 nm ($\pm$S.E.M.)) and corresponds to the diameter of actin filaments seen in electron microscopy [25,44]. The height of the supported basal cell membrane itself was in the range of 30 nm (32 ± 5.5 nm) in regions where filaments and probably

accompanying cytoplasmic material were not removed (Fig. 2b). The average thickness of the attached membranes in relation to the glass surface in areas with no peripheral proteins covering the membrane was 8 nm (8 ± 0.7 nm) (Fig. 2f). This is an indication that these membranes are composed of a single membrane layer with a small cleft to the support as shown by Fig. 1e. The thickness of basal or apical cell membranes measured by Le Grimellec et al. [45] and Larmer et al. [20] was 10 nm or 5 nm, respectively. These values are in good agreement with the thickness of lipid bilayers on mica, which had a height of 6 nm under liquid [47].

Many protocols are known to remove peripheral proteins from membrane preparations [26–30]. Incubating the attached membranes with a high salt buffer containing 2 M NaCl, which has been used to remove peripheral membrane proteins, also removes cytoskeletal material, and allowed us to resolve small spherical profiles and ridges on the membrane (Fig. 3). Fluorescence staining showed that actin filaments were considerably less abundant after high salt treatment, often with no filaments found any more (Fig. 3b). Integral membrane proteins, such as the transfected sodium phosphate transporter type II, were not removed and their distribution was identical to control membranes (Fig. 3a, b) or to the basal side of intact, subconfluent cells (not shown). The topography of high salt treated membranes obtained by AFM (Fig. 3c) was almost devoid of long filaments, but globular structures could now be recognized. Spherical structures could be resolved down to 10 nm (11 ± 0.7 nm) diameter. Similar globular structures were found by scanning areas under 1 μm on the basal MDCK membrane in contact mode by Le Grimellec et al. [45]. The shape and size of these structures is similar to protrusions found on the extracellular side of apical MDCK membranes [20]. Due to the convolution of very small structures by the scanning tip [48], the spherical structures may represent proteins having smaller diameters than currently can be resolved using routinely available AFM tips and these structures correspond probably to membrane proteins or protein complexes [49]. The cytoplasmic side of basal cell membranes also has protrusions aligned to ridges running in a direction different from the scan direction (Fig. 3c). These could represent filaments left over after stripping, or could be structures organized by the underlying extracellular matrix and corresponding adhesion molecules or by cytoskeletal components [50]. The high resolution which can be obtained by AFM on supported cell membranes without overlying cytoskeleton should now open the possibility of getting structural information as well as identifying proteins in the membrane by means of appropriate labels (e.g. binding of antibodies), thus providing insights into the organization of membrane proteins in their native environment.

4. Conclusion

In this work we show that squirting adherent cells with hypotonic buffer generates extended areas covered with basal cell membranes firmly attached to a glass surface, which is necessary for the investigation with SPM in physiological buffer solutions. The surface of the attached membranes can be further modified by changing buffer conditions. Highly resolved AFM images of the actin cytoskeleton lining the inside of the cell membrane have been obtained in buffer solution. Therefore, AFM or more generally SPM could become a tool for the analysis of receptor distribution, structure of protein complexes, or processes involved in channel or pore formation, even in native, unfixed basal cell membranes. This technique could very well complement results obtained by other methods. The resolution of protein structures in physiological buffer solutions with SPM opens the possibility of detecting 'life' on the inner side of native unfixed membranes.

Acknowledgements: We like to thank Marina Balzer for her excellent support preparing the cells, Philip Berger for providing COS-7 cells, Frank Zaugg for discussing various aspects of AFM, Miriam Erni for preparing samples for transmission electron microscopy, Heinz Sonderegger and Anja Burse for assistance in preparing figures as well as Jan Loffing and Deborah Stroka for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation, 4036-044059 and the Rudolf und Fridl Buck Stiftung.

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