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Potassium-Selective Atomic Force Microscopy on Ion-Releasing Substrates and Living Cells

Prisca Schär-Zammaretti,*,†,‡,§ Urs Ziegler,§ Ian Forster,|| Peter Groscurth,§ and Ursula E. Spichiger-Keller‡

Center for Chemical Sensors/Biosensors and Bioanalytical Chemistry, Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, CH-8005 Zürich, Switzerland, and Institute of Anatomy and Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

A new method for simultaneous mapping of cell topography and ion fluxes was developed. A highly sensitive ion sensor system was generated by coating atomic force microscopy tips with a PVC layer containing valinomycin, an ionophore for potassium. The activity of specific ions was traced on artificial ion-releasing PVC substrates. A boundary potential was generated owing to the selective exchange of a specific ion between coated tip and ion-releasing substrate. The boundary potential was detectable as a force induced by ion-selective electrostatic interactions. The selectivity coefficient of valinomycin for potassium against sodium ($K^{f}_{K,Na}$) was -2.5 ± 0.5. Potassium efflux was measured on living MDCK-F1 cells expressing BK_{Ca} channels. We could demonstrate localized areas of high potassium concentrations at the cell surface. The potassium efflux could be reversibly inhibited by thapsigargin, which is known to inhibit the efflux of potassium from BK_{Ca} channels by suppression of calcium ATPase.

Ion-selective channels play central roles in living systems. They generate and regulate signal transduction events or maintain the ion balance to mention few examples.[1] To obtain insight into the function of such channels, the determination of their activity as well as their localization provides important information. A number of techniques have been developed to detect ion exchange over biological membranes as well as between cells and cell compartments. A very well-established method is the patch clamp technique.[2,3] Local ion currents through ion channels are detectable, and spatially well-resolved measurements can be performed depending on the size of the patch clamp pipet. In contrast to patch electrodes, ion-selective electrodes have the possibility to detect ions selectively. However, the spatial resolution is usually restricted to the micrometer range. So far, the most successful technique was established by Denuault et al.[4] with scanning electrochemical microscopy (SECM), which allows identifying a single ion exchange by electrochemical detection of molecules based on redox systems and amperometric microtips.[5−8] However, the high noise recorded affects the spatial resolution, which is limited to 800 nm[9] and ∼100 nm for the amperometric SECM.[6] Another approach leading to the detection of ion gradients through channels is to scan with a micropipet, analogous to a patch clamp pipet, over cells.[10] This method allows measurements of ion currents with a resolution in the range of 50 nm (Korchev, personal communication). However, the selective detection of ions is not possible with this procedure.

On the other hand, ions can be selectively detected with atomic force microscopy (AFM) by coating AFM tips with an ion-selective polymer layer.[11] Ion-selective membranes can recognize specific ions. The transduction of the signal given by the recognition can be achieved by potentiometry. Since in physics a force is the derivative of the potential and AFM allows the measurement of interaction forces, it is possible to measure these specific interactions by using chemically selective AFM tips. AFM has several advantages: first, it can be used in physiological buffer solutions, which allows the analysis of living cells,[12,13] and second, the interaction forces between tip and sample as well as the topography can be simultaneously obtained.[14] Furthermore, the resolution is in the nanometer range and measurements are nondestructive to the sample. Recently, the very sensitive detection system of the AFM has been used to generate different AFM-based sensors,[15] e.g., ion conductance measurements.[9]

* To whom correspondence should be addressed. Tel: ++41 21 785 81 46. Fax: ++41 21 785 85 54. E-mail: prisca.zammaretti@rdls.nestle.com.

† Present address: Nestle Research Center, CP 44, Vers chez les Blanc, 1000 Lausanne 26,

‡ Swiss Federal Institute of Technology.

§ Institute of Anatomy, University of Zürich.

|| Institute of Physiology, University of Zürich.

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In contrast to patch clamp or ion-selective electrodes, ion-selective AFM tips offer the possibility to detect ion exchange with high spatial resolution combined with high selectivity and sensitivity to a chosen ion.[11] Ion-selective density measurements are possible by measuring forces generated during the reversible interaction of the detected ion with the ion-selective coating.

In the following, we demonstrate the feasibility of using polymer-coated tips to selectively trace local ion activities. The modified tips were first tested on plasticized ion-releasing PVC versus blank PVC substrates.[11] In the present study, ion-sensitive AFM measurements were performed on living cells. We assessed the potassium efflux with potassium-selective AFM tips in vitro on MDCK-F cells, which were shown to have an oscillating activity of Ca^{2+}-sensitive potassium channels with a conductance of 53 pS.[17] MDCK-F cells are known to express BK_{Ca} channels, which have a high potassium conductance around 250 pS.[16] The combination of high spatial resolution and selectivity to potassium allowed us the observation of a potassium gradient in MDCK-F, which could be reversibly inhibited by the Ca-ATPase inhibitor thapsigargin.

MATERIAL AND METHODS

Substrates. The potassium-releasing substrate (KR) was composed of the following: 1.3 (2 wt %, 2.31×10^{-2} mol kg^{-1}) or 6.5 mg (10 wt %, 10.9×10^{-2} mol kg^{-1}) of potassium tetrakis(4-chlorophenyl)borate (Fluka, Buchs, Switzerland), respectively, 56 mg of PVC (high molecular weight, 100 000; Fluka), 56 mg of 1-nitrophenyl octyl ether (o-NPOE; Fluka), and 2 mL of tetrahydrofuran (THF; Fluka).

The sodium-releasing substrate (NaR) was composed of the following: 1.3 (2 wt %, 2.31×10^{-2} mol kg^{-1}) or 6.5 mg (10 wt %, 10.9×10^{-2} mol kg^{-1}) of sodium tetrakis[3,5-bis(trifluorophenyl)]-borate, respectively, 56 mg of PVC (high molecular weight, 100 000; Fluka), 56 mg of o-NPOE, and 2 mL of THF.

Tip Coating. The potassium-selective membrane coating the tip consisted of the following: 2.3 mg of valinomycin (1.16×10^{-2} mol kg^{-1}; Fluka), 54.7 mg of PVC (high molecular weight, 100 000; Fluka), and 122.9 mg of bis(1−butylpentyl)adipate; Fluka) dissolved in 3 mL of cyclohexanone (Fluka).

The blank membrane consisted of the following: 56 mg of PVC and 120 mg of bis(1-butylpentyl)adipate dissolved in 3 mL of cyclohexanone.

Coating Technique for AFM Tips. Initially, the cantilever was immersed in 3 mM $(HS(CH_2)_{15}COOH$ synthesized according to ref 17 in THF for 10 min to protect its reflecting surface from the coating membrane. Subsequently, the chip was washed with ethanol, followed by 1,4-dioxan. The cantilever and tip were then immersed in 10 μL of the tip coating membrane solution and centrifuged at 160*g*.[7]

Atomic Force Microscope. Force versus distance curves were acquired with a scan rate of 10 Hz using a Bioscope (Digital Instruments, Santa Barbara, CA) with silicon cantilevers (Ultralevers, ThermoMicroscopes, Geneva, Switzerland) or silicon nitride cantilevers (Nanoprobes, Digital Instruments, Santa Barbara, CA), respectively, with a nominal force constant of 0.06 N/m. The force constant of cantilevers after coating was determined according to Gibson et al.[50]

Selectivity Factor. The selectivity factor is a parameter that gives information about the affinity of an ionophore to the target ion in comparison to a competing ion. Knowledge of the selectivity allows estimation of the error that one can have while measuring with a chemically selective tip with decisive concentrations of interfering ions being present. For valinomycin, the target ion is potassium and one of the disturbing ions is, for example, sodium. The equation for the calculation of the selectivity of valinomycin is[25]

$$\text{selectivity (valinomycin)} = \log[\text{force}_{(\text{NaR/KCl})}/\text{force}_{(\text{KR/NaCl})}] \quad (1)$$

Cell Culture. Transformed MDCK-F cells[19] were cultivated in minimum essential medium with Earle's salts supplemented

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with 10% fetal calf serum (FCS), nonessential amino acids, and L-glutamine (MEM, Gibco, Basel, Switzerland) at 37 °C in a humidified atmosphere of air with 5% CO_2.

AFM Experiments on Cells. For AFM measurements, cells were plated on glass slides coated with 100 μg/mL poly(L-lysine) (Sigma, Buchs, Switzerland).[52] The cells were localized by light microscopy and imaged first in contact mode at room temperature immersed in the culture medium described previously. The cultivation medium has an ionic strength of ~151 mM, which is similar to the one used for the experiments done in salt solutions.[11] The scan rate was 1−2.5 Hz in contact mode. Since we found a high concentration of BK_{Ca} channels on the lamellopodium by immunofluorescence and the cells are relatively flat in this region, force volume images with potassium-selective tips were recorded on this area of the cells.

For experiments inhibiting the potassium exchange, 2.7 nM thapsigargin (Alomone Lab, Jerusalem, Israel) was added. Thapsigargin was dissolved in DMSO at 1 mM. In thapsigargin inhibitor experiments, 4 μL of this DMSO stock solution was added directly to the medium. The tip movement allows a quick diffusion of thapsigargin in the medium.

Immunofluorescence. Cells were fixed with 3% formaldehyde in PBS for 15 min. BK_{Ca} channel protein was detected with an anti-BK_{Ca} antibody (Alomone Lab) followed by a Texas red-labeled secondary goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.). For controls, primary antibody absorbed with BK_{Ca} antigen (Alomone Lab) was used. Images were aquired using a confocal laser scanning microscope (TCS SP, Leica, Heidelberg, Germany); experiments were performed at room temperature.

Patch Clamp Electrophysiology Experiments. Patch clamp experiments were performed as described.[20] Pipets were pulled using thin-walled borosilicate glass (Hilgemann) and had tip opening diameters of ~1 μm. Recordings were made using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) interfaced to a laboratory-built PCX-driven interface controlled by DATAC software.[21] Currents were filtered with the internal filter of the 200A unit and sampled at twice the bandwidth. Solutions had the following compositions: (bath solution) 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM Hepes, titrated to pH 7.4 with NaOH; (pipet) 140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, titrated to pH 7.4 with KOH. All experiments were performed at room temperature. After obtaining a gigaseal, cells were voltage clamped to 0 mV.

RESULTS AND DISCUSSION

In contrast to other methods, the use of ion-selective AFM tips allows the detection of ion gradients on living cells simultaneously with topography. In addition, elasticity information can be obtained, which has been shown in the literature,[13,31] and can be applied to a range of different cells including migrating cells[22] or beating myocytes.[23] An important advantage over other ion detection techniques is that ion-selective AFM measurement can be performed in standard physiological buffer solution without adjusting the buffer composition. At the time of writing, we have developed potassium- and sodium-sensitive tips.[25] An advantage of incorporating an ionophore into a polymeric membrane used as a tip coating is the flexibility to use other ionophores and modify the sensitivity of the tip to another target ions. However, the immobilization of an ionophore on the tip surface could compromise its activity[11] and its selectivity, which are directly related to the flexibility in conformational changes of the dye.

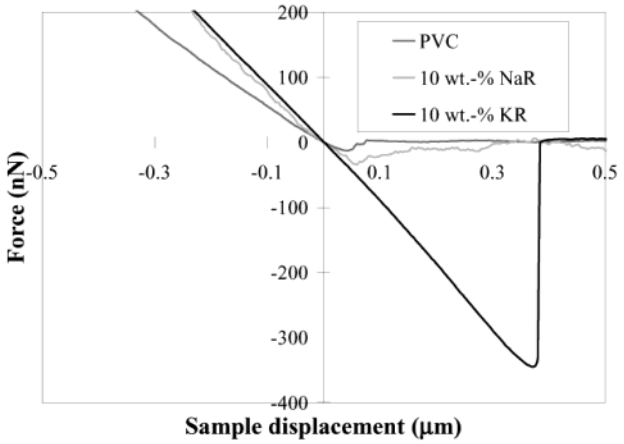

Figure 1. AFM measurements on synthetic substrates. Typical experimental force vs distance curves in an intermittent-contact regime taken on a plasticized PVC substrate with two different lipophilic salts: 10 wt % KTpClPB (KR) and 10 wt % NaR, and the reference PVC substrate without salt. A typical shape was observed during the retraction phase, where the indentation is reduced and the adhesion forces increase with presence of potassium in the substrate.

AFM Measurements on Synthetic Substrates: Background for the Biological Experiments. Artificial substrates were used to test the efficacy in detecting ion gradients on the surface of selective AFM tips. The substrate, in contrast to the coating membrane, has a higher viscosity and contains the ion-exchanger KR for K^+/Na^+ exchange or NaR for Na^+/K^+ exchange. The plasticizer used in the membrane gives the difference between the viscosity of the tip coating material and that of the substrate. The tip coating mixture must be as elastic as possible, to affect only minimally the spring constant of the cantilever. The nominal spring constant of the uncoated tip was of 0.06 N/m, but the spring constant of the coated cantilever was 0.9 ± 0.3 N/m according to the method of Gibson et al.[50] over 20 coated tips. Despite the higher force constants, adhesion forces could be accurately measured (see Figure 1). The selective exchange of a specific ion between coated tip and ion-releasing substrate occurs when the boundary potential is generated. The boundary potential has been found to be detectable as an adhesion force induced by ion-selective Coulomb interactions.[11] The adhesion forces in the retraction phase measured with a valinomycin-coated tip on a substrate of 10 wt % KR were 340 ± 113 nN (Figure 1). On a blank PVC substrate, the adhesion forces were in the range of 9.8 ± 3.3 nN whereas on 10 wt % NaR substrate they were 15.1 ± 4.8 nN. The slight increase of the adhesion forces between the PVC and the 10 wt % NaR substrate may be due to the polarization of the ion-containing substrate layer. Such a polarization is not possible for a blank PVC substrate as shown in refs 11 and 25. The 20−30 times larger adhesion forces measured on a potassium-

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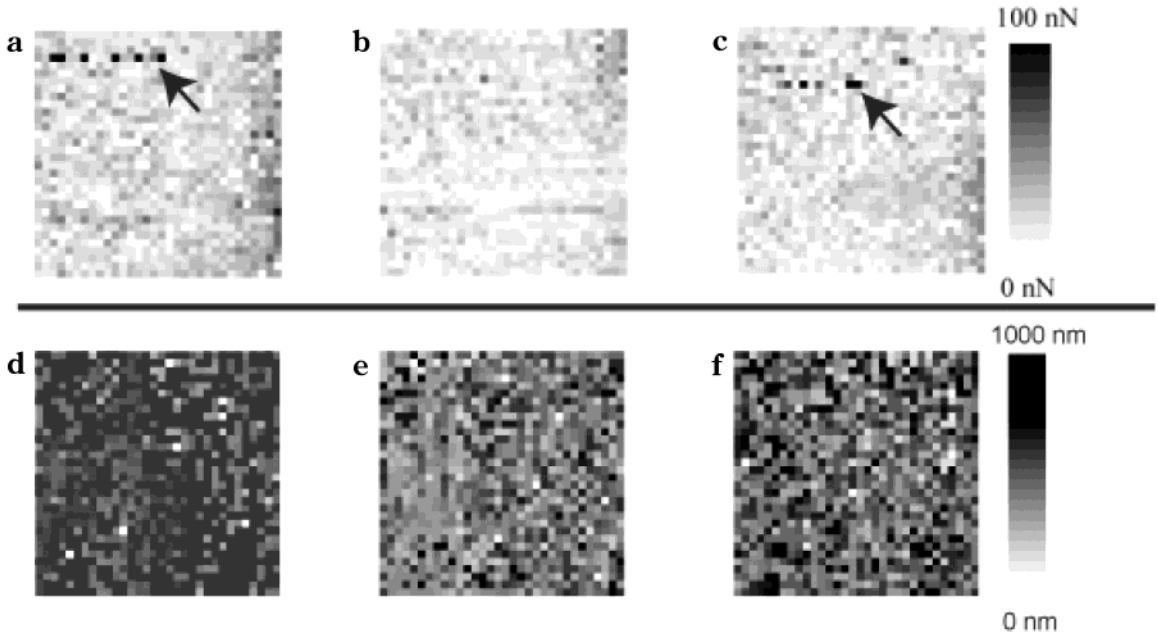

Figure 2. Localization of the BK_{Ca} channels with AFM. The force−volume files are elaborated as adhesion force maps. The arrow points to a localized area with raised adhesion forces (a). The signal was eliminated after the addition of 2.7 nM thapsigargin (b) but reappeared after substitution with fresh medium (c). Similar results were observed in 50 independent experiments. The image size is 3 μm by 3 μm, and the forces are presented in a 256 gray scale, where white corresponds to 0 nN and black to 100 nN. Images d−f show the corresponding topography to images a−c. The height is presented in a 256 gray scale, where white corresponds to 0 nm and black to 100 nm.

containing substrate can be interpreted as specific interaction between valinomycin and potassium. This is also an indication that the activity of the ionophore is not compromised by the incorporation in a polymeric membrane. It has to be remarked that the adhesion forces measured on the sodium-releasing substrate are slightly higher than the one measured on the PVC blank substrate. This could be due to the different surface charge densities of the two substrates, which cause a polarization of the tip with, consequently, an increase of the forces measured, 9.8 ± 3.3 and 15.1 ± 4.8 nN, respectively. Nevertheless, the forces measured on the potassium-releasing substrate are 1 order of magnitude higher compared to the other two substrates, which shows the effect of the selectivity of these modified tips to their specific ion-releasing substrate.

The selectivity in the detection of potassium ions is influenced by interferences with other ions in the buffer solution. The selectivity factor of potassium over sodium for a valinomycin-coated AFM tip has been found to be −2.5 ± 0.5,[11] which fits nicely within the range of −1.9 to −3.6 with comparable membrane compositions.[24]

For accurate ion detection, the nanosensors require a high selectivity as well as retention of the ionophore in the tip coating film. For the detection of ion gradients on cells, it is important that the ionophore does not leach out of the membrane coating the AFM tip. Ionophores can compromise cells and cell membranes.[51] For example, valinomycin can form pores in the cell membrane, which causes osmotic intracellular disequilibria and, consequently, cell death. Therefore, initial experiments studying the loss of valinomycin were carried out with the AFM by adhesion forces. The tip membrane was incubated continuously in salt solution and force versus distance curves were measured over a period of 8 days. These measurements were performed in a 150 mM NaCl solution on a potassium-releasing substrate. The adhesion forces recorded were plotted versus the time. The leaching out of valinomycin from the tip membrane was first detectable, with adhesion force measurements, after 10 h,[25] as a reduction of the force measured. Between 0 and 10 h, the adhesion forces did not decrease, which suggests that valinomycin was not released in the environment. Therefore, we can conclude that during the first 2 h of measurements no detectable amount of valinomycin had leached out of the membrane. Since measurements on cells were carried out during 1 h, due to the integrity of the cells at room temperature, we assumed that no valinomycin was lost from the tip and inserted into the cell membrane. In addition, if small amounts of valinomycin, which might form pores, would be transferred from the tip coating to the cell membrane, a potassium efflux would be detected even if thapsigargin is added (see Figure 2b), which is not the case. Thapsigargin inhibits the Ca-ATPase (see below). Therefore, we can consider that no valinomycin is released into the media or that the amount is insufficient to cause damages to the cell membrane.

Detection of Potassium Channels in MDCK-F Cells by Immunofluorescence and Patch Clamp Experiments. Several potassium channels have been described in MDCK-F cells, including the calcium-activated potassium channel BK_{Ca}. They appear to play a role in the migration of MDCK-F cells.[26] In our study, MDCK-F cells labeled with antibodies to BK_{Ca} for immunofluorescence show strong signals in the cell membrane and also the cytoplasm (Figure 3). The staining of the BK_{Ca} channel confirmed the presence of this channel in MDCK-F cells. Nevertheless, it was not possible to differentiate membrane channels.

Patch clamp experiments could confirm the activity and the presence of potassium channels under similar conditions used for AFM measurements as already shown in ref 27. Cells measured in the cell-attached configuration showed a very high spontaneous

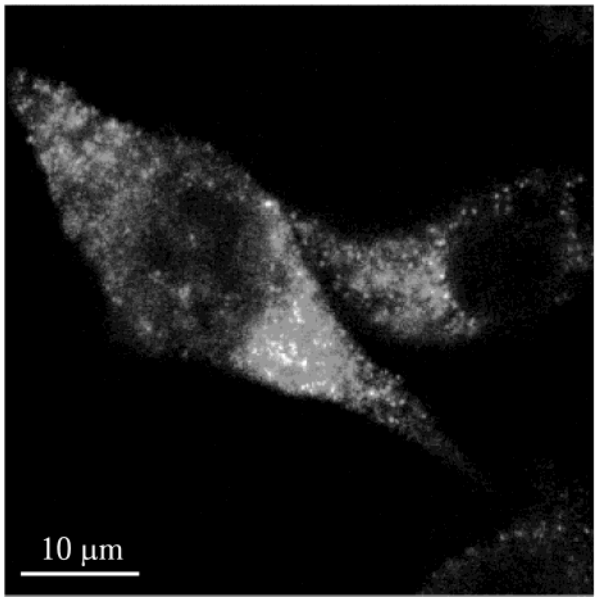

Figure 3. Detection of BK_{Ca} channel in MDCK-F cells by immunofluorescence. Antibody staining of the BK_{Ca} channels in MDCK-F cells.

activity most likely corresponding to the BK_{Ca} channels. These channels can be differentiated from other voltage-gated potassium channels due to their single channel conductance, which is 100−250 pS for the BK_{Ca} channel and ∼50 pS for the others.[43] Due to this difference, BK_{Ca} channels are also called MaxiK channels. The activity was significantly attenuated by the Ca-ATPase inhibitor thapsigargin as shown in ref 27 and repeated for confirmation (data not shown).

AFM Experiments with MDCK-F1 Cells. Elasticity and topography measurements of living cells with AFM have become standard techniques during the last years.[28−40] Reliable detection of ion gradients on cells requires the integrity of the cell membrane. Therefore, it is important that the tip does not penetrate the cell membrane during scanning. It has been shown that, during contact imaging, the tip does not damage the cell if the force is kept at minimum values.[41] Forces can be mapped with AFM by acquiring arrays of force curves. By controlling the maximal force applied to a cell during each force curve cycle the cells remain viable over extended time periods.[13] In our experiments, the cells were analyzed only for less than 1 h. During all measurements, the motility and morphology of the cells were conserved and consequently no degeneration of the cells were observed.

The detection of the potassium efflux was carried out on 3 × 3 μm^2 arrays on the lamellipodium, where the adhesion forces were measured using a potassium-selective tip. Figure 2 shows arrays of 32 by 32 force versus distance curves, where the adhesion force (a−c) and the topography (d−f) are plotted. The adhesion force values (a−c) are represented in a gray scale, where black means high adhesion and white no adhesion. The black areas show a local increase in the adhesion forces caused by an increased potassium concentration. The change in intensity of the adhesion forces and, consequently, the concentration can be related to a potassium efflux. The adhesion forces (signal) in these areas was 3−10 times higher than the forces recorded in the surrounding areas (noise). The potassium efflux is derived from the adhesion force maps; consequently, as shown in the experiments carried out on synthetic substrates, a higher adhesion force represents a higher potassium concentration at the boundary. If the signal-to-noise ratio (S/N) is greater than 1, we can conclude a higher potassium concentration was found in this area, which can be correlated with the presence of active potassium channels. The topography of the cells, which was measured simultaneously over the examined area, did not change (Figure 2d−f), indicating that the increase in the adhesion forces was not mediated by local alteration of the cell surface. In all cases studied, the addition of 2.7 nM thapsigargin to the culture medium abolished high adhesion peaks (Figure 2b). It is known that thapsigargin does not change the viability of cells.[41] This was confirmed by washing out thapsigargin, which brought back the high adhesion peaks (Figure 2c). The peaks did not remain stationary but were shifted over time because the cells were highly motile during the AFM measurements. MDCK-F cells are adherent cells. Poly(L-lysine) increases the adhesion of the cells to the substrate.[52] Nevertheless, this does not prevent the cell from moving on the substrate. The velocity of MDCK-F cells measured with contact-mode AFM imaging at room temperature was ∼0.11 μm/min (data not shown). The acquisition of an array of 32 × 32 force versus distance curves lasts ∼3 min resulting in a shift in subsequent arrays. The peaks seen in the adhesion maps probably correspond to potassium efflux from a single or several channels, which shifted due to the movement of the cell. The high adhesion peaks are recorded in a line, which is caused by the direction of the tip motion.

Figure 4 shows the adhesion force measurements over an area of 3 × 3 μm^2 on the lamellipodium as a function of time. The time can be calculated knowing that the acquisition of a force versus distance curve lasts ∼170 ms. After 3 min of measurements, 2.7 nM thapsigargin was added to the culture medium. The peak disappeared 6 min after the measurements were started. The cell medium was then regenerated and the high adhesion force peak reappeared. From Figure 4, the signal-to-noise ratio can be estimated as 3/1 for the first peak and 20/1 for the second peak, illustrating the highly sensitive detection method. The peak intensity is given by the number of potassium ions released from a potassium channel, which corresponds to 100−250 pS, in the case of the BK_{Ca} channel.[43] Thapsigargin, a Ca-ATPase inhibitor was shown to completely abolish the oscillation of the channel by changing the cytoplasmatic calcium concentration without changing the pH.[44,46] From patch clamp experiments, we know that cells show a very fast reaction to thapsigargin within 2−5 s after addition[27,44,46] by changing the calcium concentration in the cell.

Specific inhibitors such as charibdotoxin and iberiotoxin for potassium channels (data not shown) had no effect in our AFM experiments, possibly due to the very short interaction time with the channels of ∼10 ms.[47−49] This is too short to be detected by standard AFM techniques, because the acquisition of one force versus distance curve takes ∼170 ms using the force volume imaging method.

In conclusion, we consider that the potassium efflux corresponds to potassium channels present on the cells, which can be demonstrated by the application of thapsigargin, which modulates this channel by altering internal Ca^{2+}. The regeneration of the

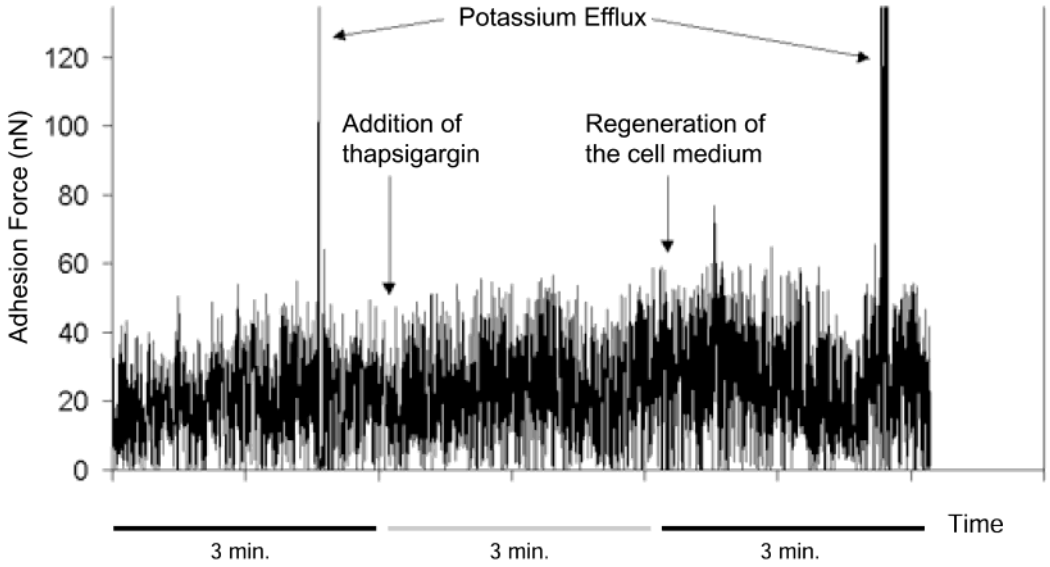

Figure 4. Time-course measurements of adhesion forces measured with a potassium-selective tip. Adhesion force measurements over an area of 3 by 3 μm^2 are displayed as a function of time. The signal given by the BK_{Ca} channel was eliminated by the addition of a 2.7 nM solution of thapsigargin in cell medium. When the environment solution was substituted with fresh cell medium, the signal of the channel reappeared. The graph was taken as a sum of three force volume images elaborated for the calculation of the adhesion force (*y*-axis) vs time (*x*-axis).

activity by substituting the culture medium containing thapsigargin with fresh cell medium proved the specific detection of potassium efflux from a living cell.

With the force array method, the resolution of the image is given by the number of force versus distance curves collected per image size. In our case, we acquired images of $3 \times 3\ \mu m^2$ with 32 by 32 force versus distance curves in order to be fast enough to follow the potassium efflux. Therefore, the digital resolution in these images is given by the pixel size, which is 97 nm and much bigger than the piezohysteresis reported for this microscope. The resolution can be improved by collecting more force versus distance curves on the same surface area ($3 \times 3\ \mu m^2$) to less than 50 nm or by scanning smaller scan sizes resulting in a resolution in the range of the diameter of the BK_{Ca} channel, which is ~50 nm.[43] Nevertheless, it is very difficult to obtain a resolution in this range on living cells.

Compared to other available techniques such as patch clamp and SECM, our technique seems to be more powerful regarding resolution. Furthermore, for patch clamp measurements, the environmental solution must be free of potassium to detect potassium channels. Nevertheless, patch clamp is more sensitive for measuring ion currents through channels. Ion detection using AFM is, currently, semiquantitative and the amount of ion measured through a channel is related to the diffusion of the ions through the tip coating polymeric membrane. Similar to the detection of ions with AFM, SECM allows collection of data on both the topography and the electrochemistry of the surface; however, the resolution achievable until now is lower than with chemically selective AFM tips. It is expected that further developments in the field of AFM techniques will offer much faster measurements in the future, allowing the investigation of faster processes or the mapping of ion gradients with a higher resolution.

In future developments, this technique could be used for the quantification of the potassium efflux from ion channels. To achieve these results, each coated tip has to be calibrated. During the first 2 h of measurements in liquid, the adhesion forces recorded onto potassium-releasing substrates are stable and the selective tip can be calibrated and applied for measurements. A calibration substrate releasing different potassium concentrations, like the PVC substrates shown in this work, might be used. After obtaining different force versus distance curves on these substrates, a calibration curve can be drawn as potassium concentration in the recorded substrate versus adhesion force. All results have to be related to the calibration curve, to obtain an absolute potassium concentration map. Nevertheless, several limitations such as, for example, constant ionic strength of the media and its pH have to be taken into account.

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