pH Nanoenvironment at the Surface of Single Melanoma Cells

Christian Stock*, Markus Mueller*, Hermann Kraehling, Sabine Mally, Josette Noël¹, Claudia Eder² and Albrecht Schwab

Institute of Physiology II, University of Münster, ¹Département de Physiologie, Université de Montréal,

²Institute of Physiology, Medical Faculty Charité, Berlin, *contributed equally to this paper

Key Words

Adhesion • Cell/matrix interaction • H⁺ gradient • Migration • NHE1

environment that promotes cell migration by facilitating cell adhesion at their front and the release of cell/matrix contacts at their rear part.

Abstract

Extracellular pH and the Na⁺/H⁺ exchanger (NHE1) modulate tumor cell migration. Yet, the pH nanoenvironment at the outer surface of the cell membrane (pH_{em}) where cell/matrix interaction occurs and matrix metalloproteinases work was never measured. We present a method to measure this pH nanoenvironment using proton-sensitive dyes to label the outer leaflet of the plasma membrane or the glycocalyx of human melanoma cells. Polarized cells generate an extracellular proton gradient at their surface that increases from the rear end to the leading edge of the lamellipodium along the direction of movement. This gradient collapses upon NHE1 inhibition by HOE642. NHE1 stimulation by intracellular acidification increases the difference in pH_{em} between the tips of lamellipodia and the cell body in a Na⁺ dependent way. Thus, cells create a pH nano-

Copyright © 2007 S. Karger AG, Basel

Introduction

Besides driving the formation and protrusion of tumor cell lamellipodia by the interaction of actin with glycolytic enzymes [1], the predominantly glycolytic metabolism of tumor cells [2] produces protons that need to be removed from the cytosol. This task is performed by transport proteins such as the Na⁺/H⁺ exchanger NHE1 and causes an acidification of the extracellular space [3]. NHE1 activity and acidic extracellular pH (pH_e) correlate with malignancy. NHE1 activation enhances the invasiveness of human breast carcinoma cells [4], and an acidic pH_e promotes experimental pulmonary metastasis of human melanoma cells [5].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1015-8987/07/0205-0679\$23.50/0

Accessible online at:
www.karger.com/cpb

Christian Stock
Institute of Physiology II, University of Muenster
Robert-Koch-Str. 27b, 48149 Muenster (Germany)
Tel. +49-251-8358155, Fax +49-251-8355331
E-Mail cmstock@uni-muenster.de

NHE1 is not distributed homogeneously in the plasma membrane but accumulates along the border of lamellipodia and near the edges of finer processes [6, 7] where it is part of focal adhesion contacts [8]. Integrins are other components of focal adhesion contacts [9]. They interact with components of the extracellular matrix (ECM) such as collagens, laminins or fibronectin [10, 11]. Focal adhesion contacts transduce the mechanical force from the cytoskeleton onto the ECM [12]. A coordinated formation and release of the integrin-mediated adhesions is a limiting process during cell migration [13].

One mechanism controlling cell adhesion depends on pH_e and involves NHE1 activity as well as $\alpha_2\beta_1$ integrins. pH_e influences the strength of cell adhesion and thus affects cell morphology and migration on a collagen I matrix [14]. A low pH_e or a high NHE1 activity lead to a tight adhesion and eventually to a decrease in cell migration, whereas an extracellular alkalosis or NHE1 inhibition prevent adhesion and migration [15]. As NHE1 and integrins are often co-localized at the leading edges of lamellipodia and within focal adhesion complexes [8, 16], the question arises whether NHE1 creates a pH nanoenvironment in close vicinity to integrin molecules, which may then stabilize the integrin/matrix bond. We therefore measured the extracellular pH (i) right at the outer surface of the plasma membrane and (ii) in the glycocalyx, i.e. within the approximately first 50 nm from the outer leaflet of the plasma membrane [17]. The cell/matrix interactions are presumed to occur within this space because the integrins' binding sites for ECM proteins stick out of the plasma membrane by about 20 nm [18, 19].

Materials and Methods

Cells and cell culture

The melanoma cell line MV3 [20] was grown in bicarbonate-buffered RPMI 1640 (Sigma, Taufkirchen, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂.

Experimental solutions

We used the same solutions as in our previous study on pH- and NHE1-dependent melanoma cell migration [14]. Since there is no difference between cell migration in Hepes-buffered and in HCO₃⁻-buffered media [14, 21, 22] only Hepes was used in the present study. Measurements were performed using Hepes-buffered Ringer solutions containing (mmol l⁻¹): 122.5 NaCl, 5.4 KCl, 0.8 MgCl₂, 1.2 CaCl₂, 1.0 NaH₂PO₄·2H₂O, 5.0 glucose, 10 Hepes. pH was adjusted by adding 1 M NaOH.

When applicable the NHE1 inhibitor HOE642 (10 µmol l⁻¹; Sanofi Aventis Pharma, Frankfurt, Germany) was added or cells

were acidified replacing 15 mmol l⁻¹ NaCl by 15 mmol l⁻¹ sodium propionate [14].

Phosphate-buffered saline (PBS) solution contained (mmol l⁻¹): 137 NaCl, 2.7 KCl, 8.1 Na₂HPO₄ and 1.5 KH₂PO₄. PBS was used as washing buffer and solvent for the secondary antibody. The primary antibody was diluted in PBS containing 2% BSA (w/v) and 0.2% (w/v) gelatine.

Immunofluorescence

Cells were treated with cold 1% Triton X-100 in PBS for 10 minutes prior to fixation. Without this permeabilization step NHE1 could hardly be labeled as the epitope was not accessible enough to the antibody. Cells were then fixed by 3.5% paraformaldehyde in PBS. Non-specific binding sites were blocked with 2% BSA (w/v) and 0.2% (w/v) gelatine in PBS. After staining the cells with the N2 polyclonal antibody to the 157 terminal amino acids of the NHE1 [7,23] for one hour (titer 1:250) and a Cy3-conjugated IgG for another hour (titer 1:400), they were washed in PBS and then covered with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Images were taken using an inverted microscope (Axiovert 200, Zeiss, Göttingen, Germany), a digital camera (Model 9.0, RT-SE-Spot, Visitron Systems, Puchheim, Germany) and the MetaVue software.

Measuring pH at the outer leaflet of the plasma membrane

Cells were resuspended in Hepes-buffered, serum-free RPMI 1640 (pH 7.0) containing 15 mmol l⁻¹ sodium propionate where applicable. They were plated onto collagen I-coated coverslips, allowed to adapt for 3 hours and then treated with a fluorescein-conjugated pH indicator for 15 to 30 minutes (Figs. 1A, B). The outer leaflet of the plasma membrane was labeled with 1 µg ml⁻¹ DHPE (1,2-dihexaeanoyl-sn-glycero-3-phosphoethanolamine, Molecular Probes, OR, USA) in 0.1% dimethylformamide (v/v) in serum-free RPMI 1640 [24]. The glycocalyx was labeled with 12.5 µg ml⁻¹ wheat germ agglutinin (WGA, Molecular Probes) in serum-free RPMI 1640 [25].

pH was measured using video imaging techniques. The coverslips were placed on the stage of an inverted microscope (Axiovert 200; Carl Zeiss, Inc.) and continuously superfused with prewarmed (37°C) Hepes-buffered Ringer solutions of various pH values containing 10 µmol l⁻¹ HOE642 and/or 15 mmol l⁻¹ sodium propionate where applicable. The excitation wavelength alternated between 440 nm and 490 nm while the emitted fluorescence intensities were monitored at 500 nm using a Photometrics camera (CoolSnapfx, Visitron Systems). A ratio was calculated from the intensities measured at 440 and 490 nm. Thus, the measurement was virtually independent of the amount of dye excited in a given region of interest and represented the local proton concentration. Filter change and data acquisition were controlled by Metafluor software (Visitron Systems). Fluorescence intensities were measured in 35 s intervals and corrected for background fluorescence by subtracting background intensities of control regions placed right next to the measured regions of interest. At the end of each experiment, the pH measurements were calibrated by successively superfusing the MV3 cells with modified Ringer

Fig. 1. Measuring pH at the cell surface. (A) Schematic model. The two fluorescein conjugates DHPE and WGA were used to measure pH either at the outer leaflet of the plasma membrane or in the glycocalyx, respectively. (B) This cell was labeled with WGA. Each of the four segments of interest is represented by a numeral (1-4; front, middle front, middle back, back). (C) Diagram of a cell with defined regions of interest. The cell is divided into four equal segments. Each segment contains four defined regions of interest (ovals and circles on the plasma membrane). The measured values of the four regions were combined in one single value representing one segment (see (B)).

solutions of pH 7.5, 7.0 and 6.5 containing (mmol l⁻¹): 125 KCl, 1 MgCl₂, 1 CaCl₂, 20 Hepes, and 10 μmol l⁻¹ nigericin (Sigma-Aldrich) [26].

To detect a pH_{em} gradient along the direction of movement cells were divided into four segments of about the same size (front, middle front, middle back, back; Fig. 1C). Each segment contained four defined regions of interest placed over the plasma membrane or the glycocalyx. The measured values of the four regions were combined in one single value representing one segment.

Fig. 2. Human melanoma cells (MV3) establish an extracellular pH (pH_e) gradient at their cell surface. A. Immunofluorescence of the NHE1 in an MV3 cell. NHE1 accumulates at the edge of the lamellipodium (asterisk). B. pH_{em} is lower at the cell front than at the rear end. The gradient is steeper at the cell membrane (DHPE) than in the glycocalyx (WGA). C. The gradient weakens as the Ringer pH of the surrounding bulk solution (pH_e) decreases. pH_{em} was measured when a steady state was reached after 7 to 10 minutes. The plasma membrane was labeled with DHPE (closed squares, *N* = 5 cells), the glycocalyx with WGA (open circles, *N* = 8 cells). Each MV3 cell was exposed to the two indicated pH_e values.

HOE642-induced changes are presented as the difference between pH_{em} before and pH_{em} after removal of HOE642 (ΔpH).

Statistics

Experiments were repeated three to six times. Data are presented as the mean values obtained from the combined value (consisting of four single regions) ± s.e.m. and were tested for significance employing Student's unpaired *t* test or paired *t* test where applicable. The level of significance was set at *p* < 0.05.

Fig. 3. The Na^+/H^+ exchanger NHE1 is more active at the leading edge than at the rear end of melanoma cells. In MV3 cells exposed to the NHE1 inhibitor HOE642 ($10 \mu\text{mol l}^{-1}$) for 15–30 minutes at a Ringer pH (pH_e) of 7.0 removal of HOE642 leads to a decrease in the cell surface pH (pH_em). Changes in pH_em induced by the removal of HOE642 (ΔpH) were more distinct (i) at the leading edge than at the rear end and (ii) right at the plasma membrane than in the glycocalyx. pH_em was measured 5–10 minutes after removal of HOE642 when a steady state was reached. The plasma membrane was labeled with DHPE (closed squares, $N = 5$ cells), the glycocalyx with WGA (open circles, $N = 7$ cells).

Results and Discussion

NHE1 is distributed inhomogeneously in the plasma membrane of MV3 cells and clusters along the border of lamellipodia (Fig. 2A). Both the inhomogeneous distribution of NHE1 and its predominant localization at sites of focal adhesions as found by Plopper *et al* [16] suggest that localized intracellular pH (pH_i) gradients might be critical for the assembly of focal adhesions [27]. However, localized pH_i gradients seem to be difficult to detect. Though in paired myocytes the generation of a local acidosis leads to an establishment of a longitudinal pH_i gradient from one to the neighbored cell mediated by cell-to-cell H^+ movement through connexin channels at gap junctions [28], and spectral imaging microscopy of pH_i in endothelial cells located at the margin of a set wound suggests that the cytosol is more alkaline at the leading than at the lagging edge [29]. But neither in single Chinese hamster fibroblasts nor in single transformed Madin-Darby canine kidney cells pH_i gradients were detected [8, 21]. We therefore examined whether there is a pH gradient in close vicinity to the outer leaflet of the plasma membrane by using proton-sensitive dyes to label either the outer leaflet of the plasma membrane (DHPE) or the glycocalyx (WGA). The pH at the plasma

membrane (pH_em) was lower at the leading edge of the lamellipodium than at the rear end of polarized melanoma cells (Fig. 2B). This difference in pH_em was higher right at the plasma membrane than in the glycocalyx. In a Ringer solution of pH 7.2 (in the following the pH of the bulk solution is referred to as Ringer pH) the difference in pH_em at the plasma membrane between the leading edge ($\text{pH } 7.46 \pm 0.06$) and the rear end ($\text{pH } 7.66 \pm 0.08$) was about 0.2 pH units ($N=5$ cells, $p=0.02$). In the glycocalyx the difference in pH_em between the leading edge ($\text{pH } 7.41 \pm 0.02$) and the rear end ($\text{pH } 7.5 \pm 0.02$) was about 0.09 pH units ($N=7$, $p=3 \times 10^{-4}$). The gradient disappeared when the Ringer pH was reduced to 6.55 (Fig. 2C). The difference in pH_em between the leading edge ($\text{pH } 6.73 \pm 0.06$) and the rear end ($\text{pH } 6.81 \pm 0.06$) of DHPE-labeled cells came to only 0.08 pH units ($N=5$, $p=0.34$). The elevated extracellular proton load at Ringer pH 6.55 leads to an acidification of the cytosol [14] which then might activate additional proton export mechanisms at the rear end accompanied by the leveling of the gradient. Alternatively, the acidic surrounding solution may reduce the driving force for NHE1-mediated proton efflux at the cell front. We conclude that the cell is surrounded by a local pH nanoenvironment exactly where integrins and ECM proteins interact. This pH nanoenvironment is regulated by the cell itself but at the same time depends on the pH of the bulk solution.

To figure out the role NHE1 plays in the generation of the pH gradient cells were treated with $10 \mu\text{mol l}^{-1}$ of the specific NHE1 inhibitor HOE642 (cariporide) for 15–30 minutes at a constant Ringer pH of 7.0. As soon as HOE642 was washed out NHE1 resumed its activity and pH_em at the cell surface decreased (Fig. 3). The decrease in pH_em was much higher at the leading edge than at the rear end and higher at the membrane than in the glycocalyx. pH_em at the plasma membrane decreased by 0.12 ± 0.02 and 0.04 ± 0.01 pH units at the leading edge and the rear end, respectively ($N=6$). The corresponding values for the glycocalyx were 0.05 ± 0.01 and 0.01 ± 0.01 pH units at the leading edge and the rear end, respectively ($N=7$). The measured acidification of pH_em upon removal of HOE642 verifies that DHPE was incorporated into the outer leaflet of the plasma membrane. Moreover, these results indicate that NHE1 (i) contributes considerably to the generation of the surface pH (pH_em) and (ii) is more active at the leading edge than at the rear end. The latter observation provides a direct functional confirmation of previous studies that showed the accumulation of NHE1 at the leading edge of lamellipodia [6–8, 16, 21]. The notion that NHE1 is the main generator of the pH gradient is

Fig. 4. Stimulation of NHE1 activity accentuates the pH_{em} gradient at the outer cell surface. In MV3 cells acidified with 15 mmol l⁻¹ propionate for 3 hours and labeled with WGA (A) pH_{em} at the tips of cell protrusions (gray bars in B) is more sensitive to the NHE1 inhibitor HOE642 than pH_{em} at the cell body (black bars in B). *N* = 5 cells with 5–10 protrusions each.

further supported by the finding that in the presence of HOE642 a pH gradient along the direction of movement could not be detected in the glycocalyx (pH_{em} 7.2±0.03 at the leading edge versus 7.15±0.02 at the rear end, *N*=7, *p*=0.29). At the plasma membrane the gradient was even reversed (pH_{em} 7.33±0.03 at the leading edge versus 7.26±0.01 at the rear end, *N*=6, *p*=0.017) highlighting the efficacy of the localized NHE1 activity at the cell front.

We then investigated the long-term stimulation of NHE1 by intracellular acidification with propionic acid. At a constant Ringer pH of 7.2 stimulation of NHE1 leads to the formation of numerous, thin lamellipodia which are very adhesive and frequently shed during cell migration

[14]. Fig. 4 shows that in propionate-treated cells the pH_{em} gradient between the tips of lamellipodia and the cell body was even more pronounced than the gradient between the leading edge and the rear end of a polarized cell. The gradient doubled and reached the same magnitude in the glycocalyx as it reached at the plasma membrane of untreated cells. Subsequent treatment with HOE642 for only 5–10 min caused the gradient to disappear: pH_{em} at the tips of cell protrusions rose from pH 7.04±0.03 to pH 7.20±0.04 (*N*=5 cells, *p*<0.01) whereas at the cell body pH_{em} did not change (pH 7.2±0.01 versus pH 7.22±0.01; *N*=5 cells, *p*=0.95). The same effect was observed when NHE1 was inhibited by removal of extracellular Na⁺ (replaced by choline): pH_{em} at the tips of cell protrusions rose also by 0.22 pH units (*N*=4 cells, *p*<10⁻¹¹) whereas at the cell body pH_{em} did not change significantly (*N*=4 cells, *p*=0.25). The changes in pH_{em} clearly depended on Na⁺ and were reversible. They indicate a higher NHE1 activity at the tips of cell protrusions than at the cell body. Remarkably, pH_{em} at the tips of lamellipodia of propionate-loaded cells was almost half a pH unit lower than that of untreated melanoma cells and was also lower than the pH of the surrounding bulk solution. This could account for the observed adhesiveness of these protrusions [14].

Our data suggest that NHE1 is crucial for the generation of the proton gradient along the direction of movement of migrating cells. There is no doubt that NHE1 is necessary for cell migration and also contributes to cell polarity [30, 31]. But what is the impact of the proton gradient on cell migration? It was shown *in vitro* [32] that the interaction between α₂β₁ integrins and collagen I or fibronectin depends on pH and that a ΔpH of 0.5 can cause a significant difference in the integrin-matrix bond. Migration of the melanoma cells used in the present study relies on α₂β₁ integrins and is modulated by the extracellular pH [14]: in surrounding solutions of pH 6.6 and 7.5 cells hardly migrate which correlates with rather tight and weak adhesion, respectively. Furthermore NHE1-inhibition or knock out lead to a significant reduction in cell migration [14, 22]. We therefore suggest that the pH_{em} gradient is physiologically relevant. The higher proton concentration at the leading edge of migrating cells promotes the establishment of new cell/matrix contacts and their subsequent stabilization. At the same time the release of cell/matrix contacts at the rear end is facilitated by a locally decreased proton concentration. That way NHE1 may contribute to cell polarization required for migration. In this regard the important finding of the present study is the NHE1-dependent extracellular nanoenvironment in a space where integrins and matrix

Fig. I. A. Exemplary calibration curves of WGA (squares), DHPE (circles) and BCECF (triangles). B. Mean ratios for calibrations of DHPE-labeled regions from a single cell at pH 7.5 (line), pH 7.0 (dotted line) and pH 6.5 (dashed line): There is no statistically significant difference between the ratios determined for the four segments from the leading edge to the rear end (four regions per segment).

proteins interact and where pH-dependent ECM-degrading matrix metalloproteinases (MMPs) [33, 34] do their work. Cells may generate and propel a proton cloud at their leading edges or at the tips of their invadopodia in order to break down the matrix first before grabbing a firm hold of the remaining matrix and migrating. Nevertheless, the role NHE1 plays in cell migration is not limited to the generation of the proton gradient at the outer cell surface but also includes its interactions with the cytoskeleton and its functions in volume regulation [15]. All three NHE1-related processes act in concert promoting the movement of the cells.

Supplementary Material

Validation of DHPE, WGA and BCECF as H^+ sensitive dyes

The pK_a value of free fluorescein is 6.4 and those for DHPE, WGA and BCECF range from 6.2 (DHPE) to 7.0 (BCECF). We checked both the linearity and the slope of each calibration for each measurement. Exemplary calibration curves (Fig. I) show that the three dyes are appropriate for the measured pH range.

DHPE is incorporated into the outer leaflet the plasma membrane

To make sure that by far most of the DHPE remains in the outer leaflet of the plasma membrane and does not

Fig. II. Removal of the NHE1 inhibitor HOE642 leads to an increase in intracellular pH as indicated by BCECF (open circles; $N=17$ cells from 5 trials) and to a decrease in pH at the outer cell surface as indicated by DHPE (closed diamonds; N =leading edges from 5 cells).

switch over to the cytosolic leaflet by flip flop we compared pH_{em} (DHPE) with pH_i (BCECF) using the same experimental protocol: removal of the NHE1 inhibitor HOE642. Removal of HOE642 leads to opposite effects on pH_{em} and pH_i (Fig. II). pH_{em} decreases while pH_i increases. The decrease of pH_{em} shows unequivocally

that DHPE is present in the outer leaflet. If DHPE was partially incorporated into the inner leaflet of the membrane the actual, masked acidification of the cell surface upon removal of the NHE1 inhibitor HOE642 would be even more significant.

Acknowledgements

Thanks go to Drs. H-J Lang and J Pünter at Sanofi Aventis who kindly provided us with HOE642. This work was supported by the fund "Innovative Medical Research" of the University of Münster Medical School (ST 2 1 06 01) and by the Deutsche Forschungsgemeinschaft (SCHW 407/10-1).

References

- Nguyen TN, Wang HJ, Zalzal S, Nanci A, Nabi IR: Purification and characterization of beta-actin-rich tumor cell pseudopodia: role of glycolysis. *Exp Cell Res* 2000;258:171-183.
- Helmlinger G, Sckell A, Dellian M, Forbes NS, Jain RK: Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism. *Clin Cancer Res* 2002;8:1284-1291.
- Tannock IF, Rotin D: Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* 1989;49:4373-4384.
- Reshkin SJ, Bellizzi A, Albarani V, Guerra L, Tommasino M, Paradiso A, Casavola V: Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell Na⁺/H⁺ exchange, motility, and invasion induced by serum deprivation. *J Biol Chem* 2000;275:5361-5369.
- Rofstad EK, Mathiesen B, Kindem K, Galappathi K: Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 2006;66:6699-6707.
- Denker SP, Huang DC, Orlowski J, Furthmayr H, Barber DL: Direct binding of the Na⁺-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H⁺ translocation. *Mol Cell* 2000;6:1425-1436.
- Lagana A, Vadhais J, Le PU, Nguyen TN, Laprade R, Nabi IR, Noel J: Regulation of the formation of tumor cell pseudopodia by the Na⁺/H⁺ exchanger NHE1. *J Cell Sci* 2000;113 (Pt 20):3649-3662.
- Grinstein S, Woodside M, Waddell TK, Downey GP, Orlowski J, Pouyssegur J, Wong DC, Foscett JK: Focal localization of the NHE-1 isoform of the Na⁺/H⁺ antiporter: assessment of effects on intracellular pH. *Embo J* 1993;12:5209-5218.
- Hynes RO: Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;110:673-687.
- Aguiar CB, Lobao-Soares B, Alvarez-Silva M, Trentin AG: Glycosaminoglycans modulate C6 glioma cell adhesion to extracellular matrix components and alter cell proliferation and cell migration. *BMC Cell Biol* 2005;6:31.
- Schwartz MA, Lechene C, Ingber DE: Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin alpha 5 beta 1, independent of cell shape. *Proc Natl Acad Sci U S A* 1991;88:7849-7853.
- Beningo KA, Dembo M, Kaverina I, Small JV, Wang YL: Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biol* 2001;153:881-888.
- Webb DJ, Parsons JT, Horwitz AF: Adhesion assembly, disassembly and turnover in migrating cells — over and over and over again. *Nat Cell Biol* 2002;4:E97-100.
- Stock C, Gassner B, Hauck CR, Arnold H, Mally S, Eble JA, Dieterich P, Schwab A: Migration of human melanoma cells depends on extracellular pH and Na⁺/H⁺ exchange. *J Physiol* 2005;567:225-238.
- Stock C, Schwab A: Role of the Na/H exchanger NHE1 in cell migration. *Acta Physiol (Oxf)* 2006;187:149-157.
- Plopper GE, McNamee HP, Dike LE, Bojanowski K, Ingber DE: Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. *Mol Biol Cell* 1995;6:1349-1365.
- Bailey J, Gingell D: Contacts of chick fibroblasts on glass: results and limitations of quantitative interferometry. *J Cell Sci* 1988;90 (Pt 2):215-224.
- Erlandsen SL, Greet Bittermann A, White J, Leith A, Marko M: High-resolution CryoFESEM of individual cell adhesion molecules (CAMs) in the glycocalyx of human platelets: detection of P-selectin (CD62P), GPI-IX complex (CD42A/CD42B alpha,B beta), and integrin GPIIb/IIIa (CD41/CD61) by immunogold labeling and stereo imaging. *J Histochem Cytochem* 2001;49:809-819.
- Takagi J, Petre BM, Walz T, Springer TA: Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* 2002;110:599-511.
- van Muijen GN, Jansen KF, Cornelissen IM, Smeets DF, Beck JL, Ruiter DJ: Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice. *Int J Cancer* 1991;48:85-91.
- Klein M, Seeger P, Schuricht B, Alper SL, Schwab A: Polarization of Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers in migrating renal epithelial cells. *J Gen Physiol* 2000;115:599-608.
- Schwab A, Rossmann H, Klein M, Dieterich P, Gassner B, Neff C, Stock C, Seidler U: Functional role of Na⁺-HCO₃⁻ cotransport in migration of transformed renal epithelial cells. *J Physiol* 2005;568:445-458.

- 23 Sardet C, Counillon L, Franchi A, Pouyssegur J: Growth factors induce phosphorylation of the Na⁺/H⁺ antiporter, glycoprotein of 110 kD. *Science* 1990;247:723-726.
- 24 Elliott JT, Tona A, Plant AL: Comparison of reagents for shape analysis of fixed cells by automated fluorescence microscopy. *Cytometry A* 2003;52:90-100.
- 25 Kovbasnjuk ON, Spring KR: The apical membrane glycocalyx of MDCK cells. *J Membr Biol* 2000;176:19-29.
- 26 Kirschberger S, Busche R, von Engelhardt W: Surface pH at the basolateral membrane of the caecal mucosa of guinea pig. *J Membr Biol* 1999;169:111-122.
- 27 Tominaga T, Ishizaki T, Narumiya S, Barber DL: p160ROCK mediates RhoA activation of Na-H exchange. *Embo J* 1998;17:4712-4722.
- 28 Swietach P, Rossini A, Spitzer KW, Vaughan-Jones RD: H⁺ ion activation and inactivation of the ventricular gap junction: a basis for spatial regulation of intracellular pH. *Circ Res* 2007;100:1045-1054.
- 29 Rojas JD, Sennoune SR, Maiti D, Bakunts K, Reuveni M, Sanka SC, Martinez GM, Seftor EA, Meininger CJ, Wu G, Wesson DE, Hendrix MJ, Martinez-Zaguilan R: Vacuolar-type H⁺-ATPases at the plasma membrane regulate pH and cell migration in microvascular endothelial cells. *Am J Physiol Heart Circ Physiol* 2006;291:H1147-1157.
- 30 Denker SP, Barber DL: Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger NHE1. *J Cell Biol* 2002;159:1087-1096.
- 31 Patel H, Barber DL: A developmentally regulated Na-H exchanger in *Dictyostelium discoideum* is necessary for cell polarity during chemotaxis. *J Cell Biol* 2005;169:321-329.
- 32 Eble JA, Tuckwell DS: The alpha2beta1 integrin inhibitor rhodocetin binds to the A-domain of the integrin alpha2 subunit proximal to the collagen-binding site. *Biochem J* 2003;376:77-85.
- 33 Egeblad M, Werb Z: New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002;2:161-174.
- 34 Fasciglione GF, Marini S, D'Alessio S, Politi V, Coletta M: pH- and temperature-dependence of functional modulation in metalloproteinases. A comparison between neutrophil collagenase and gelatinases A and B. *Biophys J* 2000;79:2138-2149.