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Intracellular pH gradients in migrating cells

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Intracellular pH gradients in migrating cells. Am J Physiol Cell Physiol 300: C490–C495, 2011. First published December 9, 2010; doi:10.1152/ajpcell.00280.2010.—Cell polarization along the axis of movement is required for migration. The localization of proteins and regulators of the migratory machinery to either the cell front or its rear results in a spatial asymmetry enabling cells to simultaneously coordinate cell protrusion and retraction. Protons might function as such unevenly distributed regulators as they modulate the interaction of focal adhesion proteins and components of the cytoskeleton in vitro. However, an intracellular pH (pHi) gradient reflecting a spatial asymmetry of protons has not been shown so far. One major regulator of pHi, the Na+/H+ exchanger NHE1, is essential for cell migration and accumulates at the cell front. Here, we test the hypothesis that the uneven distribution of NHE1 activity creates a pHi gradient in migrating cells. Using the pH-sensitive fluorescent dye BCECF, pHi was measured in five cell lines (MV3, B16V, NIH3T3, MDCK-F1, EA.hy926) along the axis of movement. Differences in pHi between the front and the rear end of migrating cells, and inhibition of NHE1 activity with HOE642 or by absence of extracellular Na+, were present in all cell lines, and inhibition of NHE1 activity was essential for cell migration and accumulates at the cell front. Hence, one essential characteristic of migrating cells is their polarization along the direction of movement (27). This morphological and functional polarization is based on an uneven distribution of adhesion proteins (23), ion channels and transporters (30), and signaling molecules (27). The importance of spatial asymmetry in migrating cells has been shown for small GTPases (Rho, Rac, and Cdc42) (27), lipid signaling (27), and cytosolic Ca2+ (31). Recent studies indicate that also the intracellular pH (pHi) may serve as a regulator of cell polarization and migration (38) because it modulates cytoskeletal dynamics directly by affecting the actin-binding proteins talin (35) and coflin (9) and indirectly by enhancing Cdc42 signaling (10, 17).

One important regulator of pHi is the Na+/H+ exchanger NHE1, which accumulates at the leading edge (16) and expels protons from the cytosol (24). As part of focal adhesion contacts (22), NHE1 activity is of fundamental importance for cell migration, e.g., in fibroblasts (4, 29) and tumor cells (39). It has been hypothesized for quite a long time that the uneven distribution of NHE1 along the cell membrane contributes to differences in the local pH, with the lamellipodia being more alkaline (4, 11). However, so far the postulated pHi gradient has not been demonstrated experimentally in migrating cells.

MATERIALS AND METHODS

Cells and Cell Culture

All cells were grown at 37°C in a humidified atmosphere of 5% CO2-95% air. A human melanoma cell line [MV3 (43)] and a murine [B16V (8)] melanoma cell line were grown in bicarbonate-buffered RPMI 1640 (Sigma, Taufkirchen, Germany), transformed Madin-Darby canine kidney epithelial (MDCK-F) cells (21) in Earle’s MEM (PA), and the EA.hy926 human endothelial cell line (7) and NIH3T3 mouse fibroblasts (14) in DMEM 41965 (GIBCO). All media were supplemented with 10% fetal bovine serum.

Experimental Solutions

Measurements of pHi were performed using HEPES-buffered Ringer solutions of pH 7.0 containing (in mmol/l) 122.5 NaCl, 5.4 KCl, 0.8 MgCl2, 1.2 CaCl2, 1.0 NaH2PO4, 2H2O, 5.0 glucose, and 10.0 HEPES. Ringer pH was adjusted by adding 1 M NaOH. For inhibition of NHE1, either HOE642 (Cariporide; 10 μmol/l) was added or the cells were superfused with a Na+-free Ringer solution containing N-methyl-D-glucamine (NMDG) instead of Na+. To check the contribution of bicarbonate (HCO3-) to the local, cytosolic pH, one set of experiments on MV3 cells was performed in HCO3−-buffered Ringer solution of pH 7.0 containing (in mmol/l) 106.0 NaCl, 5.4 KCl, 0.8 MgCl2, 1.2 CaCl2, 0.8 Na2HPO4 × 2H2O, 0.2 NaH2PO4 × H2O, 24.0 NaHCO3, and 5.5 glucose. pH was kept at 7.0 by continuous CO2-gassing during the measurements [11.7% CO2; P CO2 in the experimental solution was 89 mmHg as determined by a blood gas analyzer (modelABL5, Radiometer, Brønshøj, Denmark)].

The pH of 7.0 was chosen because both the human (MV3) and the murine (B16) melanoma cells show their maximum migratory activity (36, 42) at this extracellular pH. Moreover, B16 cells are most invasive at pH 7.0 (42). And in tumors, the extracellular pH is generally more acidic than 7.4. For these reasons it is quite relevant to assess the motility at a lower pH.

Immunofluorescence of NHE1

Murine melanoma cells. B16V cells seeded on coverslips coated with a basement membrane-like matrix (in mg/ml: 0.4 collagen type IV, 0.003 laminin, 0.03 fibronectin in HEPES-buffered RPMI, pH 7.4) were treated with cold 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min before fixation. They were then fixed in 3.5% paraformaldehyde in PBS for 45 min at 4°C. Nonspecific binding sites were blocked with 2% BSA (wt/vol) and 0.2% (wt/vol) gelatin in...
PBS. After the cells were stained with the primary antibody (hNHE1-mAb, BD Biosciences Pharmingen) for 1 h and a Cy3-conjugated secondary antibody for 45 min, the slide preparations were fixed once again, washed in PBS, and covered with Vectashield (Vector Laboratories, Burlingame, CA). Images were taken using an inverted microscope (Axiovert200, Carl Zeiss, Göttingen, Germany), a digital camera (model 9.0, RT-SE-Spot, Visitron Systems), and MetaVue software.

**Murine fibroblasts.** Immunofluorescence labeling of NHE1 in NIH3T3 cells was carried out essentially as previously described (26). Cells were fixed in 2% paraformaldehyde (15 min room temperature, 30 min on ice), washed in Tris-buffered saline (TBS), permeabilized for 10 min in 0.2% Triton X-100 in TBS, blocked for 30 min in 5% BSA in TBST (TBS + 0.1% Triton), incubated with rabbit polyclonal NHE1 antibody (a kind gift from Mark Musch, University of Chicago) 1:100 in TBST + 1% BSA (TBST-BSA) overnight at 4°C, washed in TBST-BSA, and incubated with Alexa Fluor488-conjugated anti-rabbit secondary antibody (Invitrogen, 1:400 in TBST-BSA, 1 h), followed by further washes in TBST-BSA and mounting in N-propyl-gallate mounting medium (2% wt/vol in PBS-glycerine). Cells were visualized using ×100/1.4 numerical aperture plan-apochromat objectives and the 488 nm Ar/Kr and 365 UV laser lines of a Leica DM-IRB/E microscope with a Leica TSC-NT confocal laser-scanning unit (Leica, Heidelberg, Germany). Optical slice thickness was 1 μm and pinhole size was 1 airy disc. Images were collected and frame-averaged using Leica software and processed (overlays and brightness/contrast adjustment only) using Adobe Photoshop.

Essentially, no labeling was detectable in the absence of primary antibody (not shown).

**Measuring pHi**

pHi was measured using video imaging techniques and the fluorescent pH indicator BCECF (Molecular Probes, Eugene, OR). MV3, MDCK-F, and NIH3T3 cells were plated onto coverslips coated with collagen type I (Biochrom, Berlin, Germany). B16V cells were plated onto coverslips coated with a basement membrane-like matrix (see above). Before polymerization, pH of this mixture was adjusted to 7.4 by adding 1 M NaOH. Cells were allowed to adapt for 3 h and were then incubated in the respective, HEPES- or HCO3- buffered, control solution containing 5 μg/ml BCECF-AM for 5 min. The coverslips were placed on the stage of an inverted microscope (Axiovert 200; Carl Zeiss) and continuously superfused with prewarmed (37°C) HEPES-or HCO3- buffered Ringer solution. Before ratiometric pH-measurement, the direction of migration, i.e., cell polarity, was checked by observing morphological changes in the moving cells, while during the actual pH-measurement the migratory activity was not further assessed. The excitation wavelengths were 440 nm and 490 nm, and the emitted fluorescence was monitored at 500 nm using a Photometrics camera (CoolSnapFx, Visitron Systems, Puchheim, Germany). The different wavelengths were generated by a high-speed polychromator system (Visichrome, Visitron Systems). Polychromator and data acquisition were controlled by Metafluor software (Visitron Systems). Fluorescence intensities were measured at 37°C in 35-s intervals and corrected for background fluorescence by subtracting intensities obtained from adjacent, extracellular regions. Exposure times were 100 and 250 ms for the 490- and the 440-nm image, respectively, and the camera gain used was 2. Flat field correction was not needed because the illumination across the visual field was uniform. At the end of each experiment, the pH measurements were calibrated by successively superfusing the cells with modified, high-K+ Ringer solutions of pH 7.5, 7.0, and 6.5 containing (in mmol/l) 125 KCl, 1 MgCl2, 1 CaCl2, 20 HEPES, and 10 μmol/l nigericin (Sigma) (6).

To quantify pH along the direction of movement, the image of each cell was divided into three segments that represented the functionally different regions of a migrating cell: the lamellipodium, the middle segment containing the nucleus, and the uropodium (Fig. 1). Each segment contained at least three defined regions of interest placed over the cytosol, excluding the nucleus region. The measured values for the three regions were combined in one single value representing one segment. Only adherent and polarized cells were measured. Since B16V cells adhere but do not polarize on collagen type I, the experiments with those cells were performed on a basement membrane-like matrix on which they do polarize.

NHE1 activity was assessed by using the NH4+-prepulse technique (33). Exposing cells to 20 mmol/l NH4+ for 100 s leads to an alkalization of the cytosol. Upon removal of NH4+ and Na+ (isosmotically substituted by NMDG+) pH decreases rapidly. pH recovers when Na+ is added again to the superfusate. The initial slope of the change in pH, after readdition of Na+ was taken as a measure of NHE1 activity, because these experiments were carried out in absence of HCO3-, precluding contributions from Na+-HCO3- cotransporters (29, 40).

**Statistical Analysis**

All experiments were repeated at least eight times except for the migration experiments mentioned above (N = 4). Data are presented as means ± SE and were tested for significance employing Wilcoxon signed-rank test for paired samples, Mann-Whitney U-test for independent samples, and Friedman test when more than two groups were compared with each other. If normal distribution could be verified, Student’s unpaired t-test was applied. The level of significance was set at p < 0.05 unless otherwise stated.

**RESULTS**

**Immunolocalization of NHE1**

As shown for various other cell lines including MV3, MDCK-F, and endothelial cells (3, 11, 16, 37), NHE1 also accumulated at the leading edge of migrating murine melanoma cells and NIH3T3 fibroblasts (Fig. 2).

**Intracellular pH**

Local pH measurements revealed significant differences in pH between the front and the rear end (ΔpH, front-rear) in all
investigated cell lines (Fig. 3). Measured in HEPES-buffered Ringer solution, the mean ΔpH front-rear averaged 0.16 ± 0.02 pH units in MV3, 0.15 ± 0.05 pH units in B16V, 0.05 ± 0.02 pH units in both NIH3T3 and MDCK-F, and 0.07 ± 0.02 pH units in EA.hy926 cells. Except in the NIH3T3 cells, the pH value of the middle segment was always found between the pH values of the front and rear segment, indicating that the measured differences in cytosolic pH mirror a pH gradient along the longitudinal axis of the cell body. To exclude that the gradient is caused or enhanced by the absence of HCO3− in the experimental solution, pH of MV3 cells was measured also in HCO3−-buffered Ringer solution. Under these conditions, pH front-rear averaged 0.18 ± 0.04 pH units, underlining the physiological significance of the gradient.

NHE1 Dependence of the pH Gradient in MV3 and B16V Cells

Because pH gradients were most distinct in the two melanoma cell lines, these cells were used to investigate whether NHE1 activity contributes to the generation of the pH gradients. Upon inhibition of NHE1 by applying either the specific NHE1 inhibitor HOE642 or a Na+−free Ringer solution, the cells acidified. Acidification of the cytosol was most prominent at the leading edge corresponding to the localization of NHE1 at the cell front. In MV3 cells the ΔpH front-rear decreased from 0.16 ± 0.02 pH units to 0.08 ± 0.03 pH units when exposed to HOE642 and to 0.09 ± 0.02 pH units when exposed to a Na+−free solution. In B16V cells exposed to HOE642, no differences in pH between the front and the rear end were detectable (Fig. 4). Thus, inhibition of NHE1 caused the pH gradient to flatten or disappear, accompanied by a general decline in pH, demonstrating that the establishment of pH gradients to a great extent depends on NHE1 activity.

Subcellular Distribution of NHE1 Activity in MV3 and NIH3T3 Cells

Since pH gradients depended on NHE1, an NHE1 activity gradient seemed supposable. Applying the NH4+−prepulse technique and using the same arrangement of regions as for the pH measurements, NHE1 activity was quantified along the longitudinal axis of the cell. Corresponding to the NHE1 accumulation at the leading edge, its activity was found to be the highest at the cell front (Fig. 5B). After an acid load, MV3 cells recovered at a rate of 0.37 ± 0.07 pH units/min at their
front, 0.32 ± 0.06 pH units/min in the middle segment, and 0.3 ± 0.06 pH units/min at their rear. NIH3T3 cells recovered at a rate of 0.29 ± 0.06 pH units/min in the middle segment, and 0.26 ± 0.06 pH units/min at their trailing end. In addition to immunofluorescence analysis of NHE1 distribution, both the NHE1 inhibition experiments and the subcellular activity measurements provide physiological evidence for the uneven NHE1 distribution in migrating cells and its contribution to the establishment of an intracellular pH gradient.

DISCUSSION

The hypothesis that NHE1 activity may generate small but still important pH gradients either extracellularly or intracellularly has been around for some time (11, 34). The presence of an extracellular pH gradient at the cell surface and its functional importance in facilitating cell migration has already been shown for human melanoma cells (37, 40). Cells that do not express a functional NHE1 or whose NHE1 is inhibited do not establish a pH gradient at the cell surface nor do they migrate. In human melanoma cells that exhibit normal NHE1 activity, partial removal of the glycocalyx causes the extracellular pH gradient to collapse and the migratory activity to decrease considerably (18). In these cells, however, the cell surface pH-gradient as well as the migratory activity can be restored by stimulating NHE1 activity (18). Here, we demonstrate a complementary, NHE1-dependent, intracellular pH gradient not only present in melanoma but also in other migrating cells. Upon inhibition of NHE1, both the pH-gradient (this study) and the cell surface pH-gradient (37) flatten and melanoma cell migration is hindered (36). Changes in the overall extracellular pH cannot compensate for the loss of the cell surface pH-gradient (40). Correspondingly, Tominaga and Barber (41) found that, in the absence of NHE1 activity, cell adhesion and spreading were impaired and pH decreased. An overall alkalization of the cytosol did not rescue the delay in cell adhesion or spreading (41). These results demonstrate the importance of local pH regulation in facilitating cell migration. Since both the extracellular pH-sensitive cell surface/matrix interaction (18, 19) and many components of the intracellular migratory machinery are regulated in a pH-dependent manner, the existence of both pH gradients seems to be most advantageous for migrating cells.

After adhering to the surrounding matrix, migrating cells polarize, defining the direction of movement. As a major regulator of this polarization, the small GTPase Cdc42 accumulates at the cell front (27), where it induces actin polymerization via the Wiskott-Aldrich syndrome protein (WASP) and the Arp 2/3 complex (12, 25) as well as through the Rac/Cdc42 effector IQ-motif-containing GTPase-activating protein 1 (IQGAP1) (2). As shown for migrating fibroblasts, activation of Cdc42 requires H⁺ efflux by NHE1 (10), which implies a fundamental role of pH in positive feedback loops at the leading edge necessary for enduring cell polarization and directed movement.

Furthermore, the polymerization and depolymerization of actin filaments are coordinated pH-dependently. The generation of free barbed filament ends by the activity of the actin-severing protein cofilin promotes dynamic actin polymerization (45) and membrane protrusion at the cell front. The ability...
of coflin to sever F-actin is stronger at more alkaline pH values (46) due to an increased inhibition of coflin by pH-dependent phosphoinositide binding at more acidic pH (9). Another actin-binding protein regulating actin assembly and disassembly, gelsolin, is activated by more acidic pH values (1) as can be found at the rear end of migrating melanoma cells. It is tempting to assume that gelsolin is involved in actin recycling at the rear end of migrating cells whereas coflin regulates actin dynamics at the leading edge. Those are only two of several examples of how migratory events depend on an optimal pH environment (13, 35, 38).

Thus, the pH gradient appears to be a prerequisite for cell migration, and the question arises as to whether its slope might function as a “cruise control system.” As a matter of fact, H-ras-transfected NIH3T3 migrate faster than wild-type NIH3T3 (28), show an increased net acid extrusion (15, and tend to establish a steeper pH gradient \( \Delta p_{H} \) front-rear: 0.07 ± 0.01 for NIH3T3-ras (n = 9) vs. 0.05 ± 0.02 for wild-type NIH3T3 (n = 10; \( P = 0.14 \)). On the other hand, a comparison of the five investigated migration.

hypothesize that the establishment of this pHi gradient represents one mechanism by which NHE1 activity promotes cell migration.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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