Polarization of Na^+/H^+ and Cl^-/HCO_3^- exchangers in Migrating Renal Epithelial Cells

Magnus Klein,* Ponke Seeger,* Barbara Schuricht,* Seth L. Alper,‡ and Albrecht Schwab*

From the *Physiologisches Institut, D-97070 Würzburg, Germany; and ‡Departments of Medicine and Cell Biology, Harvard Medical School and Molecular Medicine and Renal Units, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

abstract   Cell migration is crucial for processes such as immune defense, wound healing, or the formation of tumor metastases. Typically, migrating cells are polarized within the plane of movement with lamellipodium and cell body representing the front and rear of the cell, respectively. Here, we address the question of whether this polarization also extends to the distribution of ion transporters such as Na^+/H^+ exchanger (NHE) and anion exchanger in the plasma membrane of migrating cells. Both transporters are required for locomotion of renal epithelial (Madin-Darby canine kidney, MDCK-F) cells and human melanoma cells since their blockade reduces the rate of migration in a dose-dependent manner. Inhibition of migration of MDCK-F cells by NHE blockers is accompanied by a decrease of pH_i. However, when cells are acidified with weak organic acids, migration of MDCK-F cells is normal despite an even more pronounced decrease of pH_i. Under these conditions, NHE activity is increased so that cells are swelling due to the accumulation of organic anions and Na^+. When exclusively applied to the lamellipodium, blockers of NHE or anion exchange inhibit migration of MDCK-F cells as effectively as when applied to the entire cell surface. When they are directed to the cell body, migration is not affected. These data are confirmed immunocytochemically in that the anion exchanger AE2 is concentrated at the front of MDCK-F cells. Our findings show that NHE and anion exchanger are distributed in a polarized way in migrating cells. They are consistent with important contributions of both transporters to protrusion of the lamellipodium via solute uptake and consequent volume increase at the front of migrating cells.

key words: migration • Na^+/H^+ exchanger • Cl^-/HCO_3^- exchanger • cell volume • pH

INTRODUCTION

Cell migration plays a central role for such diverse physiological and pathophysiological processes as embryogenesis, wound healing, immune defense, and tumor metastases. A typical feature of migrating cells is their polarization within the plane of movement into lamellipodium (front) and cell body (rear) reflecting underlying cytoskeletal "polarization." Distinct cytoskeletal mechanisms underlie the protrusion of the lamellipodium and the retraction of the trailing end of migrating cells (for review, see Condeelis, 1993; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996).

Whereas the role of the cytoskeleton in cell migration has been studied in great detail, limited information is available on the role of ion transporters and ion channels in cell migration and on their distribution in crawling cells. In neutrophil granulocytes, chemotaxis is regulated by activation of the Na^+/H^+ exchanger (NHE)[1]

(Rosengren et al., 1994; Simchowitz and Cragoe, 1986). At least two explanations can account for the permissive effect of NHE activity on chemotaxis: regulation of intracellular pH (pH_i) or of cell volume. Accordingly, hypotonic swelling of granulocytes mimics the stimulatory effect of NHE-mediated cell swelling on chemotaxis (Rosengren et al., 1994). Both cell volume and pH_i also regulate cytoskeletal architecture and cell migration (Hallows et al., 1991; Van Duijn and Inouye, 1991; Schwab et al., 1999).

Previously, we studied the role of a Ca^{2+}-sensitive K^+ channel for migration of transformed renal epithelial (Madin-Darby canine kidney, MDCK-F) cells (Schwab et al., 1994, 1995). We suggested that the intermittent activity of this K^+ channel supports the cytoskeletal mechanisms of migration by inducing a localized cell shrinkage at the trailing end of crawling MDCK-F cells (Schwab et al., 1999). However, these experiments left the following question open: by which mechanism is MDCK-F cell volume restored after K^+ channel-mediated cell shrinkage? Previous studies on cell volume regulation (Lang et al., 1998) suggested NHE, anion exchanger, and $Na^+/K^+/2Cl^-$ cotransporter as good candidates for this function. If volume uptake mediated by these transporters is required for migration, their inhibition should also impair locomotion. We showed earlier that this is indeed the case for $Na^+/K^+/2Cl^-$

cotransport (Schwab et al., 1994). In the present study, we set out to define the roles of the Na^+/H^+ and anion exchangers in cell migration. In particular, we wished to elucidate whether these transporters affect migration by modulation of pH_i or by regulation of cell volume.

In addition, we studied the distribution of NHE and anion exchanger in migrating cells. Prompted by our own observation of polarized K^+ channel activity (Schwab et al., 1995; Reinhardt et al., 1998) and by the finding that NHE is concentrated at the leading edge of fibroblasts (Grinstein et al., 1993), we tested the hypothesis that NHE and anion exchanger support protrusion of the lamellipodium by mediating solute uptake–induced localized volume increase at this cell pole. Swelling due to this uptake of salt and water would then act in concert with the gel-osmotic swelling postulated to occur as a consequence of actin filament assembly at the cell's leading edge (Oster and Perelson, 1987).

METHODS

Cell Culture

Experiments were carried out on alkali-transformed MDCK-F cells (Oberleithner et al., 1991) and with human melanoma cells transfected with actin-binding protein 280 (ABP280; Cunningham et al., 1992). Both cell types were kept at 37°C in humidified air containing 5% CO_2. Cells were grown in bicarbonate-buffered MEM, pH 7.4, with Earle's salts (Biochrom) supplemented with 10% fetal calf serum (Biochrom). To maintain a constant selection pressure on ABP280-transfected melanoma cells, their culture medium contained geneticin (0.5 g/liter; Gibco Laboratories). For experiments, cells were plated on poly-1-lysine–coated coverslips (0.1g/liter; Serva Biochemicals). Experiments were performed between 1 and 2 d after seeding.

Migration Experiments

Migration of individual MDCK-F cells and human melanoma cells was monitored in paired experiments with video microscopy as described previously (Schwab et al., 1994). Migration was quantitated from calibrated videoprints as the distance lamellipodia advanced with time. During the entire course of the experiments, cells were superfused with prewarmed (37°C) Ringer solution that contained (mmol/liter): 122.5 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.2 $CaCl_2$, 1.0 NaH_2PO_4, 5.5 d-glucose, 10.0 HEPES, pH 7.4, titrated with 1 mol/liter NaOH. Indicated cells were alternatively superfused with CO_2/HCO_3^--buffered Ringer solution (equilibrated with 5% CO_2, pH 7.4) containing 24 mmol/liter $NaHCO_3$ (isomotically replacing NaCl). When applicable, 15 or 30 mmol/liter NaCl were replaced by Na-formate or Na-propionate. The protocol of all migration experiments was such that a 15-min control period was followed by a 10-min experimental period. The rate of migration during the experimental period is always normalized to that of the preceding control period.

Local Superfusion Experiments

Migration experiments with local superfusion of either cell body or lamellipodium were performed as described previously (Schwab et al., 1997). A micropipette (2–3-μm tip diameter) was connected to a pressure-driven microinjection device (Eppendorf). The pipette was brought into close vicinity of either the cell body or lamellipodium of MDCK-F cells. Local superfusion was started by elevating the "injection" pressure. Then a well-defined stream of control Ringer's solution or Ringer's solution supplemented with ethylisopropylamiloride (EIPA), Hoe 694, or 4,4'-diisothiocyanate-stilbene-2,2'-disulfonic acid (DIDS) flowed over one pole of the cell under study. Simultaneously, cells were superfused "systemically." The correct positioning of the local superfusate was verified after each experiment by superfusing the cell with a lissamine green–containing solution (Schwab et al., 1997). Drug-induced changes in rate of cell migration were interpreted as functional evidence for altered activity of NHE or anion exchangers in the membrane of the cell pole under study.

Measurements of pH_i

pH_i of MDCK-F cells was measured by using video imaging techniques and the fluorescent pH indicator BCECF (Molecular Probes, Inc.). For dye-loading, MDCK-F cells were incubated in culture medium containing 2 μmol/liter BCECF-AM for 1–2 min. Coverslips were placed on the stage of an inverted microscope (Axiovert TV 100; Carl Zeiss, Inc.) and continuously superfused with prewarmed (37°C) Ringer's solution. Excitation wavelength alternated between 488 and 460 nm, respectively. The emitted fluorescence was monitored at 500 nm with an ICCD camera (Atto Instruments). Filter change and data acquisition were controlled by Attofluor software (Atto Instruments). Average fluorescence intensities (corrected for background fluorescence) were measured at 10-s intervals in several demarcated regions of interest placed over the projected cell surface.

At the end of each experiment, pH_i measurements were calibrated by superfusing MDCK-F cells with a modified Ringer solution containing (mmol/liter): 125 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 20 HEPES, and 10 μmol/liter nigericin using either two-point calibrations (pH 7.5 and 6.5) or four-point calibrations (pH 8.0, 7.5, 6.5, and 6.0).

The intracellular buffer capacity, β, was determined by a modification of the method described by Weintraub and Machen (1989). Cells were superfused with solutions in which Na^+ was replaced by NMDG. After a 3-min pulse with 40 or 20 mmol/liter NH_4Cl (isosmotically replacing NMDG-Cl), extracellular NH_4Cl was reduced in a stepwise fashion while recording pH_i. NH_4^+ transport was minimized by supplementing these solutions with 1 mmol/liter $BaCl_2$ and 1 μmol/liter bumetanide. The ensuing changes of pH_i and the calculated changes of the intracellular NH_4^+ concentration were used to determine the intracellular intrinsic (CO_2/HCO_3^--independent) buffering capacity β.

Cell Volume Measurements

The effect of propionate on the volume of freshly trypsinized, suspended MDCK-F cells was determined electronically with a Coulter Counter. To obtain maximal propionate-induced swelling, we adopted a protocol described previously (Grinstein et al., 1984). Cells were immersed in a solution containing (mmol/liter): 140 Na-propionate, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 10 HEPES, pH 6.7. When needed, this solution was substituted with 10 μmol/liter EIPA.

Immunofluorescence

MDCK-F cells growing on glass cover slips were rinsed twice with prewarmed PBS and fixed for 30 min with 3% paraformaldehyde in PBS. Fixed cells were washed with PBS, pH 7.4, PBS supplemented with 1% SDS (Brown et al., 1996), and PBS (pH 8.0) containing 50 mmol/liter glycine and permeabilized with 0.1% Triton X-100 (4 min). Cells were incubated with primary antibody directed against the COOH-terminal peptide of the $Cl^-/$

HCO$_3^-$ exchanger AE2 (Stuart-Tilley et al., 1994) for 1 h at room temperature. Alternatively, cells were incubated with a mixture of antibody and blocking peptide (12 μg/ml) against which this antibody was raised or with an unrelated peptide (12 μg/ml). Peptide and antibody were preincubated overnight at 4°C. Before and after adding the Cy3-labeled secondary antibody (goat anti–rabbit IgG; Dianova), cells were washed with PBS, pH 7.4, supplemented with 2.7% NaCl. Cells were mounted in Vectashield (Vector Laboratories, Inc.) for conventional or confocal immunofluorescence microscopy. Specific staining of the anion exchanger AE2 is represented by the difference between immunofluorescence in the presence and absence of the blocking peptide.

Statistics

All data are presented as mean ± SEM. Paired or unpaired Student's *t* test were performed where applicable. Significance was assumed when $P < 0.05$.

RESULTS

NHE Modulates Migration and pH$_i$

MDCK-F cells migrate at a rate of 0.92 ± 0.02 μm/min ($n = 320$) under control conditions. The specific NHE blocker EIPA (100 nmol/liter–25 μmol/liter) reduces the rate of migration to 52.0 ± 7.3% of control (Fig. 1 A) in a dose-dependent manner. 25 μmol/liter EIPA elicits indistinguishable inhibition of migration when MDCK-F cells are superfused with CO$_2$/HCO$_3^-$-buffered Ringer solution (48.2 ± 5.8% of control; $n = 13$; data not shown). Another specific NHE blocker, Hoe 694, has a similar effect. 1 and 10 μmol/liter Hoe 694 reduce the rate of migration to 76.9 ± 7.7% ($n = 20$) and 63.2 ± 5.6% of control ($n = 18$), respectively. Together, these data suggest a requirement for NHE activity for normal migration of MDCK-F cells. The half maximal inhibition in the low micromolar range points to the involvement of NHE1 (Noel and Pouyssegur, 1995). In the presence of EIPA, NHE activity in MDCK-F cells may be partially replaced by K$^+$/H$^+$ exchange. Simultaneous application of 10 μmol/liter EIPA and 10 μmol/liter omeprazole, a blocker of the H$^+$/K$^+$-ATPase, causes a stronger inhibition of migration than EIPA alone (36.3 ± 8.1% of control; $n = 12$). Migration of ABP280-transfected human melanoma cells also depends on NHE activity. Under control conditions, melanoma cells migrate at a rate of 1.17 ± 0.18 μm/min. 25 μmol/liter EIPA reduces the rate of migration to 16.4 ± 5.9% of control ($n = 15$; Fig. 1 A).

As shown in Fig. 1 B, NHE blockade with EIPA or Hoe694 is followed by a dose-dependent reduction of pH$_i$ from the control value of 7.28 ± 0.01 ($n = 91$). There is no detectable standing gradient of pH$_i$ between cell body and lamellipodium. Both NHE inhibitors reduce pH$_i$ to similar extents. Half maximal intracellular acidification and half-maximal inhibition of migration exhibit similar IC$_{50}$ values, consistent with the involvement of the same NHE isoform in both pro-
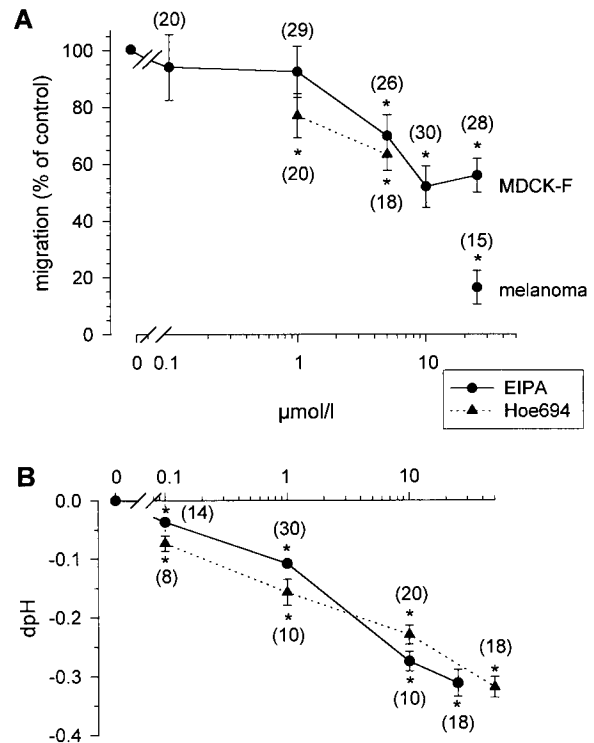

Figure 1. (A) Migration of MDCK-F cells is inhibited by EIPA or Hoe 694 in a dose-dependent manner. Migration of human melanoma cells is almost completely inhibited by 25 μmol/liter EIPA. (B) EIPA and Hoe 694 also elicit a dose-dependent intracellular acidification in MDCK-F cells. Although the IC$_{50}$ values for the inhibition of migration and for the decrease of pH$_i$ are similar, it is noteworthy that 1 μmol/liter EIPA has no effect on migration of MDCK-F cells despite lowering pH$_i$ by 0.1 pH units.

cesses. However, careful inspection of Fig. 1, A and B, reveals subtle differences between the inhibition of migration by EIPA and the decrease of pH$_i$. Whereas 1 μmol/liter EIPA has no effect on migration, it reduces pH$_i$ by 0.1 pH units. This suggests that moderate intracellular acidification does not suffice to inhibit cell migration, and that the Na$^+$/H$^+$ exchanger may modulate migration by mechanisms other than its impact on pH$_i$. Nonetheless, these experiments are consistent with inhibition of migration by EIPA or Hoe694, reflecting blockade of the Na$^+$/H$^+$ exchanger.

Migration of MDCK-F Cells Depends on NHE Activity Rather than on pH$_i$

If inhibition of migration due to NHE blockade is due to the reduction of pH$_i$ below a threshold of 0.1 pH units acidification, this effect should be mimicked by acidifying MDCK-F cells with weak organic acids such as propionic and formic acid. Their protonated forms permeate the cell and rapidly dissociate (Boron, 1983), in turn activating Na$^+$/H$^+$ exchange and leading to cellular accumulation of Na-propionate or Na-formate. Hence, application of weak organic acids should cause intracellular

acidification and an increase of cell volume due to osmotically obliged water uptake (Grinstein et al., 1984). As shown in Fig. 2 A, application of Na-propionate or Na-formate is indeed followed by rapid acidification of up to 0.5 pH units. The ensuing pH_i recovery is abolished when Na-propionate is applied in the presence of 10 μmol/liter EIPA. Cell volume rises from 2,230 ± 80 fl to 2,420 ± 90 fl, or by 8.4 ± 1.0% within 5 min after the application of propionic acid ($n = 10$). When Na-propionate is applied in the presence of 10 μmol/liter EIPA, volume does not change (+1.7 ± 0.9%; $n = 10$).

As shown in Fig. 2 B, the marked acidification of MDCK-F cells with Na-propionate or with Na-formate does not inhibit migration. To test whether propionate-induced activation of NHE is responsible for the pH insensitivity of MDCK-F cell migration, we applied 15 mmol/liter Na-propionate in the presence of 10 μmol/liter EIPA. Under these conditions, migration is inhibited to the same extent as with EIPA alone (51.7 ± 7.6% of control; Fig. 2 B).

Fig. 3 plots the migration of MDCK-F cells as a function of the decrease of pH_i. It is apparent that migration of MDCK-F cells does not solely depend on pH_i, but rather on NHE activity per se. We made a qualitatively similar observation in ABP280-transfected melanoma cells. Whereas NHE blockade with EIPA almost completely inhibits migration (16.4 ± 5.9% of control), melanoma cells are slowed down only to 54.8 ± 14.5% of control rates ($n = 14$) in the presence of 15 mmol/liter propionate (Fig. 2 B). These observations raise the possibility that NHE activity may modulate migration due to its effect on cell volume. That increased NHE activity in the presence of propionate or formate is not followed by a stimulation of migration is probably due to the potentially inhibitory effect of lowered pH_i on migration. However, this negative effect can be compensated for by increased NHE activity in MDCK-F cells.

Anion Exchange Is Required for Migration

Removal of extracellular Cl^- in CO_2/HCO_3^--buffered Ringer's solution (isosmotically replaced by gluconate) demonstrates the existence of an anion exchanger in MDCK-F cells. Fig. 4 A reveals that pH_i immediately rises at a rate of 0.11 ± 0.01 pH units/min upon removal of Cl^- ($n = 36$) and returns to baseline upon readdition of Cl^- to the bath. The pH recovery upon addition of Cl^- is sensitive to the anion exchange inhibitor, DIDS. Anion exchange activity is also evident in HEPES-buffered Ringer's solution. pH_i rises at a rate of 0.06 + 0.01 pH units/min ($n = 8$) upon removal of extracellular Cl^- (data not shown). Taking the intracellular buffering power into account, which is lower in the absence of CO_2/HCO_3^-, this corresponds to an approximately 10-fold lower transport rate of the anion exchanger than in the presence of exogenous CO_2/HCO_3^-.
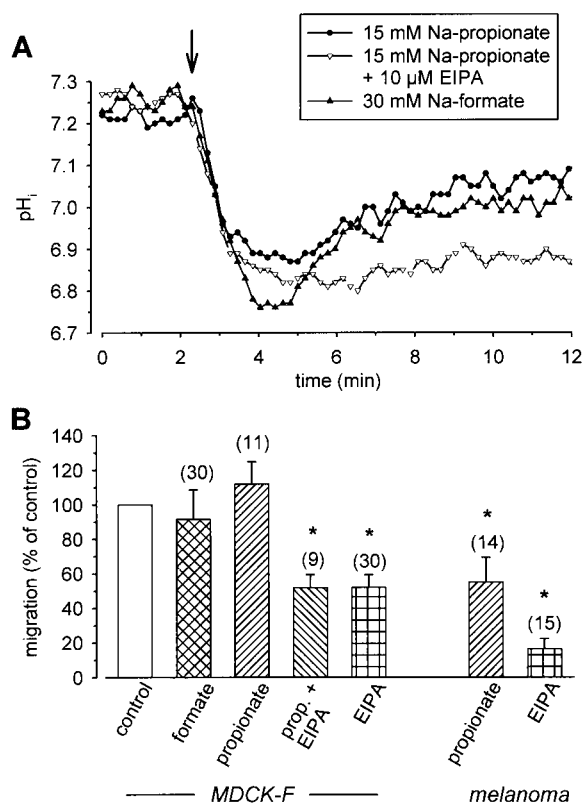

Figure 2. Effect of weak organic acids on pH_i and migration of MDCK-F cells and of human melanoma cells. (A) Formate and propionate induce a marked intracellular acidification of up to 0.5 pH units. Partial pH recovery from the acid load requires intact NHE activity. (B) Despite massive intracellular acidification, formate and propionate alone do not inhibit cell migration of MDCK-F cells. Migration of MDCK-F cells is impaired only when NHE is blocked with EIPA. Human melanoma cells behave qualitatively in a similar way. While NHE blockade with EIPA is followed by an almost complete inhibition of migration, intracellular acidification with 15 mmol/liter propionate causes only a partial inhibition of migration.

As summarized in Fig. 4 B, DIDS slows migration of MDCK-F cells in a dose-dependent manner. 100 and 500 μmol/liter DIDS almost completely inhibit migration of MDCK-F cells (22.3 ± 7.7% and 12.5 ± 5.7% of control, respectively). The sensitivity to DIDS is enhanced in the absence of exogenous CO_2/HCO_3^-. 25 μmol/liter DIDS elicit an almost complete inhibition of migration in HEPES-buffered Ringer's solution (20.7 ± 7.2% of control; Fig. 4 C). This is consistent with the lower anion exchange activity in the absence of exogenous CO_2/HCO_3^- being a rate-limiting step for normal cell locomotion. To test for the contribution of endogenously produced HCO_3^-, we also studied the effect of 100 μmol/liter acetazolamide, an inhibitor of carbonic anhydrase. Acetazolamide has no effect on migration by itself (104.7 ± 11.8% of control), nor does it accentuate the inhibition caused by 25 μmol DIDS in
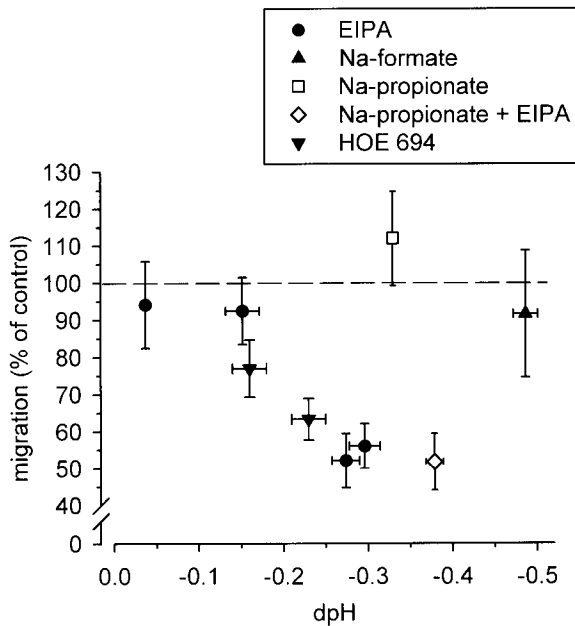
Figure 3. Cell migration plotted as a function of pH_i. When NHE is active, pH_i can vary over a wide range (0.5 pH units) without inhibiting migration. Migration is only impaired when the decrease of pH_i is due to blockade of NHE.

HEPES-buffered Ringer's solution ($16.8 \pm 5\%$ of control; Fig. 4 C). DNDS (100 μmol/liter), another inhibitor of anion exchange, also slows MDCK-F cells to $68.3 \pm 8.3\%$ of control values ($n = 11$; data not shown). DIDS (100 μmol/liter) almost completely inhibits migration of human melanoma cells as well ($24.8 \pm 9.6\%$; $n = 12$; Fig. 4 B, inset). Thus, parallel operation of NHE and anion exchangers appears to be required for efficient locomotion of both cell types tested.

NHE Activity in MDCK-F Cells

We next determined the transport rate of the Na^+/H^+ exchanger in MDCK-F cells. Monitoring changes of pH_i after stepwise reduction of extracellular NH_4^+ allowed calculation of the intrinsic buffer capacity β. In the physiological range of pH_i, β is $\sim$5 mmol^{-1} pH unit^{-1} (data not shown). NHE-mediated Na^+ flux was calculated by multiplying β by the initial change of pH (dpH/dt) after application of EIPA or after removal of extracellular Na^+ (0.03 pH units/min). Under basal "resting" conditions (pH_i between 7.2 and 7.3), with the assumption of 1:1 Na^+/H^+ transport stoichiometry, NHE mediates uptake of 1.5 mmol/liter Na^+ per min, corresponding to $\sim$1% of the total cellular cation content per min.

NHE activity in MDCK-F cells was also measured as Na^+-dependent pH_i recovery from imposed acid load. After acidifying cells in Na^+-free Ringer's solution by removal of extracellular NH_4^+, readdition of extracellu-
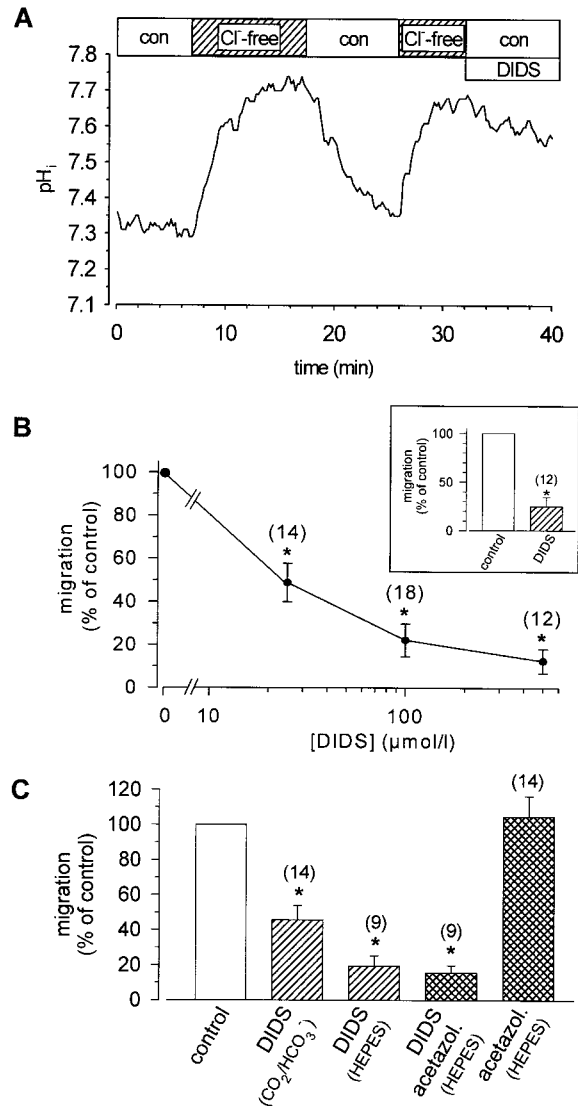

Figure 4. (A) Demonstration of Cl^-/HCO_3^- exchange in MDCK-F cells. Removal of extracellular Cl^- is followed by an intracellular alkalinization. pH recovery after readdition of Cl^- is inhibited by DIDS. (B) DIDS impairs migration of MDCK-F cells in CO_2/HCO_3^--buffered Ringer's solution in a dose-dependent manner. The inset shows that 100 μmol/liter DIDS inhibit migration of human melanoma cells to the same extent as that of MDCK-F cells. (C) Migration experiments testing for the contribution of endogenous HCO_3^- production. The sensitivity to DIDS (25 μmol/liter) is increased in HEPES-buffered Ringer''s solution. Endogenous HCO_3^- production appears not to drive the anion exchanger since acetazolamide (acetazol.; 100 μmol/liter), an inhibitor of carbonic anhydrase, has no effect on migration of MDCK-F cells when applied in HEPES-buffered Ringer's solution.

lar Na^+ initiated pH_i recovery at a rate of 0.28 ± 0.03 pH units/min ($n = 23$; data not shown).

Local Superfusion Experiments

To contribute to cell migration, cell volume regulation should optimally be polarized within the cell. This hy-

pothesis should be reflected by polarized distribution of volume regulatory ion transporters in the migrating cell. Therefore, we functionally tested the distribution of NHE and anion exchange activity in MDCK-F cells with a local superfusion technique (Schwab et al., 1997). When control Ringer's solution is directed to lamellipodium or to cell body, MDCK-F cells migrate at their normal rate (data not shown).

Fig. 5 summarizes the experiments with local superfusion of NHE inhibitors. When the lamellipodium is superfused with 25 μmol/liter EIPA, cells are slowed to the same extent as with superfusion of EIPA over the entire cell ($50.5 \pm 4.2\%$ of control; $n = 11$). In contrast, when the cell body of migrating MDCK-F cells is exposed to EIPA, locomotion is not affected and cells move normally ($103.4 \pm 7.9\%$ of control; $n = 10$; Fig. 5 A). Hoe 694 (50 μmol/liter) has the same differential effect as EIPA when consecutively superfused over both cell poles of one cell. As shown in Fig. 5 B, Hoe 694 slows migration only when applied to the lamellipodium of MDCK-F cells ($41.2 \pm 8.9\%$ of control; $n = 6$), while it does not impair migration when directed to the cell body of the same cell ($105.3 \pm 4.2\%$ of control; $n = 6$).

We next investigated whether the anion exchanger is also unevenly distributed in migrating MDCK-F cells by local superfusion of DIDS (25 μmol/liter) in CO_2/HCO_3^--buffered Ringer's solution (Fig. 6). When DIDS is applied to the lamellipodium of migrating MDCK-F cells, they are slowed to $48.6 \pm 6.4\%$ of control. This degree of inhibition is identical to that produced by application of DIDS to the entire cell (compare with Fig. 4 B). In contrast, when the cell body is exposed to DIDS, migration of MDCK-F cells is not impaired. MDCK-F cells move at the same rate ($85.3 \pm 5.8\%$ of control; $n = 9$) of migration as when the cell body is superfused with control Ringer's solution. Taken together, these experiments suggest that functionally important NHE and anion exchangers are concentrated at the leading edge of migrating MDCK-F cells.

Immunocytochemical Localization of AE2 Anion Exchanger in Crawling Cells

As a correlate of the functional data on the localization of the anion exchanger, we visualized its distribution in MDCK-F cells with an anti–AE2 antibody (Stuart-Tilley et al., 1994). As evident from Fig. 7, functional and morphological localization of the anion exchanger match well in MDCK-F cells. In addition to staining within the cell body, there is a strong signal at the leading edge of migrating MDCK-F cells. Staining is specific since it can be almost completely blocked by incubating the antibody with the antigenic peptide (Fig. 7 C). Staining is not affected when the antibody is incubated with an unrelated peptide (not shown).
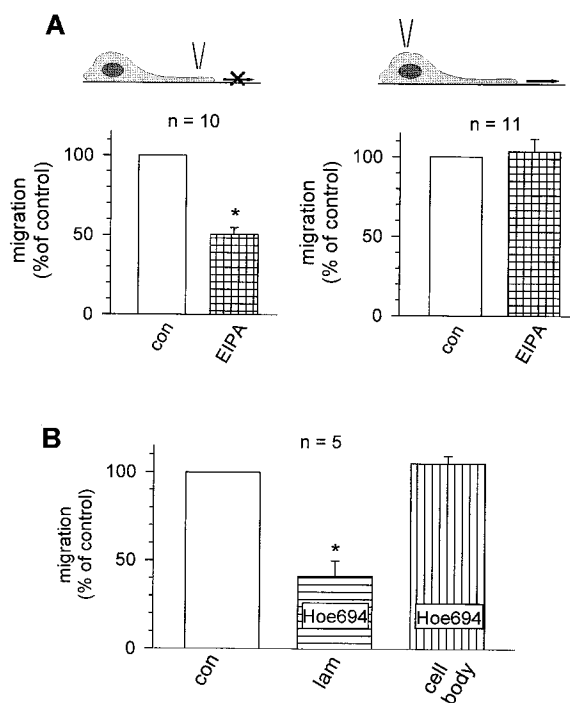

Figure 5. Local superfusion of NHE inhibitors. (A) EIPA (25 μmol/liter) inhibits migration only when applied to the lamellipodium of migrating MDCK-F cells. Exposing the cell body to EIPA does not impair migration. (B) Hoe 694 (50 μmol/liter) was sequentially directed to lamellipodium and cell body of the same cells, respectively. Migration is only inhibited when the lamellipodium is exposed to Hoe 694.

DISCUSSION

We combined migration experiments with measurements of pH_i and indirect immunofluorescence to study the functional roles of NHE and anion exchanger in cell migration. Our experiments clearly show that both transport activities are required for efficient locomotion of transformed renal epithelial cells and of human melanoma cells. NHE dependence of locomotion is also known for other cell types such as granulocytes (Simchowitz and Cragoe, 1986; Rosengren et al., 1994; Ritter et al., 1998) and keratinocytes (Bereiter-Hahn and Voth, 1988). Thus, it appears that NHE activity is a general requirement for locomotion. To our knowledge, the role of the anion exchanger in cell migration has not yet been studied in detail.

NHE and anion exchanger are both involved in regulation of intracellular pH and cell volume homeostasis, both of which have been shown independently and in different cell types to be important for cell migration (Simchowitz and Cragoe, 1986; Rosengren et al., 1994; Van Duijn and Inouye, 1991; Ritter et al., 1998; Schwab et al., 1999). Similarly, pH_i and cell volume modulate the organization of the cytoskeleton, which provides the directed force for locomotion (Tonetti et al., 1990;
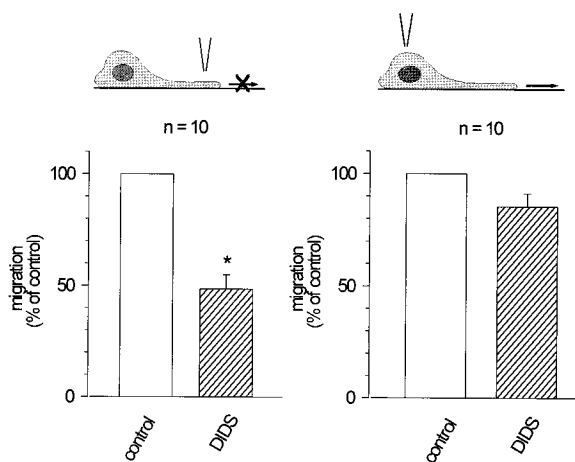
Figure 6. Local superfusion of DIDS, an inhibitor of the anion exchanger, in CO_2/HCO_3^- buffered Ringer's solution. Migration of MDCK-F cells is only inhibited when DIDS (25 μmol/liter) is directed towards the lamellipodium.
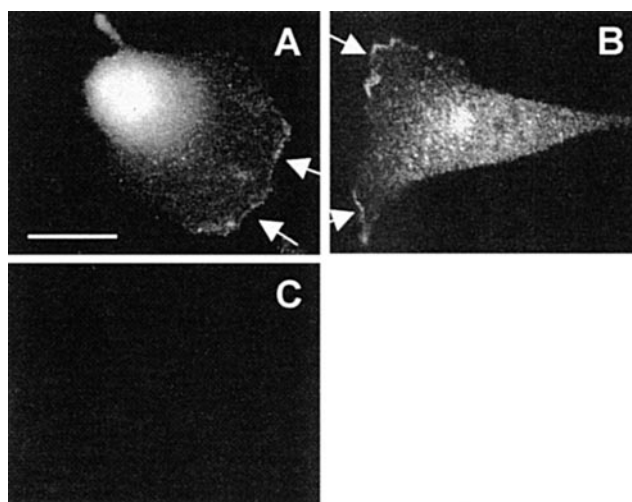

Figure 7. Visualization of the anion exchanger in MDCK-F cells. Both conventional (A) and confocal microscopy (B) show that in addition to intracellular staining, the specific antibody reveals concentration of the anion exchanger AE2 at the leading edge of MDCK-F cells (arrows). (C) When the antibody is blocked with the antigenic peptide, staining at the leading edge is completely abolished.

Hallows et al., 1991; Edmonds et al., 1995; Schwab et al., 1999). In particular, NHE activity has been linked to RhoA-induced stress fiber formation (Vexler et al., 1996). The intimate relation between pH and volume regulation complicates efforts to determine which, if either, might be responsible for inhibition of migration after NHE blockade. Our experiments provide circumstantial evidence favoring cell volume as being of greater importance in mediating the effect of NHE on migration. 1 μmol/liter EIPA reduces pH_i by 0.1 pH units, but migration is not inhibited. Even when cells are acidified by 0.5 pH units with 30 mmol/liter formate, they crawl at their normal rate. Conversely, the degree of inhibition of migration by 10 μmol/liter EIPA is not further increased by simultaneous application of EIPA and propionate, although pH_i is 0.1 pH units more acidic in the latter condition. Finally, parallel operation of NHE and anion exchangers in the lamellipodium suggests that they might modulate migration via their influence on cell volume. Thus, our experiments indicate that increased NHE activity uncouples cell migration from pH_i. This observation may be of pathophysiological relevance for leukocyte or tumor cell migration, since these cells often migrate into or out of acidotic tissue.

How can changing cell volume modulate migration? In our view, the distribution of NHE and anion exchanger points to a possible explanation. They are both localized at the leading edge of the lamellipodium. One of the mechanisms involved in the protrusion of the lamellipodium is gel-osmotic water flow into migrating cells at their leading edge (Oster and Perelson, 1987). Due to their localization in the same region of the cell, NHE and anion exchanger can support the protrusion of the lamellipodium by causing osmotic water uptake

(a form of isosmotic regulatory volume increase) at the leading edge. NHE mediates the uptake of ~1% of the cellular cation content per minute. Taking the osmotically obliged water uptake into account, the transporters in tandem would account for the uptake of 1% of total cell volume per minute at the leading edge of the lamellipodium. If, for the sake of argument, the resulting swelling exclusively affects the protruding leading edge [a cross-linked actin filament network resists osmotic water flow to some extent (Ito et al., 1992)], one can calculate that the lamellipodium will advance at a rate of ~1 μm/min, exactly the normal rate of migration. Although an oversimplification, this example demonstrates that salt and water uptake at the leading edge of migrating cells can be of a magnitude sufficient to contribute to lamellipodial protrusion.

By measuring cell volume of MDCK-F cells with the atomic force microscope, we provided indirect support for the idea of localized osmotic swelling at the leading edge of migrating cells. The volume of lamellipodium and cell body can change independently from each other since activating or inhibiting a Ca^{2+}-sensitive K^+ channel exclusively affects the volume of the cell body (Schneider et al., 2000). Physiologically, the Ca^{2+}-sensitive K^+ channel is responsible for intermittent shrinkage of the cell body (Schwab et al., 1995; Schneider et al., 2000). Thus, with respect to salt and water transport, migration can be modeled as regulatory volume increase at the leading edge of the cell coordinated with regulatory volume decrease at the cell body (Lang et al. 1998).

It is notable that NHE blockade inhibits migration to the same degree whether in HEPES buffer or in $CO_2/$

HCO$_3^-$-buffered Ringer's solution. The absence of exogenous HCO$_3^-$ might be expected to inhibit anion (Cl$^-$/HCO$_3^-$) exchange in a HEPES-buffered solution and diminish the impact of NHE activity on cell volume. Intracellular HCO$_3^-$ production appears not to drive Cl$^-$/HCO$_3^-$ exchange activity since blockade of carbonic anhydrase with 100 μmol/liter acetazolamide has no effect on cell migration in nominally CO$_2$/HCO$_3^-$-free solutions and since it does not pronounce the inhibitory effect of DIDS in HEPES-buffered Ringer's solution. However, we showed that the anion exchanger in MDCK-F cells also accepts OH$^-$ ions as counterions, although at an $\sim$10-fold lower transport rate than its Cl$^-$/HCO$_3^-$ exchange rate. A similar observation was made for recombinant AE2 Cl$^-$/HCO$_3^-$ exchanger, which can also function at ambient CO$_2$ conditions (Jiang et al., 1994). This lower transport rate of the anion exchanger is of a magnitude similar to that of NHE and thereby suffices for normal locomotion in HEPES-buffered solution. Moreover, AE2-mediated Cl$^-$/base exchange in *Xenopus* oocytes exhibits nearly equivalent rates at room air and in 5% CO$_2$ when assessed during the hypertonic phase of secondary regulatory volume increase (RVI; Jiang et al., 1997). It is possible that the lamellipodium of a crawling MDCK-F cell, undergoing a form of localized RVI, presents a similar local environment, either accelerating Cl$^-$/OH$^-$ exchange or enhancing its affinity for intracellular HCO$_3^-$.

While we think that local regulation of cell volume is of major importance for the effect of NHE and anion exchanger on migration, we do not know the exact molecular mechanism by which these transporters elicit their action. Presently, we cannot rule out the possible involvement of changes in pH$_i$. In amoeba, bundling and cross linking of actin filaments by elongation factor 1α is increased when pH$_i$ falls (Edmonds et al., 1995). Thus, pH$_i$ can affect the structural plasticity of the cytoskeleton, which is important for its remodeling during migration. The relative role of pH$_i$ for migration probably varies between different cell types. Melanoma cells appear to be more sensitive than MDCK-F cells to propionate-induced intracellular acidification since propionate inhibits migration of human melanoma cells, but not of MDCK-F cells.

NHE1 is concentrated at sites of focal contact (Grinstein et al., 1993; Plopper et al., 1995). This localization appears to be important for RhoA-dependent formation of stress fibers (Vexler et al., 1996) and for integrin-mediated signaling events during cell adhesion (Schwartz et al., 1991). Recently, NHE was shown to interact via a regulatory cofactor, NHE-RF, with merlin. Both NHE-RF and merlin colocalize to membrane ruffles, filopodia, and microvilli; i.e., to similar structures, as shown for NHE1 in fibroblasts (Grinstein et al., 1993; Murthy et al., 1998). Merlin and related ERM proteins

have actin binding sites (Martin et al., 1997), and they have been implicated in Rho-dependent cytoskeletal reorganization (Mackay et al., 1997). Thus, it appears feasible that NHE exerts its effect on the actin-based cytoskeleton through a physical interaction with NHE-RF and MERM proteins. In gastric parietal cells, the anion exchanger AE2 colocalizes at the basolateral membrane with a form of ankyrin (Jöns and Drenckhahn, 1998) to which it may (Jöns and Drenckhahn, 1998) or may not (Morgans and Kopito, 1993) bind. A similar scaffolding or binding protein may serve to concentrate both ion exchangers at the front of MDCK-F cells. However, despite the many open questions that remain, our findings of the polarization of NHE and anion exchanger in MDCK-F cells emphasize that distinct membrane domains are present not only in sessile neuronal or epithelial cells, but also in migrating cells.

REFERENCES

Bereiter-Hahn, J., and M. Voth. 1988. Ionic control of locomotion and shape of epithelial cells: II. Role of monovalent cations. *Cell Motil. Cytoskelet.* 10:528–536.

Boron, W.F. 1983. Transport of H$^+$ and ionic weak acids and bases. *J. Membr. Biol.* 72:1–16.

Brown, D., J. Lydon, M. McLaughlin, A. Stuart-Tilley, R. Tyszkowski, and S. Alper. 1996. Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem. Cell Biol.* 105:261–267.

Condeelis, J. 1993. Life at the leading edge: the formation of cell protrusions. *Annu. Rev. Cell Biol.* 9:411–444.

Cunningham, C.C., J.B. Gorlin, D.J. Kwiatkowski, J.H. Hartwig, P.A. Janmey, H.R. Byers, and T.P. Stossel. 1992. Actin-binding protein requirement for cortical stability and efficient locomotion. *Science.* 255:325–327.

Edmonds, B.T., J. Murray, and J. Condeelis. 1995. pH regulation of the F-actin binding properties of *Dyctyostelium* elongation factor 1α. *J. Biol. Chem.* 270:15222–15230.

Grinstein, S., M. Woodside, T.K. Waddell, G.P. Downey, J. Orlowski, J. Pouyssegur, D.C.P. Wong, and J.K. Foskett. 1993. Focal localization of the NHE-1 isoform of the Na$^+$/H$^+$ antiport: assessment of effects on intracellular pH. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:5209–5218.

Grinstein, S., J.D. Goetz, W. Furuya, A. Rothstein, and E.W. Gelfand. 1984. Amiloride-sensitive Na$^+$–H$^+$ exchange in platelets

and leukocytes: detection by electronic cell sizing. *Am. J. Physiol. Cell Physiol.* 247:C293–C298.

Hallows, K.R., C.H. Packman, and P.A. Knauf. 1991. Acute cell volume changes in anisotonic media affect F-actin content of HL-60 cells. *Am. J. Physiol. Cell Physiol.* 261:C1154–C1161.

Ito, T., A. Suzuki, and T.P. Stossel. 1992. Regulation of water flow by actin-binding protein-induced actin gelation. *Biophys. J.* 61:1301–1305.

Jiang, L., M.N. Chernova, and S.L. Alper. 1997. Secondary regulatory volume increase conferred on *Xenopus* oocytes by expression of AE2 anion exchanger. *Am. J. Physiol. Cell Physiol.* 272:C191–C202.

Jiang, L., A. Stuart-Tilley, J. Parkash, and S.L. Alper. 1994. pH_i and serum regulate AE2-mediated Cl^-/HCO_3^- exchange in CHOP cells of defined transfection status. *Am. J. Physiol. Cell Physiol.* 267:C845–C856.

Jöns, T., and D. Drenckhahn. 1998. Anion exchanger 2 (AE2) binds to erythrocyte ankyrin and is colocalized with ankyrin along the basolateral plasma membrane of human gastric parietal cells. *Eur. J. Cell Biol.* 75:232–236.

Lang, F., G.L. Busch, M. Ritter, H. Völkl, S. Waldegger, E. Gulbins, and D. Häussinger. 1998. Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* 78:247–306.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell.* 84:359–369.

Mackay, D., F. Esch, H. Furthmayr, and A. Hall. 1997. Rho- and rac-dependent assembly of focal adhesion complexes and actin filaments in permeabilized fibroblasts: an essential role for ezrin/radixin/moesin proteins. *J. Cell Biol.* 138:927–938.

Martin, M., C. Roy, P. Montcourrier, A. Sahuquet, and P. Mangeat. 1997. Three determinants in ezrin are responsible for cell extension activity. *Mol. Biol. Cell.* 8:1543–1557.

Mitchison, T.J., and L.P. Cramer. 1996. Actin-based cell motility and cell locomotion. *Cell.* 84:371–379.

Morgans, C.W., and R.R. Kopito. 1993. Association of the brain anion exchanger, AE3, with the repeat domain of ankyrin. *J. Cell Sci.* 105:1137–1142.

Murthy, A., C. Gonzalez-Agosti, E. Cordero, D. Pinney, C. Candia, F. Solomon, J. Gusella, and V. Ramesh. 1998. NHE-RF, a regulatory cofactor for Na^+/H^+ exchange, is a common interactor for merlin and ERM (MERM) proteins. *J. Biol. Chem.* 273:1273–1276.

Noel, J., and J. Pouyssegur. 1995. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am. J. Physiol. Cell Physiol.* 268:C283–C296.

Oberleithner, H., H.-J. Westphale, and B. Gaßner. 1991. Alkaline stress transforms Madin-Darby canine kidney cells. *Pflügers Arch.* 419:418–420.

Oster, G.F., and A.S. Perelson. 1987. The physics of cell motility. *J. Cell Sci. Suppl.* 8:35–54.

Plopper, G.E., H.P. McNamee, I.E. Dike, K. Bojanowski, and D.E. Ingber. 1995. Convergence of integrin and growth factor receptor signalling pathways within the focal adhesion complex. *Mol. Biol. Cell.* 6:1349–1365.

Reinhardt, J., N. Golenhofen, O. Pongs, H. Oberleithner, and A. Schwab. 1998. Migrating transformed MDCK cells are able to

structurally polarize a voltage-activated K^+ channel. *Proc. Natl. Acad. Sci. USA.* 95:5378–5382.

Ritter, M., P. Schratzberger, H. Rossmann, E. Wöll, K. Seiler, U. Seidler, U. Reinisch, C.M. Kahler, H. Zwierzina, H.-J. Lang, et al. 1998. Effect of inhibitors of Na^+/H^+ exchange and gastric H^+/K^+ ATPase on cell volume, intracellular pH and migration of human polymorphonuclear leukocytes. *Br. J. Pharmacol.* 124:627–638.

Rosengren, S., P.M. Henson, and G.S. Worthen. 1994. Migration-associated volume changes in neutrophils facilitate the migratory process in vitro. *Am. J. Physiol. Cell Physiol.* 267:C1623–C1632.

Schneider, S.W., P. Pagel, C. Rotsch, T. Danker, H. Oberleithner, M. Radmacher, and A. Schwab. 2000. Volume dynamics in migrating epithelial cells imaged with atomic force microscopy. *Pflügers Arch.* 439:297–303.

Schwab, A., J. Reinhardt, P. Seeger, B. Schuricht, and P.C. Dartsch. 1999. K^+ channel activity modulates migration and the actin cytoskeleton of transformed renal epithelial cells by controlling cell volume. *Pflügers Arch.* 438:330–337.

Schwab, A., F. Finsterwalder, U. Kersting, T. Danker, and H. Oberleithner. 1997. Intracellular Ca^{2+} distribution in migrating transformed renal epithelial cells. *Pflügers Arch.* 434:70–76.

Schwab, A., K. Gabriel, F. Finsterwalder, G. Folprecht, R. Greger, A. Kramer, and H. Oberleithner. 1995. Polarized ion transport during migration of transformed Madin-Darby canine kidney cells. *Pflügers Arch.* 430:802–807.

Schwab, A., L. Wojnowski, K. Gabriel, and H. Oberleithner. 1994. Oscillating activity of a Ca^{2+}-sensitive K^+ channel—a prerequisite for migration of alkali-transformed Madin-Darby canine kidney (MDCK-F) cells. *J. Clin. Invest.* 93:1631–1636.

Schwartz, M.A., C. Lechene, and D.E. Ingber. 1991. Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin alpha5 beta 1, independent of cell shape. *Proc. Natl. Acad. Sci. USA.* 88:7849–7853.

Simchowitz, L., and E.J. Cragoe, Jr. 1986. Regulation of human neutrophil chemotaxis by intracellular pH. *J. Biol. Chem.* 261:6492–6500.

Stuart-Tilley, A., C. Sardet, J. Pouysségur, M.A. Schwartz, D. Brown, and S.L. Alper. 1994. Immunolocalization of anion exchanger AE2 and cation exchanger NHE1 in distinct, adjacent cells of gastric mucosa. *Am. J. Physiol. Cell Physiol.* 266:C559–C568.

Tonetti, M., M. Budnick, and R. Niedermann. 1990. Receptor-stimulated actin polymerization requires acidification in human PMNs. *Biochim. Biophys. Acta.* 1054:154–158.

Van Duijn, B., and K. Inouye. 1991. Regulation of movement speed by intracellular pH during *Dictyostelium discoideum* chemotaxis. *Proc. Natl. Acad. Sci. USA.* 88:4951–4955.

Vexler, Z.S., M. Symons, and D.L. Barber. 1996. Activation of Na^+-H^+ exchange is necessary for RhoA-induced stress fiber formation. *J. Biol. Chem.* 271:22281–22284.

Weintraub, W.H., and T.E. Machen. 1989. pH regulation in hepatoma cells: roles for Na-H exchange, Cl^--HCO_3 exchange, and Na-HCO_3 cotransport. *Am. J. Physiol. Gastrointest. Liver Physiol.* 257:G317–G327.