Na+/H+ exchange and pH regulation in the control of neutrophil chemokinesis and chemotaxis

Hisayoshi Hayashi,1 Orit Aharonovitz,1 R. Todd Alexander,1,2 Nicolas Touret,1 Wendy Furuya,1 John Orlowski,3 and Sergio Grinstein1,4

1Program in Cell Biology and 2Department of Pediatrics, the Hospital for Sick Children, Toronto, Ontario; 3Department of Physiology, McGill University, Montreal; and 4Department of Biochemistry, University of Toronto, Toronto, Canada

Submitted 28 May 2007; accepted in final form 17 December 2007

Abstract

Three lines of evidence suggest the involvement of NHE1 in motility. First, removal of extracellular Na+ was found to impair cell motility (15, 20). A similar effect was noted when Na+/H+ exchange was inhibited by amiloride and by analogs of this diuretic that specifically inhibit NHE1 (14, 15). Finally, cell migration was found to be markedly reduced in mutant cells lacking functional NHE1 (4). Although its role is well documented, the manner whereby Na+/H+ exchange initiates and/or supports cell migration is not clearly defined. Several possible mechanisms of action can be envisaged. The cytosolic alkalization that results from stimulation of NHE1 may signal the remodeling of the cytoskeleton. Indeed, artificially imposed changes in intracellular pH (pHi) have been demonstrated to alter the state of actin polymerization (24). Another possibility is that NHE1 induces a gain of osmotolites and water and that the associated swelling propels the extension of pseudopods (19, 22). Net osmotic gain can result from Na+/H+ exchange, because hydrogen ions that are extruded from the cytosol can be replaced by intracellular buffers. Moreover, the eventual alkalization drives chloride into the cells in exchange for bicarbonate, ultimately yielding NaCl uptake. A third potential mechanism was suggested by Denker and Barber (5), who found that Na+/H+ exchange-deficient cells adhered abnormally to the substratum. These authors proposed that NHE1 is required for the proper assembly of focal adhesion complexes, by a mechanism requiring ezrin. The integrins that anchor such focal adhesions are thought to sense not only the physical presence of the exchanger but also its activity. In this regard, Schwab and colleagues (19, 22) believe that NHE1 activity at the leading edge of migrating cells produces a localized extracellular acidification that facilitates adhesion via integrins. Finally, it is conceivable that NHE1 supports cell motility indirectly, by maintaining pHi in the physiological range while the contractile apparatus of the cells generates excess metabolic acid. Although less attractive, this housekeeping effect should not be disregarded.

Much of the work to date analyzing the involvement of NHE1 in cell motility has been performed using immortalized cells in culture. To facilitate the analysis of migration, mutant clones of melanoma or Madin-Darby canine kidney (MDCK) cells with particularly high mobility have been selected for study. Much less is known about the behavior of primary cells, because most of these migrate poorly and are difficult to isolate. Polymorphonuclear leukocytes (neutrophils) are an exception. These cells play a central role in innate immunity by...
sensing and migrating to sites of infection, where they ingest and destroy invading microorganisms. Circulating neutrophils are readily isolated from blood and, when challenged in vitro by chemoattractants, show prodigious rates of stimulated migration (chemokinesis). Directed migration (chemotaxis) is observed when the cells are exposed to a gradient of the attractant. For these reasons, we chose to study human neutrophils to reexamine the role and mechanism of action of NHE1 in cell migration. To better correlate the activity of NHE1 with cell migration, we used a modified microscopy setup that facilitates the simultaneous measurement of pH by fluorescence ratio imaging while visualizing the cells by differential interference contrast (DIC) to monitor and quantify motility.

MATERIALS AND METHODS

Materials and solutions. Heparin was obtained from Organon Teknika (Toronto, ON). Dextran T500 and Percoll were purchased from Pharmacia (Milwaukee, WI). Nigericin, dihydrodorhamidine 123 (DHR), and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-ace toxy methylester (AM), and SNARF-5F-AM were obtained from Molecular Probes (Eugene, OR). N-formyl-Met-Leu-Phe (fMLP), ethylisopropyl amiloride (EIPA), and cytochalasin D were purchased from Sigma Chemical (St. Louis, MO). Fibronectin was obtained from Roche Diagnostics (Laval, Quebec). Collagen G was acquired from Biochrome. All other chemicals were of analytical grade and were obtained from Aldrich Chemical (Milwaukee, WI). Compound HOE694 was a generous gift from Hoechst (Zurich, Switzerland).

The isotonic Na⁺-rich medium contained (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 5.5 glucose, and 10 HEPES, pH 7.4. Isotonic Na⁺-free medium contained (in mM) 140 KCl, 1 CaCl₂, 1 MgSO₄, 5.5 glucose, and 25 N-methyl-D-glucammonium (NMG)-HEPES, pH 7.4. Phosphate-buffered saline (PBS) contained (in mM) 150 NaCl, 10 KCl, 8 sodium phosphate, and 2 potassium phosphate, pH 7.4.

Cells. Human neutrophils (>98% pure) were isolated from heparinized whole blood obtained by venipuncture, using dextran sedimentation and discontinuous plasma-Percoll gradients as described by Böyum (1). Alternatively, they were isolated by differential sedimentation of whole blood on PolyPorphrep (Axis Shield), as described by the manufacturers. After isolation, cells were resuspended in HEPES-buffered RPMI 1640 at 10⁷ cells/ml and kept at room temperature, rotating slowly until used (generally ≤4 h). Similar results were obtained when the cells were stored on ice. MDCK-F cells were the kind gift of Prof. A. Schwab (University of Münster, Münster, Germany). They were cultured in MEM with 5% serum.

Chemokinesis and chemotaxis assays. To measure neutrophil migration, round glass coverslips (25-mm diameter; Thomas Scientific, Swedesboro, NJ) coated with 5 μg/cm² fibronectin were mounted in Leiden chambers, overlaid with 1 ml of the indicated solution, and placed on the heated stage of the microscope. After 100-μl aliquot of the neutrophil suspension was added, after which the cells were allowed to settle and adhere for 10 min. Nonadherent cells were allowed to settle and adhere onto the coverslip, and images of the migrating cells were acquired every 2 min, during incubation at 37°C. All measurements were performed at 37°C.

Video microscopy and pH measurements. To measure cytosolic pH (pHi), 1-ml aliquots of the cell suspension were incubated for 20 min with 2 μM BCECF-AM or 10 μM SNARF-5F-AM and washed once. Simultaneous imaging of cellular BCECF or SNARF fluorescence and of cell morphology by DIC was performed as described (3). Selection of excitation filters and image acquisition were controlled by the Metafluor software (Universal Imaging, West Chester, PA) running on a Dell Optiplex DGX 590 computer interfaced with a Photometrics Micromax camera via a 12-bit GPIB/IIA board (National Instruments, Foster City, CA).

For image processing, areas corresponding to individual migrating neutrophils were selected as regions of interest, and fluorescence intensity at 515 nm was measured with excitation at 490 and 440 nm, respectively, representing the fluorophores BCECF and SNARF, respectively. The pH gradient was generated by addition of K⁺-free media buffered to varying pH values (between 6.3 and 7.7) or by addition of media of pH 7.4 with varying concentrations of K⁺ that contained the K⁺/H⁺ exchanging ionophore nigericin (5 μg/ml). Because at equilibrium the ratios of intracellular and extracellular K⁺ and H⁺ concentrations are equal ([K⁺]i/[K⁺]o), the desired pHi can be calculated from the imposed [K⁺]i gradient and pHo, assuming a [K⁺]i of 140 mM. Based on these considerations, the solutions used contained the following: for pHi 6.8, 35 mM K⁺ and 102 mM NMG⁺; for pHi 6.4, 14 mM K⁺ and 123 mM NMG⁺; and for pHi 6.0, 5.6 mM K⁺ and 131.4 mM NMG⁺. The KCI product was maintained constant to preclude changes in cell volume and glucanate, and HEPES was used to balance the osmolarity.
and buffer the pH. In addition, all solutions contained 1 mM CaCl₂, 1 mM MgSO₄, and 5.5 mM glucose.

Other methods. Superoxide production was measured in individual neutrophils using DHR. The fluorescence of rhodamine 123, the oxidized product of DHR, was measured using the optical system described above.

All ion substitutions and the addition of inhibitors were made immediately before the observation period, to avoid unwanted effects of pH changes that may develop gradually upon inhibition of NHE1. Experimental data are presented as the mean ± SE of the indicated number of experiments. When two populations were compared, data were analyzed by either unpaired or paired Student’s t-test, as applicable. Multiple populations were compared using a one-way ANOVA and Dunnett’s multiple comparison test (Prism; GraphPad, San Diego, CA).

RESULTS

Changes in pHobserved during the induction of chemokinesis with fMLP. To serve as signals that initiate cell motility, changes in pHi must precede the onset of migration. We therefore analyzed the temporal relationship between these events. Cell migration and pHi were monitored simultaneously in human neutrophils by a combination of DIC microscopy and dual-excitation fluorescence ratio imaging. All measurements were performed in media that were nominally bicarbonate free, to minimize the contribution of anion exchangers and cotransporters. Freshly isolated cells were allowed to settle onto fibronectin-coated coverslips. Shortly after adherence, such cells from 3 separate experiments) that was random. The pH under these conditions was 7.38 ± 0.07. Chemokinesis was next stimulated by the addition of 10⁻⁸ M fMLP. The chemooattractant induced a biphasic change in motility: a rapid and transient change that was followed by a more gradual and sustained increase (Fig. 1). The stimulation of cell migration was accompanied by similarly biphasic changes in pHi; a small (0.05 ± 0.05 pH units; >75 cells from 5 determinations), yet reproducible acidification accompanied the initial burst in chemokinesis, while a marked alkalinization developed in parallel with the secondary increase in mobility. On average, pHi increased by 0.26 ± 0.07 pH units within 5 min of stimulation and remained elevated for the duration of the experiment. The origin of these pH changes and their significance to the chemokinetic response was studied next.

Stimulation of Na⁺/H⁺ exchange during chemokinesis. Two independent approaches indicate that Na⁺/H⁺ exchange is responsible for the increase in pHi observed in Fig. 1. First, the fMLP-induced alkalinization was absent when Na⁺ was omitted from the bathing medium. Instead, a monophasic, pronounced acidification was recorded under these conditions (Fig. 2). In four such experiments, pHi dropped by 0.6 ± 0.02 pH units (n > 60 cells) over 5 min and failed to recover thereafter. Virtually identical results were obtained when chemokinesis was activated in cells bathed in Na⁺-rich solutions that contained the inhibitor HOE-694 (Fig. 2). These observations not only confirm that alkalinization requires Na⁺/H⁺ exchange but also indicate that NHE1 is the isoform involved. At the concentration used (10 μM), HOE-694 completely inhibits this isoform, but not NHE3 (2). Accordingly, NHE1 has been detected in neutrophils by both RT-PCR and immunochemical methods (6).

Fig. 1. Neutrophil chemokinesis and associated intracellular pH (pHi) changes. Freshly isolated human neutrophils were loaded with BCECF, plated onto fibronectin-coated coverslips, and allowed to adhere. The initial location of the adherent cells was determined by differential interference contrast (DIC) microscopy (A), and the resting pHi was determined by ratio imaging of the BCECF fluorescence. Next, chemokinesis was stimulated by addition of 10⁻⁸ M N-formyl-Met-Leu-Phe (fMLP), and DIC and fluorescence images were acquired at 15-s intervals for the next 600 s. The position of the cells at the end of this interval is shown in B, where the trajectories of the cells, traced as described in MATERIALS AND METHODS, are also shown. Bar, 20 μm. The relationship between pH, (■) and the rate of migration (▲) is shown in C. The addition of 10⁻⁸ M fMLP is indicated by the arrow. Data are means ± SE of over 15 cells from 3-5 different experiments.
NADPH oxidase activation contributes to the fMLP-induced intracellular acidification. Inhibition of Na\(^+\)/H\(^+\) exchange in migrating neutrophils unmasked a pronounced acidification (Fig. 2), suggesting that fMLP stimulated the net generation of acid. The chemoattractant is known to activate the NADPH oxidase, an enzymatic complex that generates acid equivalents during the course of superoxide synthesis (3). Indeed, the formation of reactive oxygen intermediates during fMLP-induced chemokinesis was verified using DHR, as illustrated in Fig. 3A.

In the next series of experiments, chemokinesis was stimulated in cells treated with diphenylene iodonium (DPI), a potent antagonist of the NADPH oxidase, to assess whether this enzyme and the associated metabolic pathways contribute to the acidification. As anticipated, the inhibitor virtually eliminated the production of reactive oxygen intermediates (Fig. 3B), and this effect was specific, since fMLP-induced chemokinesis was unaffected (Fig. 4). The effect of DPI on the pH\(_i\) changes associated with chemokinesis is illustrated in Fig. 3D. The acidification induced by fMLP in Na\(^+\)-free solution was partially inhibited by DPI (by 45%, ΔpH = 0.33 ± 0.05 over 5 min), implying that a fraction of the acid produced in response to the chemoattractant is generated by the NADPH oxidase.

At least part of the remaining acid accumulated during chemokinesis is likely to have originated from metabolic energy consumed by the contractile machinery of the cells. This notion was tested by pretreating the cells with cytochalasin D, which precludes the remodeling of the actin cytoskeleton. The effectiveness of the inhibitor was ascertained before the determinations of pH\(_i\) by directly measuring its effects on cell motility (Fig. 4). As illustrated in Fig. 3D, the acidification caused by fMLP in Na\(^+\)-deficient medium was greatly reduced when the cells were treated with cytochalasin (ΔpH = 0.12 ± 0.05 over 5 min). Of note, the generation of oxygen intermediates was also virtually eliminated by cytochalasin D (Fig. 3C), consistent with earlier observations that the respiratory burst in suspended neutrophils is small and transient and that substantial amounts of superoxide are generated only after the cells spread on the substratum in an actin-dependent manner (3).

Together, the preceding results indicate that during chemokinesis acid is generated by at least three sources: the largest fraction is contributed by the metabolic pathways that supply the contractile machinery; a second component is associated with the NADPH oxidase, while a small residual amount of acid is generated by other, unidentified sources.

**Relationship between pH\(_i\) and neutrophil chemokinesis.** The preceding data indicate that, despite production of sizable amounts of metabolic acid equivalents, neutrophils bathed in physiological (Na\(^+\) rich) media undergo a conspicuous cytosolic alkalinization when stimulated by fMLP. Moreover, the close temporal relationship between pH\(_i\) and the sustained phase of chemokinesis suggests that these events may be functionally linked. This premise was tested by measuring the motility of cells suspended in Na\(^+\)-free medium. Figure 4 shows that, despite the failure of the cells to become alkaline, their ability to migrate randomly in response to fMLP was not significantly altered. In three separate experiments the rate of migration, measured over 10 min, was 0.13 ± 0.01 and 0.12 ± 0.01 μm/s in Na\(^+\)-containing and Na\(^+\)-free media, respectively (P > 0.2). Very similar results were obtained when chemokinesis was measured in neutrophils bathed in Na\(^+\)-rich medium containing HOE-694. As before, the cells migrated at near normal rates, despite the pronounced cytosolic acidification seen in the presence of the NHE1 inhibitor (Figs. 2 and 4). The statistically insignificant decrease noted in both instances occurred only toward the end of the observation period, when pH\(_i\) reached its most acidic level.

**Chemotaxis does not require Na\(^+\)/H\(^+\) exchange activity.** The preceding results were unexpected, to the extent that both spontaneous and stimulated cell migration have been reported to depend on Na\(^+\)/H\(^+\) exchange (4, 11, 21). It is conceivable that in neutrophils the exchangers may be required for directed, but not for random, migration. To test this possibility, we measured the rate of migration toward a point source of fMLP in the presence and absence of Na\(^+\) and in the presence of NHE1 inhibitors. To measure directed migration (chemotaxis), fMLP was allowed to diffuse at a constant rate from the tip of an agar-filled glass micropipette. As illustrated by the image sequence in Fig. 5, A–D, neutrophils detect and move in the direction of the gradient of the chemoattractant emanating from the pipette. Figure 5 summarizes measurements of both directed migration (the net distance traveled toward the chemotactic source) and total migration (a measure of the overall distance traveled by the cells, irrespective of direction). The total migration was only slightly greater than the net migration, implying that the neutrophils directed themselves efficiently toward the pipette. The ratio of net to total migration in control cells was 0.93 ± 0.02. Importantly, neither the motility of the cells nor their ability to orient themselves in the direction of the chemoattractant gradient required the presence of extracellular Na\(^+\). Therefore, cytosolic alkalinization is not indispensable for chemotaxis, a conclusion that is also supported by the insensitivity of the process to HOE-694 and to another potent NHE1 inhibitor, EIPA (Fig. 5E). The ratio of net to total migration was 0.89 ± 0.03 and 0.93 ± 0.03 in cells incubated with EIPA and HOE-694, respectively, and 0.78 ± 0.07 in Na\(^+\)-free medium.
The preceding results are seemingly at odds with earlier literature suggesting that alterations in pH are essential to trigger the migratory response. Our measurements of pHi reflect the average of the entire cell. It is conceivable that these measurements differ from the submembranous pH, which would be affected more markedly by the activity of NHE1, and that this local pH is the main determinant of cell motility. To analyze this possibility, we performed experiments where the intracellular pH was clamped at the desired level using nigericin. When added at sufficiently high concentrations, this electroneutral K⁺/H⁺-exchanging ionophore will outstrip the activity of the endogenous H⁺ (equivalent) transporters and will therefore be the primary determinant of pHi. By setting the transmembrane K⁺ gradient and pHₑ to predetermined levels, pHi can be clamped to any desired value. Importantly, because the ionophore operates at the plasma membrane, this procedure ensures that the submembranous pH is controlled most effectively.

In the experiments of Fig. 6A, [K⁺]ₑ was set to a value identical to that found intracellularly, determined earlier using flame photometry (12), and pHₑ was 7.3. The effectiveness of the pH clamp was verified by monitoring pHₑ before and during the induction of chemokinesis. The biphasic pH changes that normally accompany stimulation by fMLP were obliterated by the combined treatment with nigericin and elevated [K⁺] (cf. Figs. 1C and 6A). Despite the stability of pHₑ, which remained invariant at 7.3 throughout the experiment, the neutrophils displayed normal chemokinetic behavior. The two distinct phases of motility seen under physiological conditions (Fig. 1C) were faithfully replicated in the presence of nigericin/K⁺.
Fig. 5. Role of NHE1 in fMLP-induced chemotaxis. Freshly isolated human neutrophils were plated onto fibronectin-coated coverslips and allowed to adhere. A micropipette filled with agar containing 10^{-7} M fMLP was then lowered onto the visual field, and the response of the cells was monitored by DIC microscopy. Representative images, acquired at the indicated times after exposure to the chemoattractant, are shown in A–D. The distance between the centroid of the cells and the mouth of the pipette is indicated by the double-headed arrows; the thin lines in D show the cell tracks. Bar, 20 μm. E: summary of measurements of total and directed (net) migration during chemotaxis from experiments like that in A–D. Cells were bathed in Na^{+}-rich medium containing EIPA (10 μM) or HOE-694 (10 μM) or in Na^{+}-free medium [replaced with equimolar N methyl-D-glucammonium (NMG^+)] and tracked for up to 5 min. The distance migrated per 100 s is shown. Data are means ± SE of at least 7 cells from 3–5 experiments for each condition.

(Fig. 6A). These experiments argue against the hypothesis that localized submembranous pH changes caused by NHE1 are critical for cell migration.

The apparent discrepancy between our data and some reports in the literature may be explained by the different cell types and conditions used. In particular, it is noteworthy that because neutrophils migrate very rapidly, our experiments were comparatively short (5–10 min), whereas those performed by others required much longer times, due to the slower rates of migration of the cells under investigation. It is possible that following prolonged inactivation of NHE1, the pH gradually drifted to acidic levels that compromised cell motility. To evaluate this possibility, we measured the pH dependence of chemokinesis. To this end, we resorted once more to nigericin/K^+ to fix and maintain pH_i to the desired values. In the experiments illustrated by the open bars in Fig. 6B, [K^+]_i was fixed at the intracellular level, as above, and pH_i was driven to the value of choice by modifying pH_o. Figure 6B shows that although chemokinesis was robust when the pH was slightly alkaline (physiological), cell motility decreased markedly at more acidic levels.

In the preceding experiments, manipulation of pH_i required the imposition of parallel changes in pH_o. The observed effect cannot therefore be attributed with certainty to the changes in cytosolic pH. To circumvent this limitation, we devised a protocol whereby pH_i could be manipulated while maintaining pH_o at the normal physiological level of 7.4. To this end, cells were treated with nigericin in media of varying [K^+]. All
media were Na⁺ free, and osmolarity was maintained using the impermeant cation NMG⁺. The results of such experiments are illustrated by the filled bars in Fig. 6B. As before, acidification of the cytosol led to a gradual inhibition of chemokinesis, but the inhibitory effect was more gradual, with little effect seen at pHᵢ 6.8 and nearly complete inhibition at 6.0. These observations confirm that chemokinesis is sensitive to the intracellular pH but also reveal that the process is even more sensitive to alterations in pHₒ.

Inhibition of chemokinesis can be exerted not only by an artificially imposed acidification (as shown in Fig. 6B) but also by the metabolic acid generated by the cells, which accumulates when extrusion via Na⁺/H⁺ exchange is impaired. This is illustrated in Fig. 6C, where motility and pHᵢ were measured concomitantly following stimulation of neutrophils by fMLP in Na⁺-free medium. As indicated in Fig. 2, cells stimulated in media devoid of Na⁺ underwent a gradual acidification following addition of the chemoattractant (filled circles in Fig. 6C). The acidification was particularly fast and profound in the experiment chosen for illustration. Note that the chemokinetic activity changed in parallel; cell motility was highest near neutral pHᵢ but declined gradually as the cells acidified (open circles). These findings confirm the pronounced pH sensitivity of chemokinesis and imply that dysregulation of pHᵢ homeostasis can lead to reduced cell motility.

*Are localized changes in pHₒ essential for migration?* Using melanoma cells, Stock et al. (21) found earlier that, as in our experiments, changes in pHₒ had profound effects on the rate of cell migration. On the basis of such observations and the effects of inhibitors of NHE1, these authors proposed that Na⁺/H⁺ exchange directs cell migration by generating a localized extracellular acidification at the leading edge of the cells, which results from focal extrusion of H⁺. This model predicts that neutralization of such localized pHₒ changes would hinder chemokinesis and chemotaxis. This hypothesis was tested in Fig. 7, where the buffering power of the extracellular solution was varied by increasing the concentration of HEPES between 2 and 40 mM. Measuring chemotaxis toward a point source of fMLP, as in Fig. 5, we found that neither the total nor the directed migration were affected by a 20-fold increase in buffering capacity (Fig. 7). These findings argue against a determinant role of pHₒ changes in cell motility.

*Spontaneous migration of immortalized epithelial cells requires Na⁺/H⁺ exchange activity.* Superficially, our data seem to be at odds with those of other investigators (1a, 8, 11, 13, 18, 19, 22) who reported that Na⁺/H⁺ exchange activity is essential for optimal motility. To ensure that our observations in neutrophils were not due to methodological differences, we...
obtained MDCK-F cells, a particularly motile clone of the MDCK line (a kind gift of Dr. A. Schwab). As reported by others (8), we found these cells to be spontaneously motile on collagen-coated surfaces, particularly shortly after plating. Motility was sustained for at least 30 min in physiological, Na+-rich medium but declined gradually when Na+ was omitted or when Na+/H+ exchange was precluded by addition of EIPA (Fig. 8). Clearly, in MDCK-F cells, continued Na+/H+ exchange activity is essential for sustained motility.

**DISCUSSION**

Previous reports showed clearly that the motility of a variety of cell types is depressed by omission of extracellular Na+, by the addition of inhibitors of Na+/H+ exchange, and by mutations that impair the expression or function of NHE1 (4, 11, 14, 21). These studies, which we were able to replicate (Fig. 8), provided compelling evidence that NHE1 is required for optimal cell migration. On the other hand, the precise mechanism underlying this novel aspect of NHE1 function remained controversial. This gap in understanding motivated our experiments.

An increase in pHi could mediate the effects of NHE1 in cell migration. Indeed, our data revealed a striking parallel between the development of cytosolic alkalinization and the sustained phase of chemokinesis (Fig. 1). An elevated pHi could alter the activity of multiple components of the adhesive and motile machinery of the cells. However, various aspects of our results disagree with this notion. First, the initial, intense burst of chemokinesis occurred while the cytosol was, if anything, slightly more acidic than the resting pHi (Fig. 1). Second, we found that neither removal of external Na+ nor pharmacological inhibition of NHE1 affected the rate of chemokinesis or chemotaxis, despite the concomitant development of a pronounced acidification (Figs. 2 and 4). Third, preventing the alkalinization by nigericin-mediated clamping of pHi similarly failed to affect chemokinesis (Fig. 6). Jointly, these findings appear to rule out NHE1-induced alkalinization as the parameter responsible for stimulation of cell motility.

An alternative hypothesis is that by extruding H+, NHE1 generates a localized extracellular acidification that favors directed motion. The proponents of this hypothesis (19, 22) have suggested that improved adherence of integrins to the substratum at a moderately acidic external pH promotes migration in a direction dictated by the polarized distribution of NHE1 in the cell. In accordance with this proposal, we found that neutrophil migration is even more sensitive to pHo than it is to pHi (Fig. 6). Nevertheless, our findings are inconsistent with a key role of external acidification. Briefly, we found that normal neutrophil migration could be initiated and persisted for several minutes under conditions where H+ extrusion was prevented by removing Na+ or by addition of HOE-694 or EIPA. Moreover, increasing the external buffering power, which would be predicted to dampen the pHo changes generated by NHE1, was similarly without effect (Fig. 7).

The entry of Na+ into the cell may be the parameter accounting for the role of NHE1 in motility. A gain in [Na+]i could force Ca2+ into the cell by altering the gradient driving Na+/Ca2+ exchange, and the divalent cation may signal actin remodeling. Alternatively, the osmotic gain may induce swelling to propel lamellipodia forward. As before, however, this possibility seems to be negated by the experiments where motility was found to be near normal in the absence of external Na+ or when NHE1 activity was blocked.

In our experiments, significant inhibition of chemotaxis and chemokinesis was observed only when the pH was manipulated. In particular, we noted that intracellular acidification impaired migration. We also noted that when Na+/H+ exchange was precluded, the cytosolic pH gradually acidified. We therefore propose that NHE1 plays a permissive role in cell migration, by ensuring the maintenance of pHi within a range compatible with normal integrin function and actin remodeling. Although the exchanger itself may not be part of the attachment, signaling, or mechanical events that underlie cell migration, its role in the process can nevertheless be critical. The activation of cells by chemoattractants is associated with a burst in metabolic acid generation. In the case of human neutrophils, the acid equivalents stem from at least three different sources: the consumption of energy associated with actomyosin contraction, the activation of the NADPH oxidase and the accompanying acceleration of the hexose monophosphate shunt, as well as a third, smaller component that remains unidentified. Under normal circumstances, the acid equivalents generated by these pathways are promptly extruded from the cell, largely via NHE1. If left unchecked, however, this ongoing acid generation forces pHi to become progressively more acidic, ultimately reaching levels that can interfere with one or more of the complex steps required for effective cell migration. This could occur when Na+ is omitted or when NHE1 activity is precluded by addition of inhibitors or by mutations in the exchanger. In this event, the rate of migration would decline gradually, and the effects should be more noticeable for prolonged experiments than for short ones, such as the ones we performed presently. Because neutrophils migrate very fast, we were able to record mobility rates accurately over short intervals, before the development of excessive acidification. The inhibitory effect we observed upon inhibition of NHE1 developed gradually (Fig. 6C) and was significant only after many minutes. Of note, the duration of our experiments was considerably shorter than that of virtually all preceding studies, which ranged from 25 min to 24 h (4, 8, 20, 21). We believe that the use of acute rather than more chronic manipulations allowed us

![Fig. 8. Sustained migration MDCK-F cells requires Na+/H+ exchange activity. Renal epithelial MDCK-F cells were plated onto collagen-coated cover-slips, and their migration was determined by DIC microscopy. The cells were bathed with either Na+-rich (●) or Na+-free (NMG+ substituted) medium (●). Where indicated, the cells were bathed in Na+-rich medium containing 10 μM EIPA (●). Data are means ± SE of 44, 27, and 18 determinations for Na+-rich, Na+-free, and EIPA-containing samples, respectively.](http://ajpcell.physiology.org/)

*AJP-Cell Physiol.* • Vol. 294 • February 2008 • www.ajpcell.org
to distinguish a permissive role for NHE1 from the more integral signaling or effector function assumed previously. Indeed, when we measured the spontaneous motility of MDCK-F cells, inhibition of NHE1 produced a comparatively small inhibition of borderline significance in the first 10 min, which became much more pronounced at longer times (Fig. 8).

Our observations do not rule out the possibility that in addition to its pH-regulatory, permissive effect, NHE1 also acts as a physical anchoring site for cytoskeletal components and that this interaction is required for optimal attachment and migration. Evidence furnished largely by Denker and Barber (5) suggests that NHE1 plays a structural role in bridging the membrane with the cytoskeleton. It is noteworthy, however, that NHE1 null mice are viable, implying that the cell migration required for morphogenesis and tissue remodeling occurs in the absence of these exchangers. No immunological complications have been associated with this genotype, which suggests that neutrophil and macrophage chemotaxis are similarly normal.

In summary, our data indicate that chemokinesis and chemotaxis are markedly sensitive to the cytosolic, and especially to the extracellular, pH. Because maintenance of pH i is critical for proper motility, NHE1 appears to be permissive for the process, especially in in vitro experiments that use nominally bicarbonate-free solutions, conditions that minimize the contribution of other pH-regulatory pathways. The exquisite sensitivity of chemotaxis to the extracellular pH may explain the inability of neutrophils to enter and eliminate abscesses and some solid tumors, which have been described to have inordinately acidic pH.

**REFERENCES**


