Vacuolar H\(^+\)-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity

Souad R. Sennoune, Karina Bakunts, Gloria M. Martínez, Jenny L. Chua-Tuan, Yamina Kebir, Mohamed N. Attaya, and Raul Martínez-Zaguilán

Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, Texas 79430-6551

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pHcyt than that of surrounding normal cells (10, 15, 40).

Moreover, tumor cells often produce acidic metabolites (39). Furthermore, tumor cells thrive in a hypoxic microenvironment with an acidic extracellular pH. To survive in this harsh environment, tumor cells must exhibit a dynamic cytosolic pH regulatory system. We hypothesize that vacuolar H\(^+\)-ATPases (V-ATPases) that normally reside in acidic organelles are also located at the cell surface, thus regulating cytosolic pH and exacerbating the migratory ability of metastatic cells. Immunocytochemical data revealed for the first time that V-ATPase is located at the plasma membrane of human breast cancer cells: prominent in the highly metastatic and inconspicuous in the lowly metastatic cells. The V-ATPase activities in isolated plasma membranes were greater in highly than in lowly metastatic cells. The proton fluxes via V-ATPase evaluated by fluorescence spectroscopy in living cells were greater in highly than in lowly metastatic cells. Interestingly, lowly metastatic cells preferentially used the ubiquitous Na\(^+\)/H\(^+\) exchanger and HCO\(_3\)\(^-\)-based H\(^+\)-transporting mechanisms, whereas highly metastatic cells used plasma membrane V-ATPases. The highly metastatic cells were more invasive and migratory than the lowly metastatic cells. V-ATPase inhibitors decreased the invasion and migration in the highly metastatic cells. Altogether, these data indicate that V-ATPases located at the plasma membrane are involved in the acquisition of a more metastatic phenotype.

metastasis; intracellular pH; migration; sodium ion/hydrogen ion exchanger; bicarbonate transport

Maintenance of cytosolic pH (pHcyt) is crucial to normal cell function because many cellular processes have a narrow pH optimum (38). Tumor cells possess high-glycolytic activity and produce acidic metabolites (39). Moreover, tumor cells often exist in a hypoxic microenvironment with a lower extracellular pH (pHex) than that of surrounding normal cells (10, 15, 40). Acidic pHex and hypoxic environment are not permissive for cell growth and have been associated with apoptosis (14, 25).

To minimize the potentially toxic reduction in pHcyt that would accompany growth in a chronically acidic environment, tumor cells must upregulate the proton extrusion mechanism(s) that maintains pHcyt. The ability to upregulate proton extrusion may be essential for tumor cell survival. The influence of pHcyt on many cellular functions has been studied with respect to cell growth (16), cell motility (27), tumorigenesis (36), metastasis (45), apoptosis (14), and drug resistance in cancer cells (28, 50).

Four major types of pHcyt regulatory mechanisms have been identified in tumor cells: Na\(^+\)/H\(^+\) exchangers, bicarbonate (HCO\(_3\)\(^-\)) transporters, proton-lactate symporters, and proton pumps (11, 38, 42).

Recently, the vacuolar H\(^+\)-ATPase (V-ATPase) has emerged as a novel and important pHcyt regulatory system in some specialized cells, including tumor (25, 26, 28). This proton pump is ubiquitously expressed (33, 35), not only in vacuolar membranes but also in plasma membranes (26, 58) of eukaryotic cells. The V-ATPase is a multi-subunit enzyme complex composed of a membrane sector (V\(_0\)) and a cytosolic catalytic sector (V\(_1\)) (35). The integral V\(_0\) domain functions in proton translocation, whereas the peripheral V\(_1\) domain hydrolyzes ATP. V-ATPase is a member of a family of ATP-driven proton pumps responsible for the acidification of intracellular compartments such as endosomes, lysosomes, Golgi-derived vesicles, and clathrin-coated vesicles (35).

In addition to the role of V-ATPase in intracellular compartments, this enzyme is important for plasma membrane functions in various specialized cells (13, 32, 54).

There are several classes of inhibitors of V-ATPase, including the macrocyclic lactones bafilomycin and concanamycin, the benzolactone enamides salicylihalamides and lobotamides, and, more recently, the macrolide lactams chondrophin and poecillastrin (3). Although they exhibit different potencies and selectivity to inhibit V-ATPases from mammals and fungal sources, they all seem to bind to subunit c to exert their effect. Because of the many isoforms, it is possible that mutations may cause resistance to V-ATPase inhibitors. Indeed, mutations in subunit c involved in binding of V-ATPase inhibitors decrease the sensitivity of bafilomycin by ~20- to 60-fold and to concanamycin by ~3-fold in Neurospora crassa (3).

V-ATPase is functionally expressed in plasma membranes of human tumor cells and may have specialized functions in cell growth, differentiation, angiogenesis, and metastasis (25-27).

Furthermore, pHcyt is critical for the cytotoxicity of anticancer agents, and V-ATPase has been implicated in the acquisition of the multidrug resistance phenotype (20, 28, 40).

Therefore, understanding the mechanisms regulating pHcyt and tumor acidity is important for developing new approaches to cancer chemotherapy, and V-ATPase may represent a potential target for cancer chemotherapy (52). We know that V-ATPases at the cell surface play a role in maintaining an alkaline intracellular environment favorable for growth, while maintaining an acidic extracellular environment favorable for invasion (28, 29).

We hypothesize that V-ATPase is important in the acquisition of a more metastatic and invasive phenotype.
Because little is known about the mechanisms of pH34 regulation in breast cancer, we employed human breast cancer cell lines with distinct metastatic potential and determined the distribution and the functional activity of V-ATPase in highly and lowly metastatic cells. For this purpose, we investigated, by immunocytochemistry and confocal laser scanning microscopy, the distribution of V-ATPase in highly and lowly metastatic human breast cancer cells. We determined the enzymatic activity at their plasma membranes, to corroborate the V-ATPase distribution, and in cell homogenates. Then, in living cells, we evaluated the proton fluxes via plasma membrane V-ATPase (pmV-ATPase), as well as via the ubiquitous Na+/H+ exchanger and HCO3-/based H+-transporting mechanisms. We also evaluated the kinetics of the migration and invasion of lowly and highly metastatic cells. We used bafilomycin and concanamycin to study V-ATPase activity, proton flux (JH+), via pmV-ATPase, and migration and invasion because they are better known inhibitors.

MATERIALS AND METHODS

Buffers and chemicals. Cell perfusion buffer (CPB) contained 1.3 mM CaCl2, 1 mM MgSO4, 5.4 mM KCl, 0.44 mM KH2PO4, 110 mM NaCl, 0.35 mM Na2HPO4, 5 mM glucose, 2 mM glutamine, and 20 mM HEPES, at 37°C, and the pH was adjusted to either 7.4 or 8.0, as needed (29). Na+-free CPB consisted of all CPB ingredients, except those containing sodium (110 mM N-methyl-glucamine was substituted for NaCl). High-K+ buffer contained 146 mM KCl, 20 mM NaCl, 5 mM glucose, 2 mM HEPES, 10 mM MES, and 10 mM bicine. The rationale for using these organic buffers was to allow for precise buffering across a wide pH, ranging from 5.0 to 9.0 (29). Bafilomycin A1 and concanamycin were obtained from Wako Chemicals (Richmond, VA). The pH fluorescent indicator carboxyseminaphthodihalo-fluor-1 (SNARF-1)-acetoxymethyl ester (AM) was obtained from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma Chemical (St. Louis, MO), unless indicated otherwise.

Cell culture. Human breast cancer cell lines, MDA-MB-231 (ATCC no. HTB-26; passage 28), MB435s (ATCC no. HTB-129; passage 239), MDA-MB-468 (ATCC no. HTB-132; passage 340), and MCF-7 (ATCC no. HTB-22; passage 148) were purchased from American Type Culture Collection (ATCC). The cells were plated in culture dishes and grown as follows. MB231 and MB468 cells were grown in Leibovitz’s L-15 (ICN Biomedical, Costa Mesa, CA) containing 2 mM glutamine, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. MB435s cells were grown as MB231 cells, except that the media were supplemented with 10 mg/ml insulin. MCF-7 cells were grown in MEM (nonessential amino acids Earle’s balance salt solution) containing 10 mg/ml insulin, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification. MCF-7 cells were grown in MEM (nonessential amino acids Earle’s balance salt solution) containing 10 mg/ml insulin, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification. MCF-7 cells were grown in MEM (nonessential amino acids Earle’s balance salt solution) containing 10 mg/ml insulin, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification. MCF-7 cells were grown in MEM (nonessential amino acids Earle’s balance salt solution) containing 10 mg/ml insulin, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification. MCF-7 cells were grown in MEM (nonessential amino acids Earle’s balance salt solution) containing 10 mg/ml insulin, 24 mM NaHCO3, 10 mM HEPES, 0.067 g/l penicillin, and 0.143 g/l streptomycin. All cell lines were supplemented with 10% FBS (Gibco, Grand Island, NY) under a 95% air-5% CO2 humidification.

plasma membranes. Plasma membranes of breast cancer cells were obtained as described elsewhere, with modifications (7). Briefly, confluent monolayers from ten 100-mm petri dishes were trypsinized three times with 0.05% trypsin, 0.1% EDTA, 0.1 mM CaCl2, 1 mM MgSO4, and 150 mM NaCl, pH 7.4. Cells were then harvested by scraping with a rubber policeman. Membranes were isolated from the resulting cell suspension after hypotonic lysis and differential centrifugation, followed by treatment with sodium iodide (7). The pellet was resuspended in homogenizer buffer (250 mM sucrose, 1 mM EGTA, and 50 mM Tris-HCl) and then applied to the top of 20–40% sucrose gradient and centrifuged at 200,000 g for 1 h at 4°C. The membranes from the interphase 20–40% sucrose were diluted in Tris-EDTA buffer and collected by centrifugation for 30 min at 100,000 g. The final pellet was resuspended in homogenizer buffer and stored at –80°C. Electron microscopy analysis of the plasma membrane fractions showed that they were free of mitochondria and other cellular organelles.

V-ATPase enzymatic activity. The V-ATPase activity in both cell homogenates and isolated plasma membranes from highly and lowly metastatic breast cancer cells was determined from the hydrolysis of radiolabeled ATP, as described (7). Plasma membranes (1 mg/ml) were treated with 0.7 mg/ml deoxycholate for 30 min at 37°C to expose V-ATPase to substrate. Detergent-treated membranes were then diluted 1:10 and incubated at 37°C for a total volume of 0.2-mL ATPase assay buffer containing 25 mM Tris-HCl, 4 mM MgCl2, 0.1 mM EGTA, 4 mM γ-[32P]ATP (Perkin Elmer/NEN Life Sciences), and 50 mM bafilomycin A1, pH 7.0. The V-ATPase activity was estimated by subtracting total ATP hydrolysis minus the bafilomycin-sensitive activity. Protein content was determined by the Lowry method by using BSA as a standard (22).

pH34 measurements in cell populations. The pH34 was determined by the fluorescence of SNARF-1 [5 (and 6-)carboxy-SNARF-1], as described (26, 29). Two coverslips (9 × 22 mm) containing cells at confluency were incubated with 7.5 μM SNARF-1-AM in CPB, at pH34 of 7.4 or 8.0, as needed. For experiments containing HCO3-, different pH34 values (i.e., 6.8 and 7.4), the HCO3- concentration was estimated as described earlier (11, 12). In these experiments, the pH34 was maintained constant by continuously bubbling the CPB containing HCO3- with 5% CO2. Cells were incubated for 45 min at 37°C on a rocker platform (Cole-Parmer, Vernon Hills, IL) followed by 30-min incubation in dye-free buffer to ensure complete ester hydrolysis and leakage of uncleaved dye. The two coverslips were placed back to back in a holder perfusion device and perfused at a rate of 3 ml/min, and the fluorescence of SNARF-1 was monitored with a SLM-8100/DM-3000 spectrophotometer (Leitz, Wetzlar, Germany) equipped for sample perfusion, at 37°C. Fluorescence was monitored in continuous-acquisition mode by using an excitation wavelength of 534 nm and monitoring emissions at 584, 600, and 644 nm, as

described (29). The fluorescence emission at 584 nm decreases and that at 644 nm increases, respectively, with increasing pH. The ratio of 644 to 584 nm was used to monitor pH changes. The 600-nm wavelength, which is insensitive to pH, was used to evaluate the efficiency of dye loading, quenching, or other artifacts (26). Fluorescence data were converted to ASCII format for subsequent analyses in SigmaPlot version 8.0 (Jandel Scientific, San Rafael, CA).

**Dye calibration.** In situ calibration curves were generated, as described previously (29). Briefly, the cells attached to coverslips were perfused with a high-K⁺ buffer (pH 5.5–8.0 at 0.2 pH intervals) containing 2 μM valinomycin and 6.8 μM nigericin. The high K⁺ is used to approximate intracellular K⁺, and nigericin sets the H⁺ gradient equal to the K⁺ gradient, with valinomycin completing the collapse of the K⁺ gradient. The ratio (644 nm/584 nm) of SNARF-1 was determined at each pH studied during in situ calibrations (29). The following parameters were obtained for SNARF-1 in MB231: acidic dissociation constant (pKₐ) = 7.934 ± 0.056, Rₐ = 0.367 ± 0.003, and Rₐ,max = 5.652 ± 0.429 (n = 39); in MCF-7: pKₐ = 8.077 ± 0.057, Rₐ = 0.429 ± 0.006, and Rₐ,max = 5.442 ± 0.049 (n = 39); in MDA-MB435: pKₐ = 7.54 ± 0.076, Rₐ,min = 0.435 ± 0.007, and Rₐ,max = 3.167 ± 0.194 (n = 39); in MDA-MB468: pKₐ = 7.822 ± 0.04, Rₐ,min = 0.389 ± 0.001, and Rₐ,max = 3.929 ± 0.186 (n = 39); where Rₐ,min is the ratio observed when the dye is fully protonated, and Rₐ,max represents the ratio of fluorescence obtained when the dye is fully unprotonated. These in situ calibration parameters were used to calculate the pH values for each individual experiment, as previously described (12, 26).

**Data analysis.** The initial rate of recovery from an ammonium chloride-induced acid load is measured as the slope of linear regression curve relating time and pHₑₓᵧ, as described previously (42). Briefly, cells were perfused with 25 mM NH₄Cl in CPB for 5 min to allow entry of NH₄⁺ and NH₄⁺ into the cell. Inside of the cell, the NH₄⁺ dissociates into NH₃ + H⁺, thus acid loading the cells. We then removed the NH₄Cl and evaluated the pHₑₓᵧ recovery from this acidification within the first 5 min. The individual pHₑₓᵧ data points are subtracted from the zenith pHₑₓᵧ, at 5 min and plotted against time to obtain the slope of the linear regression curve relating time and pHₑₓᵧ. Because the apparent H⁺ buffering capacity is different in each cell type and could result in distinct Jₑₓᵧ, we estimated these parameters as described earlier (42).

**Migration kinetics.** To measure the migratory ability of highly and lowly metastatic cells, migration was assessed in a wounded monolayer model. Briefly, cells were grown on 12-mm coverslips to confluence and subsequently wounded with a micromanipulator that induces a 250-μm gap. Following this injury, cells migrate to close the wound (Fig. 1). The inserts were visualized with a 20 objective (UPlan Fl 0.5 Ph1), and images of the bottom and top of the insert were obtained with a real-time confocal imaging system based on a rotating disk (Yokogawa Mod C-10 from McBain Instruments). Cell counts from bottom images compared with counts from top images were used to assess percent invasion and migration. Calcein was excited with the 488-nm line of a 15-mW krypton-argon air-cooled ion laser system (T643-RYB-02 from Melles Griot laser group). The emission signal was collected at 530 nm by using as a detector the Hamamatsu’s Orca-100 scan interline cooled (Peltier cooling system) charged-coupled device camera (12 bit). Five images per insert were obtained, and the experiments were done in duplicate. The images were subsequently analyzed, and cells were counted with the assistance of Scion Image software (Scion, Frederick, MD). Percent invasion was corrected for proliferation and calculated by using the following equation (29)

\[
\% \text{Inv}asion = \frac{\text{Total no. invading cells (lower well sample)/μm}^2}{\text{Total no. cells seeded (upper well sample)/μm}^2} \times 100
\]

**RESULTS**

**Distribution of V-ATPase in breast cancer cells.** Our laboratory’s previous study has shown that V-ATPases are functionally expressed in the plasma membrane of some invasive tumor cells (26). However, the location and the distribution of this enzyme in breast cancer cells are not clear. To determine the cellular localization of V-ATPase in the highly and lowly metastatic human breast cancer cells, we used a mouse antibody against the catalytic subunit A. Cells were also labeled with Alexa Fluor 488-phalloidin to delineate the actin cytoskeleton and the cell edge (Fig. 1, B and E). The immunocytochemical examination of highly and lowly metastatic breast cancer cells showed differences in the distribution of V-ATPase. These studies revealed a cytosolic distribution of V-ATPase in both cell lines (Fig. 1, A and D). This is in agreement with the recognized distribution of V-ATPase in intracellular compartments (endosomes, lysosomes, reticulum endoplasmic, Golgi apparatus). Importantly, in the highly metastatic MB231 cells, the V-ATPase was also localized at the plasma membrane (arrows, Fig. 1A). In contrast, in the lowly metastatic cells, the V-ATPase is inconspicuous at the cell surface (Fig. 1D). Using computer software, we assessed colocalization of red and green fluorescence on a pixel-by-pixel basis (shown as white dots) to confirm the plasma membrane distribution in highly and low metastatic cells (Fig. 1, C and F). Similar results have been obtained with other human breast cancer cells tested, including the highly and lowly metastatic MDA-MB435 and MDA-MB468, respectively (data not shown). In these cell types, the higher levels of expression have been found in the more metastatic cells. These data show for the first time that V-ATPase is located at the cell surface in highly metastatic breast cancer cells, in addition to its distribution in intracellular organelles. In lowly metastatic MCF-7 cells, V-ATPase is expressed predominantly in intracellular compartments and inconspicuously at the plasma membrane.

**Enzymatic activity.** To corroborate the distribution of V-ATPase at the plasma membrane, we isolated plasma membranes from highly and lowly metastatic breast cancer cells and then determined the specific enzymatic activity of V-ATPase. Figure 2A shows pmV-ATPase activity in highly (MB231) and...
lowly (MCF7) metastatic cells, determined as the bafilomycin-sensitive ATP hydrolysis. Experiments performed in other human breast cancer cells indicated a similar trend. Specifically, in highly metastatic MB435s and in the lowly metastatic MB468, we determined that pmV-ATPase activities were 52.2 ± 3.66 (n = 5) and 27.12 ± 4.18 (n = 4) nmol Pi/mg protein/h, respectively. We have also measured total V-ATPase activity (expressed as nmol Pi/mg protein/h) in cell homogenates and have found that the V-ATPase activity is not significantly different between lowly (MCF7 = 2.4 ± 0.51 and MB468 = 2.80 ± 0.37; n = 5) and highly metastatic cells (MB231 = 4.29 ± 1.10 and MB435s = 5.0 ± 1.14; n = 5). These data indicate that V-ATPase activity at the plasma membrane is significantly greater in highly than in lowly metastatic cells.

Functional activity of the V-ATPase in breast cancer cells. To study the significance of V-ATPase for the regulation of proton fluxes via V-ATPase in highly and lowly metastatic cells, we monitored the rate of pH cyt recovery following an acute acid load induced by NH₄Cl (26, 42). Cells loaded with SNARF-1 were perfused with CPB in the absence of HCO₃⁻ and Na⁺ to eliminate the contribution of potential HCO₃⁻ transporters and Na⁺/H⁺ exchangers. The cells were then perfused with 25 mM NH₄Cl, which causes a rapid intracellular alkalinization. After 5 min, to allow for NH₃ and NH₄⁺ to enter into the cells, the NH₄Cl was acutely removed, resulting in intracellular acidification followed by a subsequent pH cyt recovery. As shown in Fig. 2B, the rate of H⁺ extrusion (JH⁺) was faster in highly than in lowly metastatic cells. To determine whether the pH cyt recovery in response to the acid load in the absence of HCO₃⁻ and Na⁺ is mediated via V-ATPase, we evaluated the effect of bafilomycin A₁, an inhibitor of V-ATPase (26). These experiments indicate that bafilomycin A₁ significantly inhibited the pH cyt recoveries in highly and lowly metastatic cells (Fig. 2B). The magnitude of this effect was greater in highly than in lowly metastatic cells. We have performed experiments with other human breast cancer cells with high (MB435s) and low (MB468) metastatic potential and have found a similar trend of data. In these cells, we determined that the JH⁺ were 1.05 ± 0.09 mM H⁺/min (n = 10) and 0.61 ± 0.04 mM H⁺/min (n = 10) in MB435s and MB468, respectively. The JH⁺ in these cases were significantly inhibited by the V-ATPase inhibitors concanamycin and bafilomycin.
These data indicate that pmV-ATPase can be induced by growing cells at acidic pH. Thus pmV-ATPase expression may offer an adaptive advantage for tumor cells typically exposed to an acidic pH environment.

Relative contribution of Na+/H+ exchanger and HCO3−-based H+ -transporting systems for pHcyt regulation in breast cancer cells. Most mammalian cells regulate their pHcyt by using the ubiquitous Na+/H+ exchanger and HCO3−-based H+ -transporting mechanisms (11, 38, 42). To evaluate their relative contribution to pHcyt regulation in relation to pmV-ATPase activity, we determined \( J_{\text{H+}} \) in the presence of Na+ and HCO3−. Under these conditions, all HCO3−-based H+ -transporting mechanisms should be active. For the purposes of this study, we did not attempt to discriminate among the various Na+-independent and Na+-dependent HCO3−-based H+ -transporting systems that work to either acidify or alkalinate the cytosol, but rather grouped them in a generic term of “HCO3−-based H+ -transporting systems.” These experiments

These earlier experiments were performed at pHex 8.0 because our laboratory’s previous studies in other tumor cell types showed a maximal proton pumping activity at an alkaline pHex (26, 27, 29). To further evaluate the physiological significance of pmV-ATPase, we performed experiments at “cell culture pHex = 7.4” in both highly and lowly metastatic cells. These data show that the \( J_{\text{H+}} \) are significantly faster in highly than in lowly metastatic cells (Fig. 3A). Therefore, the \( J_{\text{H+}} \) are significantly faster at pHex 7.4 than at 8.0, regardless of the metastatic phenotype (compare Fig. 2). However, studies using magnetic resonance spectroscopy have indicated that the tumor pHex is significantly more acidic than that of normal tissue (10, 15, 40). We, therefore, grew highly and lowly metastatic cells at acidic pHex 6.8, which is consistent with that found in breast cancer cells grown in nude mice (10, 40). We determined that the rates of cell growth at pHex 6.8 were similar to those at pHex 7.4. We evaluated the \( J_{\text{H+}} \) in these cells and have determined that the \( J_{\text{H+}} \) were significantly faster in cells grown at pHex 6.8 than at 7.4. The \( J_{\text{H+}} \) are faster in highly (MB435S = 2.34 ± 0.18 mM H+/min; n = 6) than in lowly (MCF7 = 1.90 ± 0.17 mM H+/min; n = 6, \( P < 0.05 \)) metastatic cells. These data indicate that pmV-ATPase can be induced by growing cells at acidic pHex. Thus pmV-ATPase expression may offer an adaptive advantage for tumor cells typically exposed to an acidic pH environment.
were performed at pH_ex 7.4, and the J_na in the presence of both Na\(^{+}\) and HCO_3\(^{-}\) were subtracted from those determined in the absence of Na\(^{+}\) and HCO_3\(^{-}\) at pH_ex 7.4 (compare Fig. 3A). We expected that, if the J_na via V-ATPase were similar to the J_na via HCO_3\(^{-}\)-based H\(^{+}\)-transporting systems, then the difference should be zero. Values below or above zero should be taken to indicate that the J_na via pmV-ATPase are respectively faster or slower than the J_na via HCO_3\(^{-}\)-based H\(^{+}\) transport. As shown in Fig. 3B, these data indicated that lowly metastatic cells have significantly faster J_na via HCO_3\(^{-}\)-based H\(^{+}\)-transporting systems than highly metastatic cells. A similar trend was observed in the highly (MB435s) and lowly (MB468) metastatic cells, as the J_na via HCO_3\(^{-}\)-based H\(^{+}\) transport were −0.34 ± 0.14 and 0.33 ± 0.11 mM H\(^{+}\)/min (n = 6), respectively.

To determine the relative contribution of Na\(^{+}\)/H\(^{+}\) exchanger to pH_cyt regulation, we determined J_na in the presence of Na\(^{+}\) and absence of HCO_3\(^{-}\) and subtracted them from the J_na in the absence of Na\(^{+}\). These data indicated that lowly metastatic cells exhibit faster J_na via Na\(^{+}\)/H\(^{+}\) exchanger than highly metastatic cells (Fig. 3C). We also determined J_na in MB435s (−0.25 ± 0.25 mM H\(^{+}\)/min; n = 6) and MB468 (1.60 ± 0.30 mM H\(^{+}\)/min; n = 6) cells and concluded that the J_na via Na\(^{+}\)/H\(^{+}\) exchanger were significantly faster in lowly than in highly metastatic cells (P < 0.05). Altogether, these data indicate that lowly metastatic cells preferentially use the ubiquitous Na\(^{+}\)/H\(^{+}\) exchanger and HCO_3\(^{-}\)-based H\(^{+}\)-transporting systems, whereas highly metastatic cells preferentially use pmV-ATPase for pH_cyt regulation.

Effect of acidic pH_ex on pH_cyt under acute and chronic conditions. We hypothesize that the presence of pmV-ATPase may allow the cells to survive the acidic environment of tumors. We, therefore, evaluated the impact of acute changes in pH_ex from 7.4 to 6.8 in the presence and absence of HCO_3\(^{-}\). These experiments indicated that, consistently, the pH_cyt is significantly more acidic at an acidic than at an alkaline pH_ex (Table 1). Furthermore, the presence or absence of HCO_3\(^{-}\) does not change pH_cyt when measured at the same pH_ex (either acidic or alkaline). The effect of acidic pH_ex on pH_cyt only occurs acutely, i.e., when cells are exposed at acidic pH_ex for 1 h. When these cells are grown chronically at acidic pH_ex (i.e., >80 h), the resting pH_cyt are significantly more alkaline than in cells grown at pH_ex 7.4, regardless of their metastatic potential. This is possibly due to overexpression of pmV-ATPases, because the Na\(^{+}\)- and HCO_3\(^{-}\}-independent J_na are significantly faster in cells grown at acidic pH_ex as than in cells grown at pH_ex 7.4 (compare Table 1).

Migration and invasion analysis. Invasion and migration are two prominent characteristics of tumor malignancy. Many mechanisms are involved in these processes. To evaluate the physiological relevance of V-ATPase located at the cell surface, we performed invasion and migration assays. First, we studied the kinetics of migratory ability of highly and lowly metastatic cells in the presence and absence of 50 nM bafilomycin A_1. Our studies have shown that this concentration is not cytotoxic to the cells. Figure 4A shows a wounded monolayer of highly metastatic cells stained with Alexa 488-phalloidin at time 0 and after 12 h to allow for "wound closure." Figure 4B shows the migration kinetics of highly (MB231) and lowly (MCF-7) metastatic cells. The data indicate that MB231 cells closed the wound faster than MCF-7 cells with a half-time of ~8 and 24 h, respectively. Bafilomycin A_1 decreased the migration in highly metastatic cells (Fig. 4C) without affecting the lowly metastatic cells (Fig. 4D). We then studied invasion and migration of MB231 and MCF-7 cells using FluoroBlock inserts. These cells were grown onto FluoroBlock inserts coated with Matrigel (invasion) or uncoated (migration) and assessed the invasion and migration of highly (MB231) and lowly metastatic (MCF-7) cells after 8 and 24 h, respectively. These different time frames are necessary because of their distinct migratory kinetics determined from cell-wounding monolayer experiments (compare Fig. 4). These studies indicated that the invasion and migration are significantly faster in highly than in lowly metastatic cells. These differences are unlikely due to distinct cell growth, because these cells have similar doubling times. To evaluate the contribution of V-ATPase to this distinct invasive and migratory potential, parallel experiments were performed with cells incubated with 50 nM bafilomycin A_1. This resulted in a significant inhibition of invasion and migration in highly metastatic cells (Figs. 5, A and B, respectively), with no significant effects in lowly metastatic cells (Fig. 5, A and B, respectively). Similar data were obtained with concanamycin. Altogether, these studies indicate that highly metastatic cells are more invasive and migratory than lowly metastatic cells, and that the invasion and migration are inhibited by the V-ATPase inhibitors in highly metastatic cells. These results suggest that V-ATPases are involved in the invasion and migration of the metastatic cancer cells.

It is possible that the effects of bafilomycin on migration and invasion may be due to the effects on the cytoskeleton dynamics rather than on pH_cyt regulation. We, therefore, evaluated the effect of V-ATPase inhibitors on microfilament structures using Alexa conjugated to phalloidin. We determined by immunocytochemistry that the actin fiber structures are similar in both treated and nontreated cells, at 50 nM and as high as 1 μM bafilomycin or concanamycin for 30 min (data not shown).

Table 1. Steady-state pH_cyt measurements in highly and lowly metastatic breast cancer cells

<table>
<thead>
<tr>
<th>pH_ex 7.4</th>
<th>HCO_3^- free</th>
<th>+ HCO_3^-</th>
<th>Grown at pH_ex 6.8</th>
<th>HCO_3^- free</th>
<th>+HCO_3^-</th>
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</thead>
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<tr>
<td>MB435s</td>
<td>7.04±0.007</td>
<td>7.05±0.012</td>
<td>7.13±0.035*</td>
<td>6.58±0.087†</td>
<td>6.64±0.025†</td>
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<td>MB231</td>
<td>7.10±0.010</td>
<td>7.11±0.021</td>
<td>7.19±0.027*</td>
<td>6.96±0.009†</td>
<td>6.87±0.034†</td>
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<tr>
<td>MCF-7</td>
<td>7.21±0.025</td>
<td>7.16±0.010</td>
<td>7.33±0.032*</td>
<td>7.01±0.027†</td>
<td>6.94±0.008†</td>
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<tr>
<td>MB468</td>
<td>7.20±0.011</td>
<td>7.17±0.014</td>
<td>7.24±0.017</td>
<td>7.01±0.012†</td>
<td>7.00±0.011†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 12 experiments. Cells were loaded with carboxyseminaphthorhodolfluor-1, as described in MATERIALS AND METHODS. Cells were continuously perfused in the fluorometer cuvette until steady-state cytosolic pH (pH_cyt) was reached, typically 5–10 min, and the pH_cyt was determined in the presence or absence of HCO_3^- at extracellular pH (pH_ex) of either 7.4 or 6.8. Cells grown at pH_ex 6.8 for >80 h were used to determine the pH_cyt. *P < 0.05: cells grown at pH_ex 6.8 vs. cells grown at 7.4 measured at pH_ex = 7.4; †P < 0.05: pH_ex 6.8 vs. pH_ex 7.4 in the presence and absence of HCO_3^-.
These studies indicated that these drugs do not alter the morphological characteristics of stress fibers in either lowly or highly metastatic cells.

**DISCUSSION**

The present study has shown by immunocytochemistry, for the first time, that the V-ATPase is located at the plasma membrane in the highly metastatic human breast cancer cells, in addition to its well-known intracellular location. Furthermore, the specific activity of V-ATPase at the plasma membrane, but not in cell homogenate, is greater in highly than in lowly metastatic breast cancer cells. This suggests that enhanced pmV-ATPase activity rather than distinct endosomal V-ATPase activity is involved in the acquisition of a more metastatic phenotype. The position of the proton pump at the cell surface suggests that, in addition to pHcyt regulation, V-ATPase may serve several functions. One possible function is in tumor metastasis, which requires secretion of digestive enzymes that degrade extracellular matrix. So far, no sufficient evidence has been documented suggesting that V-ATPase is directly related to metastasis. Our laboratory’s previous studies showed that human melanoma cells with different metastatic potential have distinct pHcyt regulatory mechanisms (27). Furthermore, there is a positive correlation between metastatic potential and the level of V-ATPase activity at the plasma membrane (26), suggesting that V-ATPases provide the acidic extracellular environment necessary for invasion. V-ATPases have also been shown to be localized to the plasma membrane of various specialized cells, such as osteoclast (54), neutrophils (14, 32, 34), kidney intercalated cells (9, 13, 47), the mitochondria-rich cells of the rat epididymis (4), and in angiogenic cells (41).

The preferential colocalization of V-ATPase with F-actin at the cell cortex observed in this study suggests that the V-ATPase may distribute to the cell surface by interacting with cytoskeleton elements. Recent studies have shown that V-ATPases bind actin filaments, suggesting that this interaction is an important mechanism controlling transport of V-ATPases from the cytoplasm to the plasma membrane (17, 56). In osteoclasts, it has been shown that the V-ATPases were localized on dotlike organelles associated with the filamentous structures of microtubule extending to the cell surface and resided on the plasma membrane of mature-nuclear osteoclast-like cells (53). Moreover, disruption of the cytoskeleton structure by transfecting highly metastatic melanoma cells with cytokeratin mutants decreases their invasion and V-ATPase expression (27).

The immunocytochemical experiments demonstrated that the V-ATPases are expressed at the cell surface. However, these studies do not address whether V-ATPase is functional. We, therefore, performed subcellular fractionation studies in...
isolated plasma membranes. The data indicated that a bafilomycin-sensitive ATP hydrolysis system is significantly greater in highly than in lowly metastatic breast cancer cells. Electron microscopy analysis of the isolated plasma membrane indicated that these membranes were free of mitochondria and other organelles. Thus it is unlikely that the V-ATPase activity found at the plasma membrane was due to endosomal contaminants. Furthermore, because the plasma membranes from lowly metastatic cells were isolated with the same protocol and the V-ATPase activity was minor, this supports our contention that a significantly higher number of bafilomycin-sensitive enzymes are present at the plasma membrane of the highly metastatic cells.

To determine whether the presence of V-ATPase at the plasma membrane plays a role in pH<sub>cyt</sub> regulation, we evaluated whether there were differences in the response to acid loads in highly and lowly metastatic cells. The recovery from acid loads showed that highly metastatic cells exhibited a pH<sub>cyt</sub> recovery that was faster than in lowly metastatic cells. Importantly, inhibition of V-ATPase with bafilomycin and concanamycin significantly decreased the J<sub>H<sup>+</sup></sub>. These data indicate that the distribution of V-ATPase at the cell surface is responsible for the increased J<sub>H<sup>+</sup></sub> observed in highly metastatic cells and that V-ATPase is positioned at the cell surface to extrude H<sup>+</sup> across the plasma membrane. Early studies from the yeast H<sup>+</sup>-ATPase-transfected cell model have suggested that overexpression of proton pumps on the cell membrane can increase the pH<sub>cyt</sub>, trigger cell proliferation, and finally causes tumorigenesis (12, 36). The present study indicates that V-ATPase overexpression at the cell surface increases the J<sub>H<sup>+</sup></sub> and is responsible for a more metastatic phenotype. Furthermore, we also determined that lowly metastatic cells preferentially used Na<sup>+</sup>/H<sup>+</sup> exchanger and HCO<sub>3</sub><sup>-</sup>-based H<sup>+</sup>-transporting mechanisms, whereas highly metastatic cells preferentially used pmV-ATPase to regulate their pH<sub>cyt</sub>.

The distribution of V-ATPase at the plasma membrane may induce other effects besides increasing the magnitude of the J<sub>H<sup>+</sup></sub> in cancer cells. Tumor invasion and metastasis are two hallmarks of the neoplasm malignancy. They are the major causes of the morbidity of the cancer patients. To understand the physiological significance of the V-ATPase expression at the cell surface in invasion and migration, we employed a wounded monolayer model to evaluate the kinetics of migration in the highly and lowly metastatic breast cancer cells. In these experiments, scraping off a 250-μm region in a confluent monolayer of cells resulted in cells migrating toward the wound. When healing was allowed to continue, the wound was completely recovered in ~18 and 72 h in highly and lowly metastatic cells, respectively. Bafilomycin A<sub>1</sub> treatment decreased wounded behavior in the highly metastatic cells with minor effect on lowly metastatic cells. These data indicate that V-ATPase expression at the cell surface is involved in the faster migratory ability of the highly metastatic cells. We also performed studies to evaluate the ability of the cells to invade through the extracellular matrix. Our data indicated that highly metastatic cells exhibiting pmV-ATPase were more invasive than lowly metastatic cells. Importantly, bafilomycin treatment decreased the migration and invasion in highly metastatic cells. These results are in agreement with a recent study that showed that bafilomycin suppressed cell motility in NIH3T3 A31 mouse fibroblasts, possibly due to alterations of pH gradients in endocytic structures, known to exhibit V-ATPase (51). Furthermore, overexpression of the ε subunit of V-ATPase in 10T1/2 fibroblasts has been shown to enhance invasion and the secretion of matrix metalloproteinase (MMP)-2, an enzyme needed for protein degradation during invasion (18). These data suggest that overexpression of V-ATPases is important for invasion.

The precise mechanism of how V-ATPase expression at the cell surface may regulate cell motility and migration is unclear. Many factors are involved in this process, such as Ca<sup>2+</sup> (24, 44), chemoattractants (23), collagenases (48), cathepsins (43), MMPs (30), and serine protease (55). No direct evidence for V-ATPase expression at the plasma membrane for tumor invasion has been documented yet, but a relationship between pHe<sub>cyt</sub> and invasion has been suggested. First, all of the proteases mentioned above are pH sensitive. Cathepsins are lysosomal enzymes that have an optimal acidic pH (57). Acidic pH induces the redistribution and release of cathepsin B from a series of metastatic human cell lines (43). Mathematical models have been used to investigate whether altered proteolytic activity at acidic pH<sub>cyt</sub> is responsible for the stimulation of a more metastatic phenotype. In these cells, the effect of culture pH<sub>s</sub> on the secretion and activity of two different classes of proteinases, the MMPs and the cysteine proteinases (such as...
These data indicate that culturing of cells at mildly acidic pH examined. Culturing of either cell line at acidic pH 6.8 increases the invasive behavior of tumor cells (29). In these studies, the in vitro invasive potential of two human melanoma cell lines, the highly invasive C8161 and lowly invasive A375, were examined. Culturing of either cell line at acidic pH 6.8 caused dramatic increases in both migration and invasion. These data indicate that culturing of cells at mildly acidic pH induces them to become more invasive (29). Thus the presence of V-ATPase at the cell surface is a significant contributor to the induction of a more invasive phenotype, because it results in an acidic pH that maintains a more alkaline pH needed for cell growth and invasion. Indeed, tumor cells in situ have a lower pH than normal cells; this is an intrinsic feature of the tumor phenotype, caused by alterations either in acid extrusion from the tumor cells or in clearance of extracellular acid (15, 28, 40). Acidic pH benefits tumor cells because it promotes invasiveness, whereas an alkaline pH gives them a competitive advantage over normal cells for growth. Furthermore, V-ATPase is anti-apoptotic in tumor cells (49, 59).

The mechanisms involved in the decreased invasion and migration following bafilomycin and concanamycin treatment are unclear. However, it is known that a critical step in directed motility and migration is the asymmetric actin polymerization at the leading edge, to establish cell polarity. Increases in pH transitional promote recruitment and actin binding of coflin at the leading edge of migratory cells (2). An increase in pH transitional also stimulates the actin-severing activity of coflin (2). Cofilin localizes at the leading edge in fibroblasts and in cancer cells (8). The complex of actin depolymerizing factor and coflin tends to bind F and G actin in a pH-dependent fashion (2). Thus disruption of pH transitional regulatory mechanisms may, in turn, affect actin polymerization and thereby cell migration. However, our data indicated that bafilomycin treatment did not affect actin cytoskeleton. Thus inhibition of pmV-ATPase at the leading edge may result in decreased cell migration. Further studies are needed to elucidate if localized pH transitional changes imposed by preferential localization of pmV-ATPase at the leading edge regulates invasion and migration in highly metastatic cells.

The cytoskeleton also contributes to the transport of biosynthetic cargo of vesicles derived from the Golgi apparatus, including endosomes and lysosomes (21), and the actin cytoskeleton is involved in the secretory vesicle transport to the plasma membrane (19). Thus inhibition of V-ATPase and pH transitional regulation may lead to a disruption of vesicle trafficking needed for cell movement. A role for cortical actin has been found in neuronal cell line PC-12, in which the motility of secretory vesicles was mediated by actin (19). The role of the endocytic pathway in cell migration is unclear. In migrating neutrophils and other cell types, integrins may recycle from the lagging to the leading through polarized endosomal recycling (5, 6, 37). Because the endosomes and lysosomes are part of the endomembranous compartments with a high turnover and are enriched with V-ATPase, it is possible that disruption of V-ATPase by bafilomycin may alter V-ATPase turnover into the plasma membrane, thus inhibiting the supply to the cell surface with components and proteins needed at the leading edge of migratory cells (31). This, however, requires further investigation.

In conclusion, V-ATPase not only takes part in pH transitional homoeostasis, but it is also involved in the acquisition of a transformed phenotype in cancer cells. The preferential expression of V-ATPase at the cell surface is important for the acquisition of invasiveness and metastasis of the tumor cells. Therefore, it appears that V-ATPase is a potential target in cancer therapy and may be an excellent candidate for anticancer drugs.

GRANTS

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