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Sodium calcium exchanger operates in the reverse mode in metastatic human melanoma cells

S. R. Sennoune[1,2], J. M. Santos[1,2], F. Hussain[2] and R. Martínez-Zaguilán[1,2]✉

[1] Departments of Cell Physiology & Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, Texas, USA
[2] Departments of Mechanical Engineering, Texas Tech University Lubbock, Texas, USA

Corresponding author: Raul Martinez-Zaguilan, Department of Cell Physiology & Molecular Biophysics, Texas Tech University Health Sciences Center, 3601 4th Street, Lubbock, Texas 79430, USA. E-mail: Raul.Martinez-Zaguilan@ttuhsc.edu

Abstract

Cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) is important in the regulation of several cellular functions involved in metastasis. We hypothesize that distinct $[Ca^{2+}]_{cyt}$ regulation explains the acquisition of a more metastatic phenotype. To test this hypothesis, we used highly and lowly metastatic human melanoma cells and $[Ca^{2+}]_{cyt}$ was monitored using Fura-2AM and fluorescence spectroscopy. Stimulation with ATP elicited a sustained increase in $[Ca^{2+}]_{cyt}$ in highly metastatic cells, but a transient increase in lowly metastatic cells. Na^+ substitution revealed Na^+/Ca^{2+} exchanger (NCX) activity in reverse mode in highly, but not in lowly metastatic cells. In highly metastatic cells, addition of Na^+ in the plateau phase of $[Ca^{2+}]_{cyt}$ increase elicited with ATP, in the absence of Na^+, resulted in a rapid return to basal, indicating that NCX can operate in both reverse and forward modes. Inhibition and knockdown of NCX, using KB-R7943 and siRNA NCX-1 respectively, supported the significance of NCX in $[Ca^{2+}]_{cyt}$ regulation in highly metastatic cells. Stimulation with UTP triggered a rapid increase in highly metastatic cells $[Ca^{2+}]_{cyt}$, but not in lowly metastatic cells suggesting that highly and lowly metastatic cells exhibit distinct purinergic receptors. These data indicate that following agonist-stimulation, NCX operates preferentially in the reverse mode to enable a sustained $[Ca^{2+}]_{cyt}$ increase in highly metastatic cells. The forward mode of NCX operation to extrude Ca^{2+} is preferred in lowly metastatic cells. The acquisition of a more metastatic phenotype involves a switch in NCX activity from forward to reverse mode that is favorable to maintain elevated $[Ca^{2+}]_{cyt}$ in response to agonist stimulation.

Key words: Intracellular Ca^{2+}, metastasis, melanoma, ATP, purinergic receptors, spectrofluorometry, FURA-2.

Introduction

Cytosolic calcium ($[Ca^{2+}]_{cyt}$) is involved in the regulation of many cellular functions including cell growth, differentiation, contraction, motility, invasion and other functions such as glycolysis, protein synthesis, DNA synthesis, exocytosis, assembly-disassembly of cytoskeleton, etc. (1-4). Thus, changes in $[Ca^{2+}]_{cyt}$ are pleiotropic in nature. Metastasis is a multistage process involving tumor cell growth and invasion through extracellular matrix to colonize distant sites (5-8). It is possible that each stage in the metastatic cascade has a distinct $[Ca^{2+}]_{cyt}$ optimum or require specific $[Ca^{2+}]_{cyt}$ regulatory mechanism. A number of studies have shown that alterations in $[Ca^{2+}]_{cyt}$ accompany the acquisition of the metastatic phenotype (9-16). We have shown that the steady-state levels of $[Ca^{2+}]_{cyt}$ in cells with low or high metastatic potential are similar (17). However, the $[Ca^{2+}]$ buffering capacity is greater in lowly than in highly metastatic melanoma cells (17). The significance of a low Ca^{2+} buffering capacity in highly metastatic cells is unclear. The mechanism responsible for the distinct Ca^{2+} regulation and distinct Ca^{2+} buffering capacity in highly and lowly metastatic cells are also unclear. It is possible, however, that this allows highly metastatic cells to maintain $[Ca^{2+}]_{cyt}$ elevated following agonist-stimulation for longer periods of time than in lowly metastatic cells. Elevated $[Ca^{2+}]_{cyt}$ following hormone stimulation may extend the time for Ca^{2+} to exert its pleiotropic effects needed for metastasis. This could also increase the probability of quantum events to occur, such as exocytosis of proteins and enzymes needed for metastasis (15, 18-20).

Steady-state $[Ca^{2+}]_{cyt}$ levels in most cells are determined by the relative rates of Ca^{2+} entry, extrusion, release and sequestration into organelles (1, 3, 18, 21). Ca^{2+} typically enters the cytosolic compartment through well-regulated Ca^{2+} channels located at the plasma membrane (PM), IP3- gated release from the endoplasmic reticulum (ER) and Ryanodine Receptor channels (RyR) channels located in the ER. Ca^{2+} entry in non-excitable cells through Ca^{2+} ion channels at the plasma membrane are activated by depletion of intracellular Ca^{2+} stores, hence known as store-operated calcium entry (SOCE) channels. ORAI 1 (also known as the calcium release-activated calcium channel protein 1) and STIM 1 (stromal interaction molecule 1) have been identified as the main constituents of the SOCE complex. ORAI 1, a highly selective Ca^{2+} channel at the plasma membrane, is activated by STIM 1 that functions as a Ca^{2+} sensor at the ER. Ca^{2+} extrusion from the cytosolic compartment occurs via PM- and ER-localized Ca^{2+}-ATPases (22-25). Ca^{2+}-binding proteins (CaBP) can also play a prominent role in regulating $[Ca^{2+}]_{cyt}$ homeostasis. In many cell types, the Na^+/Ca^{2+} exchanger (NCX) plays a key role in regulating $[Ca^{2+}]_{cyt}$ (26-29). The cardiac NCX mechanism was first demonstrated experimentally in guinea-pig atria by Reuter and Seitz in 1968 (30) and thereafter Glitsch et *al* (31) reported internal Na^+-dependent Ca^{2+} influx so that a concept of bidirectional Na^+/Ca^{2+} exchange mechanism was established (30-34). Similar systems were also found in crab nerve (35), squid axons (36) and in various other tissues (29, 37, 38). Blaustein and Hodgkin demonstrated that the electrochemical po-

tential gradient of both Na^+ and Ca^{2+} across the membrane is the energy source of the NCX (39).

The NCX transports Ca^{2+} in exchange for Na^+ in either direction, depending on the electrochemical gradients of Na^+ and Ca^{2+} (26-29, 40). In the forward mode, i.e. Na^+ entry/Ca^{2+} extrusion, the NCX represents the primary mechanism for Ca^{2+} efflux in the myocardium and thus plays a prominent role in contractile function (29, 37). During depolarization, the exchanger operates in reversed mode (Ca^{2+} entry/Na^+ extrusion) and triggers Ca^{2+}-induced Ca^{2+} release in certain cell types. The forward and reverse modes of the NCX have been observed in both excitable and non-excitable cell types. The significance of NCX for $[Ca^{2+}]_{cyt}$ regulation in human melanoma cells with distinct metastatic potential is unclear, however.

Intracellular ATP plays a fundamental role in nucleic acid synthesis, ion channel modulation, energy metabolism, and enzyme regulation - among many other physiological and biochemical processes. In addition to its role in bioenergetics, extracellular ATP also acts as an extracellular signaling molecule mediating cell-cell communication in several cell types, including tumor cells (41-44). Extracellular ATP also acts as a neurotransmitter or neuromodulator in the peripheral and central nervous systems (45, 46). At sites of tissue injury and inflammation, nucleotides, including ATP, are released from these damaged cells (47, 48) or from activated platelets or leukocytes (43). The ATP released from cells can reach concentrations sufficient to activate purinergic receptors, and damaged cells can release up to 1-5 mM ATP (49). ATP is also released by mechanical stresses, such as stretch, compression, or shear stress, in a variety of cells, including tumor cells (47, 48, 50). Tumor metastasis involves cell growth that could cause mechanical stress and compression of adjacent tissues, thus resulting in ATP release. The degradation of extracellular matrix and extravasion of tumor cells in metastasis could also release ATP, which could have significance in the acquisition of a metastatic phenotype.

Extracellular ATP exerts its effects via a family of specific receptors termed P2 purinoceptors which consist of the P2X receptors - ligand-gated ion channels, and the P2Y receptors - G protein-coupled receptors (51, 52) and several P2Y receptors (P2Y1, P2Y2, P2Y4, and P2Y6) have been cloned, and a wide range of tissue expression of P2X and P2Y receptors has been reported (51, 53). P2Y receptor stimulation results in activation of phospholipase C, elevation of inositol triphosphate, and mobilization of intracellular Ca^{2+} (54). Nucleotide binding to P2X receptors results in opening of non-specific cation channels and membrane depolarization or Ca^{2+} influx (55). In some cells, prolonged activation of the P2X7 class of channels can form large membrane pores, and the resultant leaky cells undergo lysis and ATP release (44). Cells with an invasive phenotype such as osteoclasts and macrophages express a number of P2 receptor subtypes (43). Stimulation with exogenous ATP increases bone formation and bone-resorbing activity of osteoclasts (54, 56, 57) and macrophage function (43). P2 receptors have also been found in several cancer types, including esophageal, prostate, lung, sarcoma, colorectal cancer, and melanoma (58-63). The roles of purinergic receptors in tumor cells are paradoxical in that they can either inhibit cell growth in endometrial carcinoma and melanoma cells (63-65), or increase cell growth in MCF-7 breast cancer, lung cancer and squamous cell carcinoma (61, 66, 67). The effect of exogenous ATP in human melanoma cell functioning and signaling is unclear. We investigated the cellular mechanisms underlying the distinct Ca^{2+} buffering capacity in cells with distinct metastatic potential.

Materials and methods

Buffers and chemicals

Cell superfusion buffer (CSB) contained: 0.35 mM Na_2HPO_4, 110 mM NaCl, 0.44 mM KH_2PO_4, 5.4 mM KCl, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM HEPES, 5 mM glucose, and 2 mM L-glutamine at a pH of 7.15 at 37 °C. In Na^+-free CSB, Na^+ was replaced with N-methyl-d-glucamine on an equimolar basis. KB-R7943 ((2- [2 -[4- (4- nitrobenzyloxy) phenyl] ethyl isothiourea methanesulfonate) was obtained from Calbiochem (EMD Chemicals, Inc., Gibbstone, NJ). Fluoroprobes were obtained from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cells

Lowly (A375p) and highly metastatic (C8161) human melanoma cells were grown in Dulbeccos's Modified Eagle Media high glucose (DMEM; Sigma #5648) supplemented with 24 mM $NaHCO_3$, 10 mM HEPES and 10% Fetal Bovine Serum, at 37 °C under 5% CO_2 (68).

$[Ca^{2+}]_{cyt}$ measurements by fluorescence spectroscopy

$[Ca^{2+}]_{cyt}$ measurement procedures using the fluorescence of Fura-2 have been previously described (17). Briefly, cells were grown at confluency onto 9 x 22-mm coverslips under 5% CO_2. Coverslips with cells were incubated for 30 min at 37 °C with the Ca^{2+} fluoroprobe Fura-2 (5-oxazolecarboxylic acid, 2-(6- (bis (carboxymethyl) amino)-5-(2-(2-bis (carboxymethyl) amino)-5-methylphenoxy-2-benzofuranyl), in cell superfusion buffer (CSB). We then incubated the cells for 30 min in dye-free CSB to ensure complete ester hydrolysis and leakage of uncleaved dye. The coverslips were then placed in a holder and put in a cuvette in the spectrofluorometer which is equipped with a flow-through device for buffer perfusion. Cells were continuously perfused at 3 ml/min with their respective buffers. Both the water jacket and the perfusion medium temperature was kept at 37 °C using a circulator water bath (Lauda model RM 20, Brinkman Instruments, Westbury, NY). Fluorescent ion signals were converted to $[Ca^{2+}]_{cyt}$ as described previously (17). All fluorescence measurements were performed in a temperature controlled cuvette housed in a DMX- SLM8000C spectrofluorometer (SLM, Urbana, IL), at 37 °C.

Knockdown of NCX

siRNA specific for the NCX-1 and all reagents needed for SiRNA transfection were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Transfection of cells with siRNA-NCX-1 was performed accord-

ing to manufacturer instructions. Briefly, 24 hours prior transfection, highly (C8161) and lowly (A375p) metastatic cells were plated onto rectangular coverslips (9 x 22 mm) in 60 mm petri dishes at $1x10^5$ cells in antibiotic-free normal growth medium supplemented with FBS and incubated at 37 °C in a CO_2 incubator set at 5%. Then, cells were transfected with 1 nM NCX-1 siRNA. Experiments were performed in cells transfected for up to 72 hrs. To quantify the reduction in the NCX-1 mRNA levels, quantitative RT-PCR was performed using RNA isolated from cells after siRNA treatment.

Statistical Analysis

All results are expressed as mean ± SEM. The significant differences were determined using t-test and Mann-Whitney test for normal and non-parametric distribution respectively (SigmaStat; Statistical Software, Jandel Scientific, San Rafael, CA). All statistical tests were considered significant at $p < 0.05$.

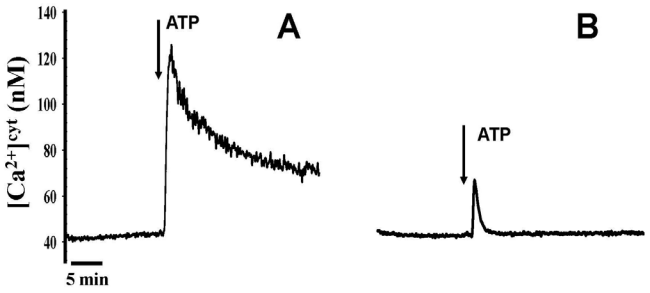
Results

ATP treatment elicits large and sustained $[Ca^{2+}]_{cyt}$ increases in highly metastatic cells, whereas in lowly metastatic cells, the $[Ca^{2+}]_{cyt}$ response is small and transient

ATP is a powerful agonist in many cell types because it binds to P2X purinergic receptors, as well as to P2Y G-protein coupled receptors located at the cell surface. Our results indicate that although the steady-state $[Ca^{2+}]_{cyt}$ in highly and lowly metastatic cells were not significantly different, their response to ATP differs significantly. As shown in Figure 1A, highly metastatic cells respond with a large and sustained $[Ca^{2+}]_{cyt}$ increase, whereas lowly metastatic cells respond to ATP with a small and transient $[Ca^{2+}]_{cyt}$ increase (Figure 1B). To evaluate the kinetics of the $[Ca^{2+}]_{cyt}$ increase in highly and lowly metastatic cells, we have fitted the complete data set ($[Ca^{2+}]_{cyt}$ changes versus time) using the following lognormal distribution:

$$y = a\exp\left[-\frac{\ln(2)}{\ln(d)^2}\ln\left(\frac{(x-b)(d^2-1)}{cd}+1\right)^2\right]$$

where *a* is the amplitude of the Ca^{2+} transient; *b* is the maximum $[Ca^{2+}]_{cyt}$ increase (herein referred as $[Ca^{2+}]_{cyt}$ peak; *c* is the full duration of the $[Ca^{2+}]_{cyt}$ increase at half maximum (herein referred as $\tau\ Ca^{2+}$); and *d* is the asymetry at half maximum $[Ca^{2+}]_{cyt}$ change. We selected this approach because the $[Ca^{2+}]_{cyt}$ increase following agonist stimulation is a stochastic phenomenon, where the magnitude of $[Ca^{2+}]_{cyt}$ increase and the duration of the $[Ca^{2+}]_{cyt}$ transient (τ) are better depicted by this approach. Using this equation and probability density function we obtained both the asymetry of the $[Ca^{2+}]_{cyt}$ increase and skewdness following agonist stimulation (*d*), as well as the shape (i.e., sustained or transient) or kurtosis of the $[Ca^{2+}]_{cyt}$ increase (*a*). For purposes of data presentation, we will only show the amplitude of the $[Ca^{2+}]_{cyt}$ increase ($[Ca^{2+}]_{cyt}$ peak) and the duration of the $[Ca^{2+}]_{cyt}$ transient ($\tau\ Ca^{2+}$). As indicated in Figure 1, this analysis indicates that the magnitude of the $[Ca^{2+}]_{cyt}$ increase is significantly larger in highly than in lowly metastatic cells. The kinetics of the $[Ca^{2+}]_{cyt}$ recovery ($\tau\ Ca^{2+}$) is ca. 13 times faster in lowly than in highly metastatic cells. The characteristics of the $[Ca^{2+}]_{cyt}$ increase following agonist-stimulation suggest that highly metastatic cells have a lower Ca^{2+} buffering capacity than lowly metastatic cells.

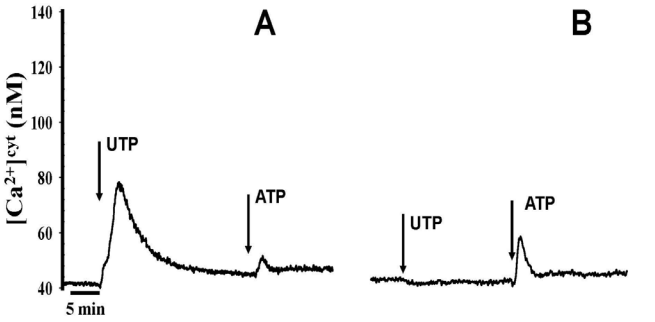


	Highly	Lowly
Ca^{2+} amplitude (nM)	71 ± 8	18 ± 2*
$\tau\ Ca^{2+}$ (sec)	407 ± 44	31 ± 3**

Figure 1. ***ATP treatment elicits large and sustained $[Ca^{2+}]_{cyt}$ increases in highly metastatic (A) human melanoma cells, whereas in lowly metastatic melanoma cells (B) the $[Ca^{2+}]_{cyt}$ response is small and transient.*** Highly (C8161) and lowly (A375p) metastatic human melanoma cells were grown at confluency onto glass coverslips, as described in Materials and Methods. Cells were loaded with 2 μM Fura-2-AM, a fluorescent Ca^{2+} indicator, for 30 min followed by 30 min incubation in dye-free CSB, to allow complete ester hydrolysis of unesterified fluoroprobe. Cells were transferred to a fluorometer cuvette equipped with a thermostated chamber, at 37 °C. Cells were continuously superfused with CSB maintained at 37 °C to obtain steady-state $[Ca^{2+}]_{cyt}$. At the time indicated with an arrow, the superfusate was changed for one containing 1 mM ATP. Each tracing is representative of the number of experiments performed in highly (n = 15) and lowly (n = 15) metastatic cells. The complete tracings were fitted to a logistic function, as described in Materials and Methods. This allowed us to obtain the parameters that describe the magnitude of the $[Ca^{2+}]_{cyt}$ increase (Ca^{2+} amplitude) and the duration of the $[Ca^{2+}]_{cyt}$ transient ($\tau\ Ca^{2+}$). The values indicated in a tabular form represent the mean ± SEM of the number of experiments previously indicated. *$p < 0.001$ when the Ca^{2+} kinetic parameters are compared in highly and lowly metastatic cells.

UTP treatment increases $[Ca^{2+}]_{cyt}$ in highly but not in lowly metastatic cells

Uridine 5'-triphosphate binds to P2Y receptors linked to phospholipase C (PLC) in a variety of cells. Activation of PLC results in elevation of inositol triphosphate (IP_3) and mobilization of Ca^{2+} from intracellular stores. Treatment of highly metastatic cells with UTP elicits a rapid increases in $[Ca^{2+}]_{cyt}$ that is smaller than that elicited with ATP. The UTP-induced $[Ca^{2+}]_{cyt}$ increase is followed by recovery towards basal with a kinetic ($\tau\ Ca^{2+}$) that is significantly slower than the observed with ATP (Figure 2A). Subsequent treatment of these cells with ATP elicits a minor increase in $[Ca^{2+}]_{cyt}$, suggesting that UTP elicits release of Ca^{2+} from intracellular stores that become desensitized to further agonist stimulation. Because these experiments are done in the presence of $[Ca^{2+}]_{ex}$ it is unlikely that the Ca^{2+} stores are depleted. Lowly metastatic cells do not respond to UTP treatment with $[Ca^{2+}]_{cyt}$ increase (Figure 2B). However, ATP treatment following UTP treatment results in a significantly smaller $[Ca^{2+}]_{cyt}$ increase associated with a slower ($\tau\ Ca^{2+}$) than the observed with ATP alone. These data sug-



	Highly		Lowly	
	UTP	ATP	UTP	ATP
Ca^{2+} amplitude (nM)	30 ± 3	6 ± 0.6	0	14 ± 1*
τ Ca^{2+} (sec)	348 ± 42	121 ± 3	0	85 ± 10**

Figure 2. ***UTP increases $[Ca^{2+}]_{cyt}$ in highly (A), but not in lowly (B) metastatic cells.*** Cells were handled as described in Figure 1. At the time indicated by the arrow, the superfusate was changed for one containing 1 mM UTP. Subsequently, the superfusate was exchanged for one containing 1 mM ATP. The traces are representative of the experiments performed in highly (n = 5) and lowly (n = 5) metastatic cells. The kinetic parameters were derived as described in figure 1. Notice that UTP desensitizes highly, but not lowly metastatic cells to further stimulation with ATP. *p < 0.01 when compared the Ca^{2+} amplitude elicited by ATP in highly and lowly metastatic cells. **p < 0.058 when compared the τ Ca^{2+} for ATP treatment in highly and lowly metastatic cells.

gest that highly and lowly metastatic cells exhibit distinct sensitivity to purinergic stimulation.

$[Ca^{2+}]_{cyt}$ increases in response to ATP derive from intracellular Ca^{2+} stores

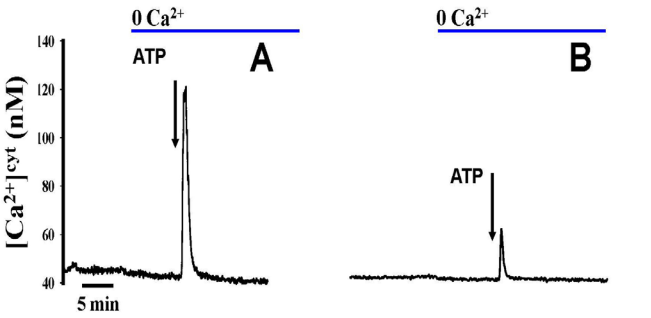
To evaluate the contribution of extracellular Ca^{2+} to the characteristics of the agonist-induced Ca^{2+} increases, we performed experiments in Ca^{2+}-free media. The removal of $[Ca^{2+}]_{ex}$ results in a minor decrease in steady-state $[Ca^{2+}]_{cyt}$ in both highly and lowly metastatic cells (Figures 3A, B). Importantly, ATP treatment in the absence of $[Ca^{2+}]_{ex}$ elicits a transient increase in $[Ca^{2+}]_{cyt}$ in highly metastatic cells (Figure 3A). The magnitude of the $[Ca^{2+}]_{cyt}$ increase is similar to the one observed in the presence of $[Ca^{2+}]_{ex}$ (cf., Figure 1A). However, the kinetics of the transient $[Ca^{2+}]_{cyt}$ increase (τ Ca^{2+}) in highly metastatic cells resemble that observed in lowly metastatic cells and is 9 times faster than that observed in the presence of $[Ca^{2+}]_{ex}$. Treatment of lowly metastatic cells with ATP in the absence of $[Ca^{2+}]_{ex}$ elicits a transient $[Ca^{2+}]_{cyt}$ increase that is significantly lower in amplitude, but similar in kinetics (τ Ca^{2+}) as that observed in the presence of $[Ca^{2+}]_{ex}$ (Figure 3B, cf., Figure 1B). Thus, ATP-stimulation in the absence of $[Ca^{2+}]_{ex}$ affects the kinetics of the $[Ca^{2+}]_{cyt}$ increase in highly metastatic cells, but less so in lowly metastatic cells.

NCX is responsible for sustained $[Ca^{2+}]_{cyt}$ increase following ATP treatment in highly metastatic cells.

To evaluate the contribution of NCX to Ca^{2+} regulation, we removed Na^+, in the presence of Ca^{2+}. This resulted in a small decrease in $[Ca^{2+}]_{cyt}$ in highly but not in lowly metastatic cells (Figures 4A, B). In highly metastatic cells, ATP stimulation in Na^+-free buffer results in a sustained increase in $[Ca^{2+}]_{cyt}$ that is similar in amplitude to that in the presence of Na^+ and Ca^{2+} (cf., Figure 1). The kinetics of the $[Ca^{2+}]_{cyt}$ recovery (τ Ca^{2+}) is however significantly faster than in the presence of Na^+. The removal of ATP in a Na^+-free buffer, to wash out ATP, does not affect the kinetics of the $[Ca^{2+}]_{cyt}$ recovery towards basal. Importantly, subsequent addition of Na^+ elicits a rapid recovery towards basal that reveals NCX operating in the forward mode, to extrude Ca^{2+}. These data suggest that following agonist stimulation, highly metastatic cells exhibit a Na^+ dependent Ca^{2+} influx pathway that is due to the NCX operating in the reverse mode, i.e, to increase $[Ca^{2+}]_{cyt}$. These data should be contrasted with that from lowly metastatic cells, where the amplitude of the $[Ca^{2+}]_{cyt}$ increase following ATP stimulation in Na^+-free buffer was similar to the one observed in the presence of Na^+ (Figure 4B). However, the kinetics of the $[Ca^{2+}]_{cyt}$ recovery is slower than in the presence of Na^+. These data suggest that in highly metastatic cells, the NCX works to maintain elevated $[Ca^{2+}]_{cyt}$ following agonist stimulation, i.e. reverse mode.

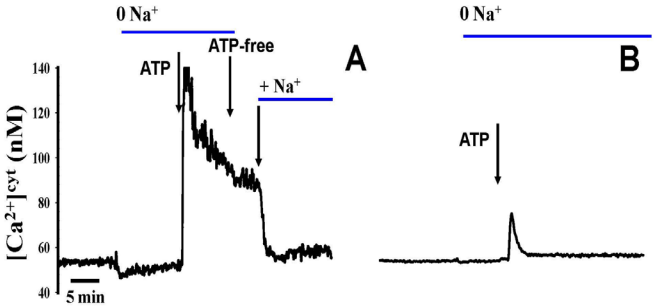
Inhibition of Na^+,K^+-ATPase followed by Na^+ removal do not reveal NCX

The typical approach to evaluate for the presence of NCX is to remove $[Na^+]_{ex}$ in the presence of $[Ca^{2+}]_{ex}$ (cf., Figure 4). Alternatively, increasing $[Na^+]_{cyt}$, by inhibiting the Na^+,K^+-ATPase with ouabain to allow Na^+ entry into the cell, should exacerbate NCX activity following Na^+ removal by increasing the Na^+ driving force to increase $[Ca^{2+}]_{cyt}$. Thus, ouabain was used to increase $[Na^+]_{cyt}$ through the inhibition of Na^+,K^+-ATPase and in turn increase $[Ca^{2+}]_{cyt}$ This treatment did not affect



	Highly	Lowly
Ca^{2+} amplitude (nM)	58 ± 5	12 ± 1.9*
τ Ca^{2+} (sec)	45 ± 3	29 ± 2.5**

Figure 3. ***$[Ca^{2+}]_{cyt}$ increases in response to ATP derive from extra- and intracellular Ca^{2+} stores in highly (A) and lowly (B) metastatic cells.*** Cells were handled as described in Figure 1. Perfusion started with CSB to obtain steady-state. The bar on the top of the tracings indicate the time where the superfusate was exchanged for a Ca^{2+}-free CSB, containing 1 mM EGTA. At the time of the arrow, the superfusate was changed for one containing 1 mM ATP, in Ca^{2+}-free CSB. The tracings are representative of the experiments performed in highly (n = 9) and lowly (n = 5) metastatic cells. Notice that the $[Ca^{2+}]_{cyt}$ responses to ATP in the absence of $[Ca^{2+}]_{ex}$ are transient in highly metastatic cells (cf., Figure 1), indicating that $[Ca^{2+}]_{ex}$ is required to maintain elevated $[Ca^{2+}]_{cyt}$ following ATP treatment. The kinetic parameters for the change in $[Ca^{2+}]_{cyt}$ were obtained as described in Figure 1. *p< 0.007 when compared the Ca^{2+} amplitude in highly and lowly metastatic cells treated with ATP. **p< 0.004 when compared the τ Ca^{2+} for ATP treatment in highly and lowly metastatic cells.



	Highly	Lowly
Ca^{2+} amplitude (nM)	75 ± 10	21 ± 0.9*
τ Ca^{2+} (sec)	303 ± 17	58 ± 7.0**

Figure 4. ***The NCX is responsible for the sustained $[Ca^{2+}]_{cyt}$ increase following ATP treatment in highly (A), but not in lowly (B) metastatic cells.*** Cells were handled as described in Figure 1. Cells were superfused with CSB to reach steady state. At the time indicated by the bar on the top of the tracings, the superfusate was changed for Na^+-free (containing Ca^{2+}). At the time of the first arrow, the perfusate was exchanged for 1 mM ATP in Na^+-free. The second arrow indicated the change in solution to ATP-free (Na^+-free). Notice that there are no apparent changes in kinetics of the $[Ca^{2+}]_{cyt}$ transient following washout of ATP. The third arrow indicate change of the superfusate to CSB (containing Na^+). The rapid decrease of $[Ca^{2+}]_{cyt}$ to basal indicates the activation of the NCX to operate in the forward mode, to extrude $[Ca^{2+}]_{cyt}$ in highly metastatic cells. The tracings are representative of the number of experiments performed in highly (n = 10) and lowly (n = 8) metastatic cells. *p < 0.001 when compared the Ca^{2+} amplitude in highly and lowly metastatic cells. **p < 0.001 when compared the τ Ca^{2+} in highly and lowly metastatic cells.

steady-state $[Ca^{2+}]_{cyt}$ in either cells (Figure 5A,B). Following ouabain pre-treament, the removal of Na^+ elicited a slight increase in $[Ca^{2+}]_{cyt}$. These data suggest that without a sufficient increase in $[Na^+]_{cyt}$, the NCX may be unable to transport Ca^{2+} into the cell at high enough rate to overcome other Ca^{2+} buffering systems. Further stimulation with ATP in Na^+-free elicited larger increase in $[Ca^{2+}]_{cyt}$ in highly (Figure 5A) than in lowly (Figure 5B) metastatic cells.

Inhibition of NCX with KB-R7943 indicates that the NCX operates in the reverse mode in highly metastatic cells

KB-R7943 has been successfully used to inhibit the reverse mode of the NCX in some cell types, albeit there is controversy (69-71). As shown in Figure 6, KB-R7943 does not affect steady-state $[Ca^{2+}]_{cyt}$ in neither highly nor lowly metastatic cells. However, treatment with ATP of highly metastatic cells, in the presence of KB-R7943, increased $[Ca^{2+}]_{cyt}$ with an amplitude that is significantly higher than in the absence of KB-R7943 (Figure 6A, cf., Figure 1A). The kinetics of the $[Ca^{2+}]_{cyt}$ recovery (τ Ca^{2+}) is 6 times faster than in the absence of KB-R7943. These data suggest that the reverse mode of the NCX is responsible for maintaining the sustained increase in $[Ca^{2+}]_{cyt}$ following agonist stimulation. These observations are in contrast to our data in lowly metastatic cells, where addition of ATP in the presence of KB-R7943 elicits a transient $[Ca^{2+}]_{cyt}$ increase that is similar in amplitude to that in the absence of KB-R7943 (Figure 6B, cf., Figure 1B). However, the kinetics of the $[Ca^{2+}]_{cyt}$ recovery following agonist stimulation is

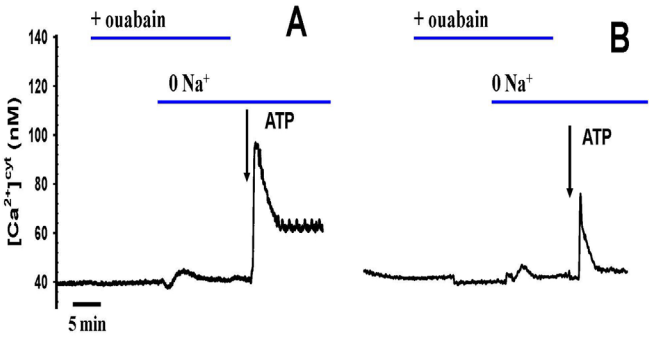
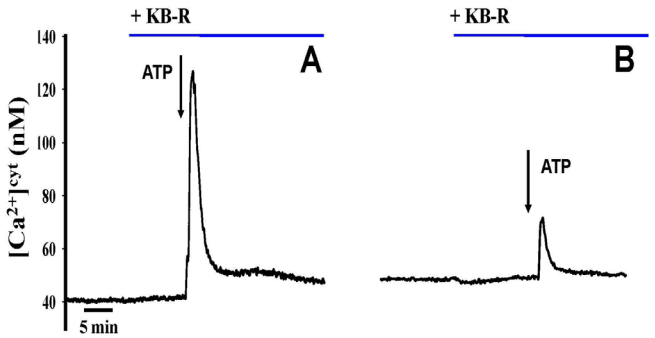


Figure 5. ***Inhibition of Na^+,K^+-ATPase followed by Na^+ removal do not reveal NCX.*** It is predicted that ouabain treatment followed by Na^+ removal should elicit an increase in $[Ca^{2+}]_{cyt}$, since this should increased the driving force for Na^+ to reveal the activity of NCX. Thus, ouabain was used to increase $[Na^+]_{cyt}$ through the inhibition of Na^+,K^+-ATPase and in turn increase $[Ca^{2+}]_{cyt}$. This treatment did not affect resting $[Ca^{2+}]_{cyt}$ in either cells. Following ouabain pretreament, the removal of Na^+ elicited a slight increase in $[Ca^{2+}]_{cyt}$. Further stimulation with ATP in Na^+-free elicited a larger increase in $[Ca^{2+}]_{cyt}$ in highly than in lowly metastatic cells. The tracings are representative of 3 experiments performed in highly and lowly metastatic cells.



	Highly	Lowly
Ca^{2+} amplitude (nM)	109 ± 15	20 ± 1*
τ Ca^{2+} (sec)	67 ± 5	41 ± 0.6**

Figure 6. ***Inhibition of NCX with KB-R7943 indicates that the NCX operates in the reverse mode following ATP-stimulation in highly (A), but not in lowly (B) metastatic cells.*** Cells were handled as described in Figure 1. Cells were superfused with CSB to reach steady state $[Ca^{2+}]_{cyt}$. At the time indicated by the bar on the top of the tracings, the superfusate was exchanged for one containing 50 μM KB-R7943. Notice that this treatment does not affect $[Ca^{2+}]_{cyt}$ in either cell type. At the time indicated by the arrow, the superfusate was exchanged for one containing KB-R7943 plus 1 mM ATP. This resulted in a rapid transient $[Ca^{2+}]_{cyt}$ increase in highly metastatic cells (cf., Figure 1). The tracings are representative of the experiments performed in highly (n = 8) and lowly (n = 5) metastatic cells. *p < 0.002 when compared the Ca^{2+} amplitude elicited by ATP in highly vs lowly metastatic cells. **p < 0.001 when compared the τ Ca^{2+} following ATP treatment in highly vs lowly metastatic cells.

significantly slower in the presence than in the absence of KB-R7943. These data support out contention that in highly metastatic melanoma cells, the NCX operates in the reverse mode to allow sustained elevated $[Ca^{2+}]_{cyt}$ increases needed for cell invasion.

NCX-1 knockdown indicates that highly metastatic cells use NCX to maintain sustained $[Ca^{2+}]_{cyt}$ increases following ATP-stimulation

siRNA offers a powerful tool to selectively suppress gene expression in mammalian cells, thus allowing

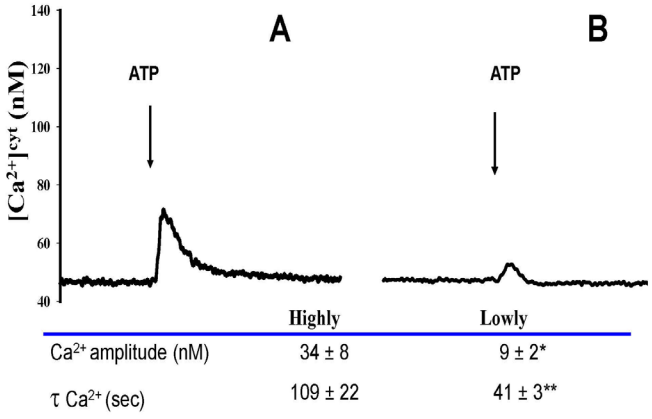


Figure 7. ***Knockdown of the NCX decreases the magnitude of the $[Ca^{2+}]_{cyt}$ increases in response to ATP in highly (A) and lowly (B) metastatic cells.*** Cells were transfected with siRNA-NCX-1 as described in Materials and Methods. Subsequent handling for $[Ca^{2+}]_{cyt}$ measurements was as described in figure 1. Superfusate started with CSB to reach steady-state. At the time indicated by the arrow, the superfusate was exchanged for one containing 1 mM ATP. Notice that the $[Ca^{2+}]_{cyt}$ increase following ATP treatment in highly metastatic cells is transient as compared to non-transfected controls (cf., Figure 1). Tracings are representative of the experiments performed in highly (n = 10) and lowly (n = 6) metastatic cells. *p < 0.044 when compared the Ca^{2+} amplitude in highly vs lowly metastatic cells. **p < 0.001 when compared the τ Ca^{2+} following ATP treatment in highly vs lowly metastatic cells.

elucidation of gene function (72-74). The siRNAs are short double stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a cellular process termed RNA interference (73). Highly and lowly metastatic human melanoma cells were transfected with siRNA-NCX1. As shown in Figure 7, the steady-state $[Ca^{2+}]_{cyt}$ in cells transfected with siRNA-NCX-1 are similar to the controls. Importantly, highly metastatic cells respond to ATP stimulation with increases in $[Ca^{2+}]_{cyt}$ that are smaller in amplitude than non-transfected controls (cf., Figure 1A). The kinetics of the $[Ca^{2+}]_{cyt}$ recovery (τ Ca^{2+}) in NCX1 knockdown highly metastatic cells are faster than in controls. Similar trend of data, i.e., smaller amplitude $[Ca^{2+}]_{cyt}$ increase with a slower $[Ca^{2+}]_{cyt}$ recovery (τ Ca^{2+}) following ATP-stimulation is also observed in NCX1 knockdown lowly metastatic cells when compared to controls.

Discussion

$[Ca^{2+}]_{cyt}$ plays a prominent role in the regulation of several cellular functions that are relevant for the acquisition of a metastatic phenotype (10, 15, 38, 75, 76). We therefore investigated if cells with distinct metastatic potential would exhibit distinct mechanisms of $[Ca^{2+}]_{cyt}$ regulation. In agreement with our previous study, steady-state $[Ca^{2+}]_{cyt}$ were similar between highly and lowly metastatic cells (17). Because steady-state levels do not address mechanisms of $[Ca^{2+}]_{cyt}$ regulation that could be different in highly and lowly metastatic cells, we performed ion substitution experiments and evaluated the cellular response to agonist-stimulation.

Stimulation of highly metastatic cells with ATP elicited a sustained increase in $[Ca^{2+}]_{cyt}$, that is in contrast to the transient increase in $[Ca^{2+}]_{cyt}$ observed in lowly metastatic cells. The significance of sustained versus transient $[Ca^{2+}]_{cyt}$ increase following agonist stimulation in metastasis is unclear. However, it may provide highly metastatic cells with prolonged elevated levels of $[Ca^{2+}]_{cyt}$ that could favor exocytosis of proteins and molecules relevant for metastasis. It is unlikely that the distinct responses to ATP between highly and lowly metastatic cells is due to distinct sensitivity to ATP, since the concentration used in this study is 1-2 orders of magnitude larger than the reported Kd for purinergic receptors in different cell types (49). It is known that exocytosis is a stochastic event that requires an increase in $[Ca^{2+}]_{cyt}$ in both excitable and non-excitable cells. Proteases released by exocytosis degrade extracellular matrix proteins during extravasation and invasion processes. Thus, sustained elevated levels of $[Ca^{2+}]_{cyt}$ in highly metastatic cells will favor exocytosis of these enzymes. Further, cell invasion needed for metastasis involves continuous addition of plasma membrane to the leading edge. The continuous addition could be provided by protein synthesis; however, this would be a slow process. Exocytosis of endosomes/lysosomes could provide an efficient mechanism for addition of plasma membrane to the leading edge, thus bypassing the need of protein synthesis.

P2 receptors are selective for ATP and are subclassified by their pharmacological properties and transduction mechanisms into P2X and P2Y receptors. P2X receptors are ligand-gated ion channels which are activated by extracellular ATP to elicit a flow of cations. The metabotropic P2Y receptor family, however, belongs to the family of G-protein-coupled receptors and the principal signal transduction pathway involves PLC which leads to the inositol 1,4,5-triphosphate (IP_3) formation and $[Ca^{2+}]_{in}$ mobilization. While ATP is an agonist over all P2Y receptors, UTP is active at P2Y2, P2Y4 and P2Y6 receptors (63). The P2Y2 receptor responds equally to extracellular UTP and ATP. In our study, the addition of UTP triggered a rapid transient increase in $[Ca^{2+}]_{cyt}$ only in highly metastatic, but not in lowly metastatic cells. Interestingly, pre-treatment with UTP decreases the response of highly metastatic cells to further ATP stimulation. In lowly metastatic cells, pre-treatment with UTP did not affect subsequent ATP stimulation, albeit it reduce the amplitude of the $[Ca^{2+}]_{cyt}$ increase, suggesting that either they do not have P2Y6 receptors or that the levels of P2Y2 receptors maybe lower in lowly than in highly metastatic cells. Further studies are needed to address these issues. Nevertheless, these data also suggest that the P2Y2 receptors are desensitized by UTP stimulation only in highly metastatic cells.

To identify the source of Ca^{2+} for the $[Ca^{2+}]_{cyt}$ increase, we performed experiments using Ca^{2+}-free media, to eliminate trans-membrane Ca^{2+} movements. Our data indicated that in highly and in lowly metastatic cells, the amplitude of the $[Ca^{2+}]_{cyt}$ increase was slightly smaller in the absence than in the presence of $[Ca^{2+}]_{ex}$. These data indicate that, the initial $[Ca^{2+}]_{cyt}$ increase derives from both extra- and intracellular stores. Importantly, in highly metastatic cells, the sustained increase in $[Ca^{2+}]_{cyt}$ disappears in the absence of $[Ca^{2+}]_{ex}$ and becomes transient, with τ Ca^{2+} that is one order of magnitude faster than in the presence of $[Ca^{2+}]_{ex}$. This indicates that $[Ca^{2+}]_{ex}$ is needed to maintain an elevated

and sustained $[Ca^{2+}]_{cyt}$ in highly metastatic cells. This should be contrasted to our data from lowly metastatic cells, where ATP stimulation in the absence of $[Ca^{2+}]_{ex}$ results in a decrease in the amplitude of the $[Ca^{2+}]_{cyt}$ increase without affecting $\tau\ Ca^{2+}$, indicating that distinct mechanisms are operational in highly and lowly metastatic cells that allow the sustained $[Ca^{2+}]_{cyt}$ increase observed in highly metastatic cells.

In order to address specific $[Ca^{2+}]_{cyt}$ regulatory mechanisms that could explain the distinct response to agonist stimulation between highly and lowly metastatic cells, we evaluated NCX activity. We focus on this exchanger, because the NCX can operate in both forward and reverse modes in different cell types, depending on the electrochemical equilibrium of Na^+ and Ca^{2+}. In the reverse mode, the NCX works to move Ca^{2+} in and Na^+ out of the cell, whereas in the forward mode it works to extrude Ca^{2+}. Thus, preferential operation of NCX in the forward mode in lowly metastatic cells could explain the rapid transient increase in $[Ca^{2+}]_{cyt}$ following agonist stimulation. In contrast, preferential operation of NCX in the reverse mode in highly metastatic cells could enable a sustained increase in $[Ca^{2+}]_{cyt}$ following ATP treatment. The two simplest approaches to evaluate the presence of NCX is to either increase cytosolic Na^+ ($[Na^+]_{cyt}$) with ouabain, which inhibits the Na^+/K^+-ATPase, or to decrease extracellular Na^+ in the presence of $[Ca^{2+}]_{ex}$. In many cell types including cardiac myocytes, either of these maneuver results in a rapid and transient $[Ca^{2+}]_{cyt}$ increase (77). However, in certain cell types such as astrocytes and endothelial cells, Na^+ removal or ouabain treatment results in only a modest increase in $[Ca^{2+}]_{cyt}$ (29, 78). Our data show that removal of Na^+ results in a minor decrease in $[Ca^{2+}]_{cyt}$ in highly, but not in lowly metastatic cells. In astrocytes, the combined use of ouabain (to increase $[Na^+]_{cyt}$) and Na^+ removal results in a rapid and transient $[Ca^{2+}]_{cyt}$ increase, indicating that increasing the Na^+ gradient can overcome the ability of Ca^{2+} regulatory mechanisms to buffer Ca^{2+} entry via NCX. We performed these types of experiments using concentrations as high as 1 mM ouabain in both highly and lowly metastatic cells, and obtained similar results as those observed with Na^+ removal alone. A negligible $[Ca^{2+}]_{cyt}$ increase was observed in both cell types. The K_i of ouabain for inhibition of Na^+/K^+-ATPase in different human cell types is in the micromolar range, which is in contrast to the K_i in rodents, where the K_i is in the millimolar range. Thus, it is unlikely that the lack of $[Ca^{2+}]_{cyt}$ change in response to ouabain treatment and Na^+ removal were due to incomplete inhibition of the Na^+/K^+-ATPase. Furthermore, the expression of the plasma membrane Na^+/K^+-ATPase ($\alpha1$ as well as $\beta1$ subunits) has been shown decreased in human prostate cancer cells as well as in several other human cancer cell lines (79) which may explain the lack of $[Ca^{2+}]_{cyt}$ changes in response to ouabain. Our studies showed that the Na^+/K^+-ATPase was expressed predominantly in the nuclear membranes as well as the endocytic pathway in several cancer lines (unpublished data).

The fact that removal of Na^+ in either the presence or absence of ouabain did not resulted increases in $[Ca^{2+}]_{cyt}$ in neither highly nor lowly metastatic cells excluded the possibility that NCX may be operational under conditions of Ca^{2+} overload. We therefore evaluated the contribution of NCX for $[Ca^{2+}]_{cyt}$ regulation in both cells following agonist stimulation in the absence of Na^+ and presence of $[Ca^{2+}]_{ex}$. These data indicate that in the absence or presence of $[Na^+]_{ex}$, the amplitude of the ATP-stimulated $[Ca^{2+}]_{cyt}$ increase is similar in highly metastatic cells. However, the kinetics of the $[Ca^{2+}]_{cyt}$ recovery ($\tau\ Ca^{2+}$) is significantly faster in the absence of $[Na^+]_{ex}$ than in the presence of both Na^+ and $[Ca^{2+}]_{ex}$. This suggests that the activity of the NCX working in reverse mode contributes to the sustained elevated $[Ca^{2+}]_{cyt}$. Importantly, re-addition of Na^+ results in a rapid return to basal, indicating that the NCX in the forward mode is also operational in highly metastatic cells. These data are in contrast to our observations in lowly metastatic cells, where in the absence of $[Na^+]_{ex}$, the amplitude of the $[Ca^{2+}]_{cyt}$ increase is similar to the one in the presence of Na^+ and $[Ca^{2+}]_{ex}$, but the $\tau\ Ca^{2+}$ is significantly slower in the absence of Na^+.

Phospholipase C (PLC) plays an important role in Ca^{2+} regulation and in many other signaling pathways. PLCs can be activated not only by the heterotrimeric G-protein-coupled receptor, but also by the protein tyrosine kinase, the small G proteins, and the Wnt/Ca^{2+} signaling pathway. PLC activation hydrolyzes its substrate, phosphatidylinositol 4,5-biphosphate (PIP_2), into two second messengers, IP_3 and diacyglycerol (DAG). Mammalian cells express six families of PLCs, each with both unique and overlapping controls over their expression and subcellular distribution (80). PLC may play a direct role in the regulation of NCX and thus promoting a metastatic phenotype. Future studies are required to investigate the significance of the interaction between PLC and NCX in metastasis. However, a study in cardiac myocytes provided evidence for a NCX-mediated Ca^{2+} influx, which is dependent on a Na^+ entry mechanism triggered by PLC-dependent activation of Transient Receptor Potential Channels-3 (TRPC3) (81). Physical interaction between TRPC3 and NCX1 and an increase of cell membrane expression of NCX upon PLC stimulation provided further evidence for these ion transport systems are associated in a cardiac signaling complex. This study demonstrated that activation of the cardiac Gq- PLC-pathway by Ang II promotes Ca^{2+} influx initiated by either Ca^{2+} re-addition or Na^+ removal consistent with an involvement of reverse mode NCX-mediated Ca^{2+} entry (81). The significance of PLC and NCX in metastasis warrants further investigation.

The pharmacological data using KB-R7943, which inhibits the reverse mode of NCX in some cell types, indicate that pre-treatment of highly and lowly metastatic cells with this drug does not affect steady-state $[Ca^{2+}]_{cyt}$. Importantly, KB-R7943 pre-treatment significantly increases both the ATP-induced $[Ca^{2+}]_{cyt}$ increase and the $\tau\ Ca^{2+}$ in highly metastatic cells when compared to the kinetics of $[Ca^{2+}]_{cyt}$ response in the absence of KB-R7943. Furthermore, highly metastatic cells expressing siRNA-NCX-1, to knockdown NCX, exhibit a smaller amplitude in $[Ca^{2+}]_{cyt}$ with a faster $\tau\ Ca^{2+}$ following ATP-treatment. The characteristics of the $[Ca^{2+}]_{cyt}$ response to ATP in NCX-1 knockdown lowly metastatic cells are affected to a lesser extent than in highly metastatic cells. Altogether, these data suggest that in highly metastatic cells, the NCX operates preferentially in the reverse mode following agonist-stimulation to

enable a sustained $[Ca^{2+}]_{cyt}$ increase, whereas in lowly metastatic cells the preferred mode of operation of the NCX is the forward mode, to extrude $[Ca^{2+}]$. These data also suggest that the transition from a lowly to a highly metastatic phenotype involves a switch in the activity of the NCX from forward mode to reverse mode that is favorable to maintain elevated $[Ca^{2+}]_{cyt}$.

Cell migration, one of the crucial hallmarks of metastasis, is regulated by intracellular calcium. In order for a cancer cell to invade it must communicate with the endothelial cells that line blood vessels. The mechanisms behind cell communication are not fully understood. We have evaluated the proximity and physical interaction of endothelial and metastatic cells. We have shown that V-ATPases at the cell surface (pmV-ATPases) are involved in angiogenesis and metastasis, and have also found that the physical interaction between endothelial and metastatic cells increase pmV-ATPase activity in both cell types (82). Our previous data indicated that interaction between endothelial and metastatic cells helps to acidify the extracellular medium and favors protease activity via V-ATPase. These data emphasize the significance of the acidic tumor microenvironment enhancing a metastatic phenotype (82). Using the same approach as previously described (82), we have evaluated the interaction of endothelial cells and metastatic cells in term of calcium signaling. In these experiments, cells were stimulated with low concentrations of ATP (500 nM) to trigger calcium oscillations in tumor cells but not in endothelial cells. However, when cells where allowed to interact, the endothelial cells showed calcium oscillation and therefore a calcium increased in the mitochondria that is known to trigger apoptosis (Sennoune et al., unpublished observations). The significance of these observations and their relationship to NCX requires further investigation.

To conclude, this study indicates that the NCX operating in reverse mode plays a prominent role in $[Ca^{2+}]_{cyt}$ regulation in highly metastatic cells, and explains the large and sustained increase in $[Ca^{2+}]_{cyt}$ that is observed following ATP-stimulation. This conclusion is supported by ion-substitution experiments, pharmacological approaches to inhibit NCX, and genetic approaches to knockdown of the expression of NCX. These data suggest that the acquisition of a more metastatic phenotype involves the preferential operation of the NCX in reverse mode to allow sustained $[Ca^{2+}]_{cyt}$ increase. The NCX could provide a molecular target to halt metastasis.

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