

The Involvement of Intracellular Calcium in the MCT-Mediated Uptake of Lactic Acid by HeLa Cells

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Abstract: The main object of this study was to evaluate the role of intracellular free calcium ion $[Ca^{2+}]_{in}$ in monocarboxylate transporter (MCT) mediated drug uptake by HeLa cells. It was hypothesized that alterations in the $[Ca^{2+}]_{in}$ levels affect Na^+-H^+ exchanger (NHE) regulated pH_{in} and thereby produce the proton-motivated driving force for monocarboxylate mediated substrate transport. The changes in intracellular pH (pH_{in}) and MCT mediated uptake rates of L-lactic acid by HeLa cells, a human cervical adenocarcinoma cell line, were evaluated under the conditions, whose $[Ca^{2+}]_{in}$ concentrations were altered by various calcium modulators, such as EGTA-AM (a chelator), nifedipine (a Ca^{2+} channel antagonist) and A23187 (an ionophore). For the purpose of comparison, the L-lactic acid uptake by HeLa cells was also evaluated under various pH_{in} conditions induced by dexamethasone. The effects of the extracellular sodium concentration on the L-lactic acid uptake by HeLa cells were evaluated to determine the involvement of NHE-regulated pH changes in the MCT mediated drug uptake process. The $[Ca^{2+}]_{in}$ concentrations and pH_{in} in HeLa were assessed using fluorescent probes fura-2 and 2',7'-bis[2-carboxyethyl-5-carboxyfluorescein] (BCECF), respectively. The treatment of HeLa cells with A23187 at concentrations of 50 and 100 μM enhanced $[Ca^{2+}]_{in}$ by 100% and 200% of the control, respectively. EGTA/AM (50 μM) or nifedipine (100 μM) did not cause any significant changes in the $[Ca^{2+}]_{in}$ levels, whereas EGTA/AM (100 μM) and nifedipine (200 μM) reduced the $[Ca^{2+}]_{in}$ levels by 30% and 25%, respectively, as compared with the control. A23187 at a concentration of 100 μM in the incubation medium lowered pH_{in} (pH 5) and subsequently the uptake rate of lactic acid by 50% ($0.47 \pm 0.03 \mu mol/mg$ protein/min) of the control. In contrast, nifedipine (200 μM) and EGTA-AM (100 μM), the calcium modulators that lowered the $[Ca^{2+}]_{in}$ levels and maintained the higher pH_{in} (pH > 6) of HeLa cells, enhanced the uptake rate of lactic acid by 60% and 130% of the control, respectively. The results of this study demonstrated that there was a close correlation between the $[Ca^{2+}]_{in}$ level and pH_{in} and that NHEs were involved with the MCT mediated uptake process in HeLa cells. An understanding of the role of $[Ca^{2+}]_{in}$ in the MCT mediated transport process could provide an efficient strategy to improve the systemic delivery of monocarboxylate substrates through the cervical mucosa.

Keywords: Calcium modulators; lactic acid; intracellular pH (pH_{in}); monocarboxylate transporters (MCTs); Na^+-H^+ exchangers (NHEs)

1. Introduction

The monocarboxylate transporters (MCTs) in normal cells play an integral role in regulating the influx and efflux of

numerous substrates containing a carboxyl group, such as L-lactic acid, acetic acid and pyruvic acid.¹⁻⁴ The uptake process of the monocarboxylate substrates was dependent on intracellular pH (pH_{in}), and the optimal pH for the lactic

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acid uptake was found to be 4.5.⁵ It was also previously reported that the activities of various epithelial transporters including peptide transporters^{6,7} and Na^+-H^+ exchangers (NHE)^{8,9} were regulated by intracellular calcium in a concentration-dependent manner. Therefore, it was hypothesized that alterations in the $[\text{Ca}^{2+}]_{\text{in}}$ levels in HeLa cells affect MCT mediated L-lactic acid uptake through Na^+-H^+ exchanger (NHE) regulated proton-motivated driving force (i.e., pH_{in} changes).

A human cervical adenocarcinoma cell line, HeLa cell, was used to evaluate the effects of the alterations of the $[\text{Ca}^{2+}]_{\text{in}}$ on the apical influx of L-lactic acid. MCT and NHE expressions in HeLa cells were identified by RT-PCR. The alterations of $[\text{Ca}^{2+}]_{\text{in}}$ in HeLa cells were induced by either a permeable Ca^{2+} chelators (i.e., the acetoxymethyl ester of EGTA [EGTA/AM]) which trapped the intracellular Ca^{2+} ions,¹⁰ a calcium channel blocker (i.e., nifedipine) which increased the supply of blood and oxygen to the heart by blocking the entry of calcium,¹¹ or a Ca^{2+} ionophore (i.e., A23187) which increased the $[\text{Ca}^{2+}]_{\text{in}}$ levels by releasing Ca^{2+} from intracellular calcium vesicles.¹²

The question of whether alterations in the $[\text{Ca}^{2+}]_{\text{in}}$ levels affect NHE regulated pH_{in} and thereby subsequently produce the proton-motivated driving force for monocarboxylate mediated substrate transport was addressed through the measurement of pH_{in} and the L-lactic acid uptake rate by HeLa cells. For the purpose of comparison, the changes in the L-lactic acid uptake rate by HeLa cells whose pH_{in} were induced by dexamethasone were also evaluated. The $[\text{Ca}^{2+}]_{\text{in}}$ concentrations and pH_{in} were assessed using fluorescent probes fura-2 and 2',7'-bis[2-carboxyethyl-5-carboxyfluorescein] (BCECF), respectively. The effects of the extracellular sodium concentration on L-lactic acid uptake by HeLa cells were also evaluated to determine the involvement of NHE-regulated pH changes in the MCT mediated substrate uptake process.

2. Materials and Methods

2.1. Materials. HeLa cell lines and the growth medium were purchased from American Type Culture Collection (ATCC, Manassas, VA). Radiolabeled ^{14}C L-lactic acid (131 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Dexamethasone was purchased from Sigma (St. Louis, MO). Trizol reagent and specific primers for NHE were purchased from invitrogen (Carlsbad, CA). Reverse transcription kit and PCR II core system were purchased from Promega (Madison, WI). All other buffers were of a reagent grade and purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell Culture. HeLa cells were cultured in the Eagle's modified minimum essential medium (MEM) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 90% fetal bovine serum and 10% penicillin/streptomycin solution. The cell lines were grown in 75 cm^2 tissue culture flasks at 37 °C in a 5% CO_2 incubator.

After cells reached confluence, the culture medium was removed and the cells in suspension were rinsed with 0.25% (w/v) trypsin-EDTA (0.53 mM) solution to get rid of all traces of serum components including trypsin inhibitors. The trypsin-EDTA solution (1.0 mL) was added to the flask, which was incubated at 37 °C until cells were fully detached. The growth medium (9.0 mL) was added to cells, which were subsequently aspirated by gentle pipetting. Appropriate aliquots of cell suspension were added to new culture vessels, which were subsequently incubated at 37 °C in a 5% CO_2 incubator. For the uptake studies, approximately 3 million cells were seeded in 12-well plates. The medium was replaced with new medium every two days.

2.3. Measurement of the Intracellular Free Calcium $[\text{Ca}^{2+}]_{\text{in}}$. To elucidate the effects of the calcium modulators on the intracellular calcium levels, the changes in the $[\text{Ca}^{2+}]_{\text{in}}$ level in HeLa cells were examined using acetoxymethyl (AM) ester of a Ca^{2+} -sensitive fluorescent dye, fura-2. HeLa cells (approximately 3.0 million) were grown in 96-well

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plates for two days. After the cell monolayer was washed with the buffer solution (pH 7.4) and its pH was adjusted to pH 4.5 with acidic buffers, cells were pretreated with varying concentrations of A23187 (50 μ M, 100 μ M), EGTA/AM (50 μ M, 100 μ M) or nifedipine (100 μ M, 200 μ M) for 15 min. Cell populations were loaded in 96-well plates with 2 μ M fura-2/AM at 37 °C for 75 min. The fluorescence of intracellular fura-2 formed within the cells through the hydrolysis process of fura-2/AM was measured using a SpectraFluor Plus fluorescent plate reader (Tecan, Research Triangle Park, NC). Fura-2/AM is a dual wavelength fluorescent dye whose excitation maximum is found at 380 nm in the absence of calcium and shifts to 340 nm upon binding calcium. In both cases, the fluorescence emission intensities were measured at 510 nm. The intracellular calcium concentration is considered to be proportional to the ratio of fluorescence intensity of the calcium-bound dye over that of the calcium-free dye (340/380).^{13,14}

2.4. Measurement of the Intracellular pH (pH_{in}). Since the Na^+-H^+ exchanger, an integral regulator of cytoplasmic pH, is expressed in various mammalian cells, the effects of the calcium modulators on intracellular pH (pH_{in}) mediated by NHEs were evaluated using a pH-sensitive fluorescent dye, 2',7'-bis[2-carboxyethyl-5-carboxyfluorescein] (BCECF). For pH_{in} measurement, HeLa cells grown in 96-well plates were preincubated with lipophilic acetoxymethyl ester of BCECF (BCECF-AM) (5 μ M) at 37 °C for 30 min. The cell monolayers were washed with pH 7.4 buffer for 5 min, and then subsequently replaced with the buffers (pH 4.5) containing A23187 (50 μ M, 100 μ M), EGTA/AM (100 μ M) or nifedipine (200 μ M).

The value of pH_{in} is calculated based on the intensity of emission measured at 538 nm by a fluorescent plate reader at predetermined time points. A calibration curve was prepared from the fluorescence ratio of the buffers with known pHs after excitation of the fluorophore at 444 nm (isosbestic point) over that at 490 nm (pH-sensitive wavelength).⁷

2.5. Effects of the Calcium Modulators on the Uptake Rate of Lactic Acid in HeLa Cells. To determine whether or not the uptake rate of the monocarboxylate substrates is closely correlated with the $[Ca^{2+}]_{in}$ levels, the changes in the uptake rate of lactic acid by HeLa cells were evaluated in the presence of various calcium modulators. HeLa cells grown in 12-well plates were washed with the buffer solution at pH 7.4 for 5 min and preincubated with those containing 100 μ M EGTA-AM, 100 μ M A23187 or 200 μ M nifedipine for 20 min. Cells were then treated with 0.5 μ Ci/mL radiolabeled L-lactic acid along with 1 mM cold L-lactic acid for 1 min at 37 °C. The amounts of L-lactic acid uptake and accumulative protein in each well were

determined using a scintillation counter (Beckman Coulter, Fullerton, CA) and Coomassie Blue and BioRad assay techniques, respectively. Bovine gamma globulin was used as a standard to quantify the amount of lactic acid uptake by each well.

2.6. Effects of the Intracellular pH (pH_{in}) on the Uptake Rate of Lactic Acid by HeLa Cells. To verify that pH_{in} change-induced proton gradient is the major driving force of the lactic acid uptake by HeLa cells, the uptake rate of L-lactic acid by HeLa cells was evaluated under various pH_{in} conditions induced by other mechanisms than the calcium modulators. HeLa cells were prepared as previously described and exposed to 2.0 or 2.5 μ mol/L dexamethasone. After HeLa cells were treated with dexamethasone for 8 h, cells were harvested and washed with Hepes-buffered Hanks' balanced salt solution (HHBSS, pH 7.4) to remove excess dexamethasone. pH_{in} was measured using a pH-sensitive fluorescent dye, 2',7'-bis[2-carboxyethyl-5-carboxyfluorescein] (BCECF). In similar studies, zinc acetate dehydrate (1 mM) was also included to check whether or not zinc ions inhibit dexamethasone-induced acidification. The uptake rates of L-lactic acid by HeLa cells were evaluated under the 3 pH_{in} conditions (pH 7.4, 6.4, 5.8) as previously described, and the results were compared with those induced by calcium modulators.

2.7. Effects of the Extracellular Sodium on the Uptake Rate of Lactic Acid by HeLa Cells. To determine the involvement of NHE-regulated pH changes in the MCT mediated lactic acid uptake process, the uptake rate of L-lactic acid by HeLa cells was measured in the presence of varying concentrations of sodium (~50 mM), which is known to be an integral component in the regulation process of NHE activities. The confluent HeLa cells were rinsed with Hepes-buffered Hanks' balanced salt solution (HHBSS, pH 7.4) in the presence or absence of sodium chloride for 30 min. A mixture of 0.5 μ Ci/mL of the radiolabeled L-lactic acid and 1 mM cold L-lactic acid was prepared in MES-HBSS (at pH 4.5). The same mixture prepared in MES-HBSS in which NaCl was replaced with choline chloride was used as a control. Cells were incubated with test samples at room temperature for 1 min, and the uptake process was terminated by rinsing cell monolayer with the ice-cold buffer. Cells were lysed in the Triton X cell lysis solution, and the amount of L-lactic acid in cells was quantified by analyzing the radiolabeled L-lactic acid using a scintillation counter.

2.8. RNA Extraction and RT-PCR Analysis. To validate the usage of HeLa cells in the MCT mediated regulation studies, MCT and NHE isoforms in RNA extracted from HeLa cells were identified using reverse transcription polymerase chain reaction (RT-PCR). About 3 million HeLa cells were seeded on a Petri dish, and the growth medium was changed every two days. Once the cells were confluent, RNA was extracted according to the procedure previously reported.¹⁵ Briefly, the growth medium was replaced with 800 μ L of TRIzol cell lysis reagent (Invitrogen, Carlsbad, CA), and RNA was extracted

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Table 1. Forward and Reverse Oligonucleotide Primer Sequences Used for RT-PCR Screening of Monocarboxylate (MCT) Isoforms as Well as Na^+/H^+ -Exchanger (NHE) Isoforms, NHE1 and NHE3, in Human Cervical Adenocarcinoma Cells

MCT isoforms	oligonucleotide sequence	
	forward	reverse
MCT-1	TCTACACTTAAATGCCACCAGCAGTTGGAGGTCC	AAAAACACCAAACACACATATCTCACACATAGCAC
MCT-5	ATCTCAGCTGCCCTCTTCATGGGTGGCAGCTT	TCAAGGCTCAGAGGTGGTCAACAATTTGGCC
MCT-6	GTTCACGTGCAAATGGAGCCGGTATGA	AACCACTTTGCCAGTTCATGTCACT

transporter	sense 5'–3'	antisense 5'–3'
NHE1 base pairs (326)	GTGGTGGTTGCTTTGGTG	ACGATGCTTGAGATAGTGGG
NHE3 base pairs (298)	CACCAAGCATGTGCGTATCAT	GAAGACCAGCGTCAGGAGC

Table 2. Calcium Modulators and Concentrations Used To Regulate Intracellular Calcium Levels^a

calcium modulators	mechanism	concentration	
		studied	selected
A23187	Ca^{2+} release from intracellular calcium stores: elevation of $[\text{Ca}^{2+}]_{\text{in}}$ concentration	50, 100 μM (22)	50, 100 μM
EGTA/AM	intracellular calcium chelation: reduction of $[\text{Ca}^{2+}]_{\text{in}}$ concentration	10, 30, 100 μM (23; 24)	50, 100 μM
nifedipine	L-type calcium channel blockade: reduction of $[\text{Ca}^{2+}]_{\text{in}}$ concentration	100 μM (10)	100, 200 μM

^a The mechanisms by which A23187, EGTA/AM and nifedipine modulate intracellular calcium levels were included.

by sequential addition of phenol, CHCl_3 and isopropanol. The samples were centrifuged at 12,500 rpm.

Reverse Transcription Kit, Polymerase Chain Reaction Kit (Promega Inc., Madison, WI) and synthetic oligonucleotides for MCT isoforms 1 to 6 as well as NHE clones 1 to 3 (Table 1) were tested according to the manufacturer's instructions. Polymerase chain reaction was run for 35 cycles with denaturation for 1 cycle at 95 °C for 2 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension 1 cycle at 72 °C for 5 min in a DNA Thermal Cycler (Midwest Scientific, St. Louis, MO). A sample (20 μL) of the polymerase chain reaction product along with loading dye was analyzed by electrophoresis performed on a prestained 3% agarose gel. The gel was photographed under ultraviolet illumination. The results of the RT-PCR reactions in the absence of RNA were used as a negative control, whereas those in the presence of RNA obtained from Caco-2 human intestinal cell line were used as a positive control.

2.9. Statistical Analyses. The differences in the $[\text{Ca}^{2+}]_{\text{in}}$ levels and changes in pH_{in} under various experimental conditions were statistically analyzed by a one-way analysis of variance (ANOVA) with pairwise multiple comparisons using the Student–Newman–Keuls method. *P* values ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of the Calcium Modulators on the Intracellular Calcium Profiles. The $[\text{Ca}^{2+}]_{\text{in}}$ levels in HeLa cells upon treatment with varying concentrations of A23187,

EGTA/AM or nifedipine were measured using a SpectraFluor Plus fluorescent plate reader. The concentrations (Table 2) used for this study were selected based on previous reports.⁵ $[\text{Ca}^{2+}]_{\text{in}}$ was obtained based on the ratio of fluorescence at 340 nm over that at 380 nm. The treatment of the cells with A23187 enhanced $[\text{Ca}^{2+}]_{\text{in}}$ by 100% and 200% of the control at A23187 concentrations of 50 and 100 μM , respectively. EGTA/AM at a concentration of 50 μM or nifedipine at a concentration of 100 μM did not cause any significant changes in the $[\text{Ca}^{2+}]_{\text{in}}$ levels, whereas EGTA/AM at a concentration of 100 μM and nifedipine at a concentration of 200 μM reduced the $[\text{Ca}^{2+}]_{\text{in}}$ levels by 30% and 25%, respectively, as compared with the control (Figure 1). The results of this study demonstrated that $[\text{Ca}^{2+}]_{\text{in}}$ in HeLa cells can be altered by externally added calcium modulators. The concentrations of 50 and 100 μM for A23187, 100 μM for EGTA/AM and 200 μM for nifedipine were selected for further studies, as these concentrations had significant effects on the $[\text{Ca}^{2+}]_{\text{in}}$ levels in HeLa cells.

3.2. Effects of the Calcium Modulators on the Intracellular pH (pH_{in}). pH_{in} of HeLa cells upon treatment with varying concentrations of nifedipine, EGTA/AM or A23187 was evaluated using a pH-sensitive fluorescent dye, BCECF. The treatment of the HeLa cells with the buffer solution (pH 4.5) alone produced intracellular acidification that reached its equilibrium (pH_{in} of 6) in about 80 min as shown in Figures 2a and 2b. A23187 at concentrations of 50 μM and 100 μM lowered pH_{in} from 7.4 to 5.4 and 5.0, respectively. In contrast, EGTA/AM (100 μM) and nifedipine (200 μM) did not cause any significant changes in pH_{in} of HeLa cells, maintaining the pH_{in} above 6.0, which was similar to the results obtained from the acidic buffer solution (pH 4.5) (Figure 2b).

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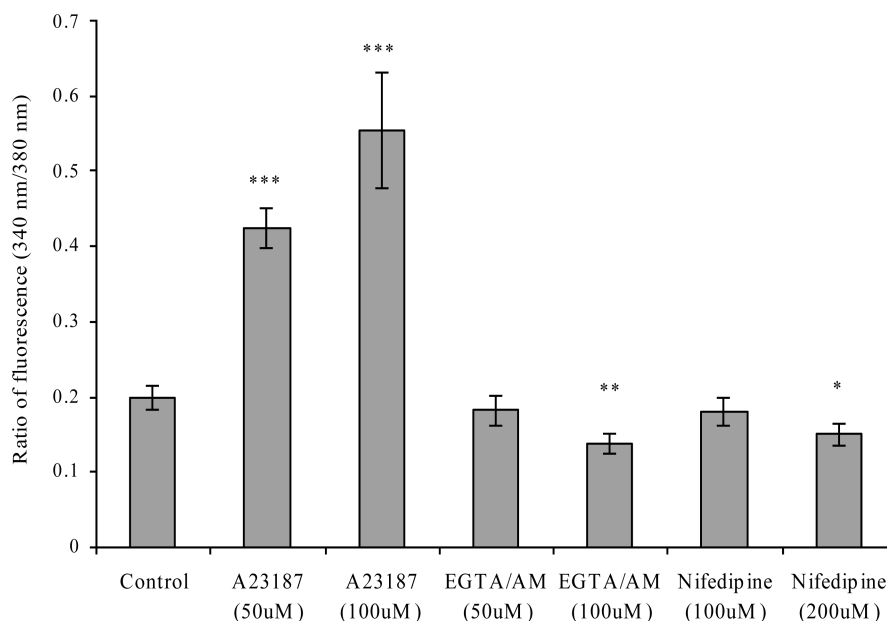


Figure 1. (a) Intracellular calcium concentrations of HeLa cells in the absence (buffer alone) or presence of the calcium ionophore A23187 (50 μ M, 100 μ M), the intracellular calcium chelator EGTA-AM (50 μ M, 100 μ M) or the calcium channel blocker nifedipine (100 μ M, 200 μ M). (b) Intracellular pH measurements in HeLa cells treated with pH 4.5 buffer (indicated by the arrow) with or without 50 μ M or 100 μ M A23187 (a calcium ionophore) for 30 min. Each point represents the mean \pm standard error of the mean ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$ significantly different from control.

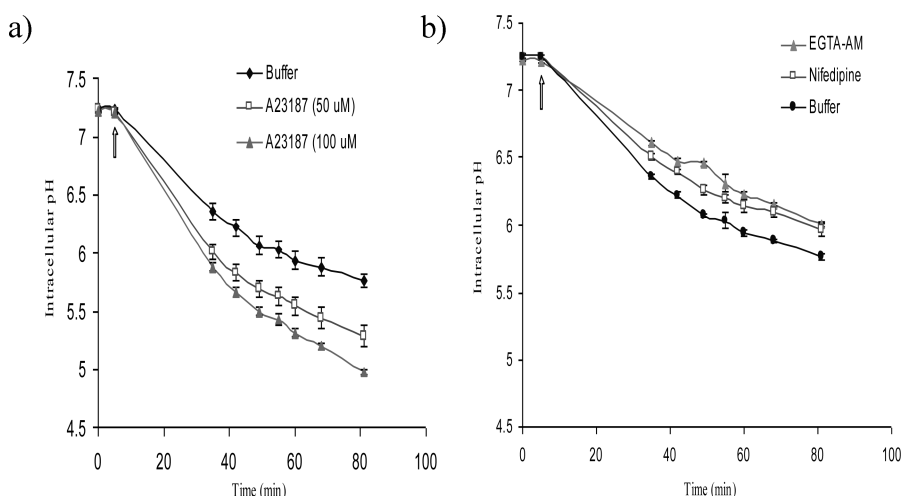


Figure 2. pH_{in} of HeLa cells in the presence of an intracellular calcium chelator, 100 μ M EGTA/AM or 100 μ M nifedipine for 30 min. Each point represents the mean \pm standard error of the mean ($n = 3$).

3.3. Effects of the Intracellular Calcium Modulators on the Uptake Rate of Lactic Acid by HeLa Cells. The uptake rate of ^{14}C L-lactic acid by HeLa cells upon exposure to the calcium modulators was measured at the apical pH of 4.5, which mimics *in vivo* vaginal conditions. A23187 at a concentration of 100 μ M in the incubation medium lowered pH_{in} (pH 5) and subsequently the uptake rate of lactic acid by 50% (0.47 ± 0.03 μ mol/mg protein/min) of the control (Figure 3). In contrast, the uptake rates of lactic acid were 1.61 ± 0.27 μ mol/mg protein/min and 2.32 ± 0.17 μ mol/mg protein/min in the presence of nifedipine (200 μ M) or EGTA-AM (100 μ M), respectively. The calcium modulators, nifedipine (200 μ M) and EGTA-AM (100 μ M), that lowered

the $[\text{Ca}^{2+}]_{\text{in}}$ levels and maintained the higher pH_{in} (pH > 6) of HeLa cells, enhanced the uptake rate of lactic acid by 60% and 130% of the control, respectively, supporting the hypothesis that the alterations of $[\text{Ca}^{2+}]_{\text{in}}$ could affect the uptake rates of lactic acid by HeLa cells, which were pH_{in} dependent.

3.4. Effect of the Extracellular Sodium on the Uptake Rate of Lactic Acid in HeLa Cells. To confirm the involvement of NHE in the regulation of the MCT mediated process, the uptake rate of ^{14}C L-lactic acid in HeLa cells in the presence or absence of sodium (~ 50 mM) was examined. As shown in Figure 3, the uptake rate of lactic acid (0.5 μ Ci/mL ^{14}C L-lactic acid plus 1 mM cold L-lactic

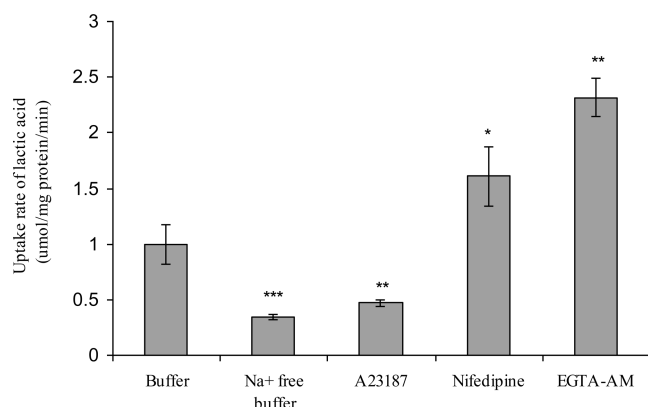


Figure 3. Effects of extracellular sodium and calcium modulators on the uptake rate of L-lactic acid by HeLa cells at pH 4.5. L-Lactate transport was measured in the absence (buffer alone) or presence of sodium, calcium ionophore A23187 (100 μ M), calcium channel blocker nifedipine (200 μ M) or intracellular calcium chelator EGTA-AM (100 μ M). Each point represents the mean \pm standard error of the mean ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from control.

acid) by HeLa cells treated with the sodium-containing solution (37.5 mM, pH 4.5) was 0.97 ± 0.28 μ mol/mg protein/min. On the contrary, the uptake rate of L-lactic acid by cells treated with the sodium-free buffer was reduced by 65% (0.34 ± 0.03 μ mol/mg protein/min) as compared with the control.

In a previous study,⁵ the classic MCT inhibitor, α -cyano-4-hydroxycinnamic acid (CHC), significantly affected the uptake rate of L-lactic acid by HeLa cells, indicating that L-lactic acid was transported through HeLa cell via the single type saturable process. The outcomes of these studies are indicative of the potential involvement of $\text{Na}^+ - \text{H}^+$ exchangers in the MCT mediated uptake process.

3.5. Effects of the Intracellular pH (pH_{in}) on the Uptake Rate of Lactic Acid by HeLa Cells. pH_{in} of HeLa cells was induced with dexamethasone (2.0 or 2.5 μ mol/L). pH_{in} in HeLa cells treated with dexamethasone was lowered from pH of 7.4 to about 5.8, supporting that dexamethasone can ensue intracellular acidification of HeLa cells. The morphological features revealed that zinc ions did not inhibit dexamethasone-induced acidification. The uptake rate of lactic acid by HeLa cells evaluated under the three pH_{in} conditions (pH 7.4, 6.4, 5.8) induced by dexamethasone followed a profile, whose rate gradually decreased as pH_{in} decreased (Figure 4). These results were in good agreement with those induced by the calcium modulators, further confirming the integral role of pH_{in} in the MCT mediated regulation of lactic acid uptake.

3.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was used to identify MCT and NHE isoforms in cumulative mRNA isolated from HeLa cells. In a previous study,⁵ the results of the RT-PCR study with specific oligoprimers for MCT isoforms confirmed the

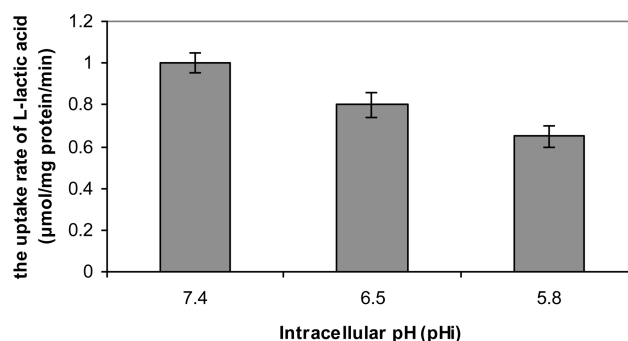


Figure 4. Effects of the intracellular pH induced by dexamethasone on the uptake rate of lactic acid by HeLa cells. Each point represents the mean \pm standard deviation of the mean ($n = 3-4$). Values at pH 6.5 and 4.8 are significantly different from that of pH 7.4, $P < 0.05$.

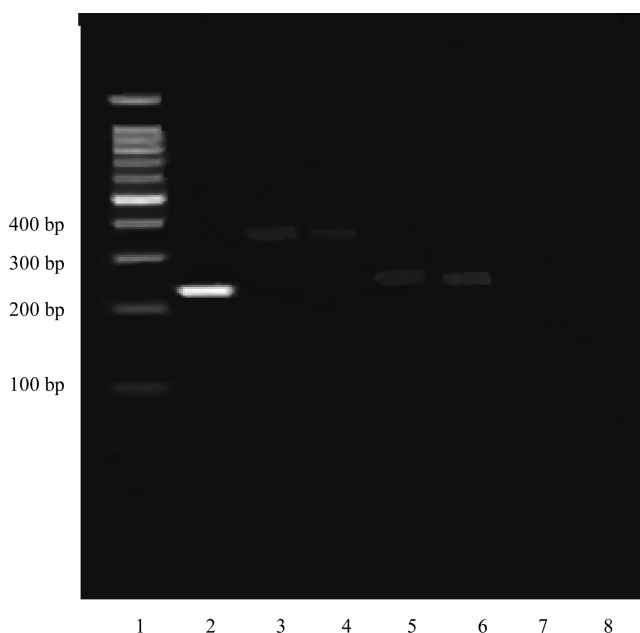


Figure 5. The results of the RT-PCR study for NHEs in HeLa Cells. Lane 1 represents 100 bp DNA ladder and lane 2 GAPDH. Lanes 3 and 4 represent NHE1 for Caco-2 (positive control) and HeLa cell, respectively. Lanes 5 and 6 represent NHE3 for Caco-2 (positive control) and HeLa cell, respectively. Lanes 7 and 8 represent negative controls for NHE1 and NHE3, respectively. bp: base pairs.

presence of MCT-1, -5, and -6 at the apical membrane in HeLa cells.

The expression profiles of NHE1 and NHE3 mRNA in HeLa cells were also characterized based on NHE isoform-specific primers (Table 1). Caco-2 mRNA was used as a positive control. As shown in Figure 5, RT-PCR yielded both NHE1 and NHE3 PCR products at 326 and 298 bp, respectively, which were identical in size to those from the previous report. NHE1 and NHE3 isoforms present in HeLa cells were detected at the same base pair levels as those in the positive control. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), which is used as a housekeeping primer in

RT-PCR, also produced a band with a known size, which was identical to the published sequence.¹⁶ An identification of NHE isoforms validated the usage of HeLa cells in the MCT mediated substrate transport studies.

4. Discussion

The cervical and intravaginal routes have been used for the delivery of locally active compounds, such as antibacterial, antifungal, antiprotozoal, antiviral, labor-inducing and spermicidal agents, prostaglandins and steroids. At present, the cervical route is broadening its role as a potential site for systemically active compounds.^{17,18} One of the major defense mechanisms of the vagina against exogenous microbes is involved with the normal microbial flora, predominantly lactobacilli, which compete with exogenous microbes by generating organic acids, such as lactic acid, and maintaining a low pH (pH range: 4.0 to 4.5).^{16,19,20} The elevated glucose consumption is a required component of the protective mechanisms of the vagina, as it produces acid and provides an evolutionary advantage to the cervical or vaginal cells similar to the function of carcinogenesis.^{21,22}

This study was conducted to determine whether or not the intracellular calcium is involved with the monocarboxylate transporter (MCT) mediated lactic acid uptake process in the cervix. A human cervical adenocarcinoma cell line, HeLa cell, which expresses MCTs, has been used for the characterization of lactic acid transport. A reduction in the $[Ca^{2+}]_{in}$ levels in HeLa cells was observed in the presence of calcium chelators like EGTA/AM or calcium channel blockers, such as nifedipine, whereas the elevated $[Ca^{2+}]_{in}$ levels were observed upon exposure to the permeable calcium ionophores like A23187.

The calcium channel blocker, nifedipine, enhanced the uptake rate of L-lactic acid by HeLa cells by 60%, which is mainly attributed to the reduction of the $[Ca^{2+}]_{in}$ levels. An

addition of EGTA-AM produced effects on the L-lactic acid uptake process similar to those produced by the channel blockers. In contrast, the Ca^{2+} ionophore, A23187, significantly reduced L-lactic acid uptake rate, which is mainly attributed to the enhancement of the $[Ca^{2+}]_{in}$ levels.

Since the alteration of the $[Ca^{2+}]_{in}$ levels was closely correlated with pH_{in} and NHE activities in intestinal epithelial cells,^{6,7,23} the intracellular pH changes in HeLa cells produced by the calcium modulators, such as calcium channel blockers, chelators or ionophores, were subsequently examined. Nifedipine and EGTA-AM maintained pH_{in} (less acidic), which is similar to those caused by the acidic buffer, and subsequently a higher electrochemical proton gradient across the apical plasma membrane, allowing faster uptake rates of L-lactic acid by HeLa cells. On the other hand, the Ca^{2+} ionophore, A23187, lowered the intracellular pH (highly acidic), thus reducing the transmembrane pH gradient, and subsequently the L-lactic acid uptake rate. MCT is a tertiary active transporter, and the MCT mediated transport processes, especially the transcellular pathway, were affected by the pH gradient. The degree of reduction of the uptake rate of L-lactic acid by HeLa cells, whose acidic pH_{in} was induced by dexamethasone, showed good correlation with those induced by calcium modulators.

The effects of $[Ca^{2+}]_{in}$ on pH_{in} appear to be best explained in terms of NHEs involvement in the pH regulation process. $[Ca^{2+}]_{in}$ was identified as an integral regulator of Na^+-H^+ exchange activity,²³ and NHEs are known to be indispensable in restoring intracellular pH toward the neutral values in response to acid-load stimulus.²⁴ Previous studies showed that NHE3 in intestinal cells and fibroblasts are activated by calcium-dependent protein kinase C at the low $[Ca^{2+}]_{in}$ concentrations.^{8,25} The involvement of NHEs in the MCT activity was also supported by the results in which the uptake rates of L-lactic acid decreased as the extracellular sodium levels decreased in the system. The sodium-free conditions prevent the NHEs from driving intracellular H^+ ions out, followed by a decreased H^+ gradient and reduction in the uptake of lactic acid.^{26,27} Successive studies are under way to elucidate the role of each NHE isoform on the transport

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process of monocarboxylate substrates across cervical epithelium. It was also considered that the use of the carcinoma cell line has a potential limitation, as the level of acidosis and the expression of transporters in normal tissues may be noticeably different, which may exert a distinctive discrepancy between in vitro outcome and clinical efficacy.

5. Conclusion

This study demonstrated that a reduction of the $[Ca^{2+}]_{in}$ level could enhance the MCT mediated L-lactic acid uptake

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by HeLa cells. A close correlation between the $[Ca^{2+}]_{in}$ level and pH_{in} was indicative of the potential involvement of NHEs in the MCT mediated uptake process in HeLa cells. An understanding of the mechanisms responsible for the alterations of the $[Ca^{2+}]_{in}$ and the MCT mediated transport process could lead to an efficient strategy to improve the uptake rate of monocarboxylate substrates through the cervical mucosa.

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