An Inhibitor of Na⁺/H⁺ Exchanger (NHE), Ethyl-Isopropyl Amiloride (EIPA), Diminishes Proliferation of MKN28 Human Gastric Cancer Cells by Decreasing the Cytosolic Cl⁻ Concentration via DIDS-Sensitive Pathways

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MAPK • Rb • p21 • Cell cycle • Anion exchanger

Abstract
Background/Aims: Tumor cells produce a large amount of acidic metabolites due to their high metabolic condition. However, cytosolic pH (pHₙ) of tumor cells is identical to or even slightly higher than that of normal cells. To maintain pHₙ at a normal or higher level, tumor cells would have to have higher expression and/or activity of H⁺ transporting systems than normal cells. The purpose of the present study was to identify effects of ethyl-isopropyl amiloride (EIPA, an inhibitor of Na⁺/H⁺ exchanger (NHE)) on proliferation of human gastric cancer MKN28 cells.

Methods: Effects of EIPA on proliferation, pHₙ, [Cl⁻]ₙ and expression of proteins regulating cell cycle and MAPKs were studied in MKN28 expressing NHE exposed to EIPA for 48 h. Results: EIPA suppressed proliferation of MKN28 cells by causing G₀/G₁ arrest without any significant effects on pHₙ but associated with reduction of [Cl⁻]. Although EIPA alone had no effects on pHₙ, EIPA co-applied with DIDS (an inhibitor of Cl⁻/HCO₃⁻ exchangers; i.e., anion exchanger (AE) and Na⁺-driven Cl⁻/HCO₃⁻ exchanger (NDCBE)) reduced pHₙ, suggesting that DIDS-sensitive Cl⁻/HCO₃⁻ transporters such as AE and/or NDCBE keep pHₙ normal by stimulating HCO₃⁻ uptake coupled with Cl⁻ release under an NHE-inhibited condition. EIPA-induced lowered [Cl⁻], up-regulated expression of p21 associated with phosphorylation of MAPKs, suppressing proliferation associated with G₀/G₁ arrest. Conclusions: EIPA suppressed proliferation of MKN28 cells through up-regulation of p21 expression via reduction of [Cl⁻], as a result from DIDS-sensitive Cl⁻/HCO₃⁻ exchanger-mediated compensation for keeping pHₙ normal under an NHE-inhibited condition. This is the first study revealing that an NHE inhibitor suppressed the proliferation of cancer cells by reducing [Cl⁻], but not pHₙ.
Introduction

Tumor cells predominantly produce energy with high-rate glycolysis followed by lactate formation in their cytoplasm due to their fast proliferation under hypoxic, hyponutrient conditions [1] differently from most normal cells producing energy with relatively low-rate glycolysis followed by oxidation of pyruvate in mitochondria [2]. This high-rate glycolysis with lactate formation of tumor cells provides a large amount of H⁺ leading to acidic microenvironments. Even in environments that tumor cells produce a large amount of H⁺ due to high-rate glycolysis, the cytosolic pH (pHᵅ) of tumor cells is maintained at a level identical to or even slightly higher than that of normal cells [3, 4]. However, if pHᵅ of tumor cells is lowered by production of a large amount of H⁺, tumor cells would induce cell cycle arrest and apoptosis [5, 6]. Therefore, regulation of pHᵅ is critical for survival of tumor cells producing a large amount of H⁺. To maintain pHᵅ at a normal level under conditions with production of a large amount of H⁺, tumor cells should have higher expression and/or activity of H⁺ transporting systems than normal cells.

Four major transporters contributing to pHᵅ regulation have been identified in tumor cells: 1) Na⁺/H⁺ exchanger (NHE), 2) Na⁺/HCO₃⁻ cotransporter (NBC), 3) Cl⁻/HCO₃⁻ exchanger including anion exchanger (AE) and Na⁺-driven Cl⁻/HCO₃⁻ exchanger (NDCBE), and 4) H⁺ pumps (v-type H⁺-ATPase, etc.) [7-9]. On the other hand, acidic microenvironments surrounding tumor cells produced by activation of H⁺ transporters provide tumor cells with advantages for migration and invasion. NHE family, which is ubiquitously expressed in most cells, is a major regulator of pHᵅ, and 10 isoforms of NHEs have been identified [10, 11]. NHE1 is the most ubiquitously expressed one among 10 isoforms, and its activity is regulated by numerous growth factors, mitogens, integrins, tyrosine phosphatases and cytokines [12-18]. NHE in tumor cells is a key transporter to maintain pHᵅ at a normal level [19], and regulation of NHE activity is one of the most important factors for proliferation and migration of many tumor cells [20-22].

Among many anticancer agents, NHE inhibitors as intelligent agents against proliferation of cancer cells in acidic environments are the most investigated ones [23]. However, the mechanism of NHE inhibitors diminishing proliferation of cancer cells is still unclear, although NHE inhibitors are generally thought to lower pHᵅ resulting in a decreased proliferation rate. In the present study, we investigated the effect of 5-(N-ethyl-N-isopropyl) amiloride (EIPA, an NHE inhibitor [24]) on the proliferation of cancer cells regarding the intracellular ionic environment in general and not exclusively the H⁺ concentration. This is the first report indicating that an NHE inhibitor induced G₁/G₀ arrest via up-regulation of MAPKs/p21 by reducing the cytosolic Cl⁻ concentration ([Cl⁻]ᵅ).

Materials and Methods

Cell culture

The moderately differentiated human gastric adenocarcinoma cell line, MKN28, were seeded into 25 cm² flasks at a density of 2.5 × 10⁵ cells/flask and incubated for 24 h in RPMI1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO₂ in air. We defined this time point as time zero (0 h). Then, cells were cultured in a culture medium containing ethyl isopropyl amiloride (EIPA, Sigma–Aldrich) or DMSO (Sigma–Aldrich) as a solvent control for EIPA.

Assay of cell proliferation

After culture under each experimental condition for 48 h, cells were detached from the flasks with trypsin–EDTA and counted with a hemocytometer. EIPA was dissolved and stocked in DMSO at concentrations 1,000 times higher than those finally applied to the cells in the culture medium. The concentrations of EIPA used in the present study were 5, 10, 25, 50, and 100 µM, and the concentration of DMSO (solvent control) was 0.1%.
Analysis of cell cycle

After culturing the cells in the culture medium containing EIPA or DMSO alone for 48 h, we detached the cells from the flasks with trypsin–EDTA treatment, and centrifuged the detached cells. Nuclear Isolation Medium of 0.5 ml (NIM-DAPI 10; Beckman Coulter, Fullerton, CA, USA) was added to cells in the pellets [25]. We determined cell cycle phases from 10,000 cells using FlowJo software (Tree Star, Inc., Ashland, OR, USA) by the Cell Lab Quanta (Beckman Coulter) with an excitation at 365 nm and emission at 450 nm for DAPI.

RT-PCR and real-time quantitative RT-PCR

At 48 h after culturing cells, we prepared total RNA from cells by using RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). Total RNA was first transcribed with Superscript TM III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) by using random hexamers for priming in accordance with the manufacturer’s instruction. The expression levels of human NHE1 mRNA were quantified by real-time quantitative PCR, using the ABI Gene Amp 5700 (Applied Biosystems, Foster City, CA, USA). We generated standard curves by using total RNA of human stomach (Cell Applications, San Diego, CA, USA) as template. The ratio of the signal of NHE to that of GAPDH was calculated for each sample. The PCR primers and probes used in the present study were as follows: TaqMan Gene Expression Assays; human NHE1 (Hs00300047_m1), human NHE2 (Hs00268166_m1), human NHE3 (Hs00188200_m1), human NHE4 (Hs00962493_m1), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) (Applied Biosystems).

Measurement of cytosolic pH (pHc)

We measured pHc of MKN28 cells using carboxy-seminaphthorhodafluor-1 (carboxy-SNARF-1) (Molecular Probes, Eugene, OR, USA), a pH-sensitive fluorescent dye, with an inverted confocal laser microscope, LSM510META (Carl Zeiss, Jena Germany). Cells were seeded into 35 mm glass bottom dishes at a density of 1.0 × 10^5 cells/dish and incubated for 24 h in RPMI1640 medium supplemented with 5% FBS in a humidified incubator at 37°C. After 24 h, cells were equilibrated at 37°C in air with 5% CO2 in air. Then, cells were cultured in the culture medium containing 25 µM EIPA with/without 50 µM 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS) (Sigma-Aldrich) or DMSO alone as a solvent control. After culture under each experimental condition for 48 h, cells were equilibrated at 37°C in air with 5% CO2 for 30 min in saline solutions (115 mM NaCl, 25 mM NaHCO3, 11 mM glucose, 4.4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, pH7.4 adjusted by CsOH) containing 10 µM carboxy-SNARF-1 of the acetoxymethyl ester form. The culture dishes were placed in an incubator, ZILCS (Tokai Hit Co., Shizuoka, Japan), on the stage of the confocal microscope and allowed to adapt for at least 20 min before starting pHc measurements. The excitation laser beam of 514 nm (Ar laser) was directed to the sample via a Plan-Apochromat 100× oil-immersion objective lens (NA 1.4, Carl Zeiss). Emitted fluorescence was simultaneously collected using a PMT, and the separated fluorescences were detected with 24 photomultiplier tubes (PMTs). Each PMT detected fluorescence in each wavelength range of 10.7 nm, and we collected two PMTs centered at 645 nm and 592 nm. The intensity of fluorescence was digitized with a META system. Several regions of interest (ROI) with a diameter of 1 µm were then randomly selected excluding nuclear regions. The emission ratio was calibrated using solutions (110 mM KCl, 25 mM KHCO3, 11 mM glucose, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES) with varying pH adjusted by CsOH that contained 10 µM nigericin (K+/H+ ionophore). The fluorescence emission ratio (645 nm / 592 nm) was calculated and used to estimate pHc from the calibration curve.

Measurement of cytosolic Cl- concentration ([Cl−]c)

We measured [Cl−]c of MKN28 cells using N-(Ethoxycarbonyl methyl)-6-methoxy quinolinium (MQAE, Invitrogen, Carlsbad, CA, USA) at 37°C in air with 5% CO2 in saline solutions (115 mM NaCl, 25 mM NaHCO3, 11 mM glucose, 4.4 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, pH7.4 adjusted by CsOH) as previously reported [26]. The MQAE is insensitive to physiological changes in pH [27]. Calibration of the fluorescence based on [Cl−]c was accomplished by 5 µM nigericin (Wako Pure Chemical Industries, Osaka, Japan), 5 µM valinomycin (K+ ionophore, Sigma-Aldrich), and 10 µM tributyltin chloride (Cl-/OH ionophore, Wako Pure Chemical Industries) in 140 mM-K+ calibration buffers (pH 7.4) with various concentrations of Cl−, which was replaced with NO3-. The ionophores were used in order to adjust the [Cl−]c to a level identical to the extracellular Cl− concentration at constant cytosolic K+ and H+ concentrations. We prepared calibration buffers by mixing 0 and 100 mM Cl− buffers. In the 140 mM K+ buffer with these ionophores, [Cl−]c depends solely on the extracellular Cl− concentration. Cellular fluorescence values observed in the 0, 50, and 100 mM
Cl buffers were processed as the fluorescence intensity (F) and incorporated into the Stern-Volmer equation as follows: 

\[ F/F_0 = 1 + K_{sv}[Cl] \]

where \( F_0 \) is the fluorescence at [Cl] = 0 mM, \( F \) is the fluorescence intensity of the cells equilibrated to a defined [Cl], and \( K_{sv} \) is the Stern-Volmer constant. \( K_{sv} \) was determined by performing a linear regression on a plot of \( F/F_0 \) vs. [Cl]. MKN28 cells were cultured in a glass bottom dish with the culture medium containing 5 mM MQAE with EIPA or DMSO for 45 min at 37°C in a CO2 incubator. MQAE-loaded cells were washed with the culture medium containing EIPA or DMSO, and incubated for 20 min at 37°C. Then, the intensity of MQAE in the chamber (ZILCS) on the stage of the microscope was measured by LSM510 META system. The sample was excited at 780 nm using a 2-photon excitation laser system (MaiTai®, Spectra-Physics, Tokyo, Japan). The laser beam was directed to the dish containing the cells via a C-Apochromat 40x water-immersion objective lens (Carl Zeiss). Emitted fluorescence was simultaneously collected by a gating, and the separated fluorences were detected by 24 PMTs. We collected one PMT at 460 nm. Then, the intensity was digitized with a META system. Several regions of interest (ROI) with a diameter of 1 µm were then randomly selected excluding nuclear regions. The [Cl] was estimated by using the Stern-Volmer equation with the determined value of \( K_{sv} \).

Western blotting
We cultured the cells in the culture medium containing EIPA or DMSO alone for 48 h. Immunoblotting was performed as previously reported [28]. The blots were incubated with a primary antibody (anti-ERK, anti-phosphorylated-ERK (Thr202/Tyr204), anti-p38, anti-phosphorylated-p38 (Thr180/Tyr182), anti-JNK, anti-phosphorylated-JNK (Thr183/Tyr185), anti-p21, anti-p53, anti-phosphorylated-p53 (Ser15), and anti-GAPDH obtained from Cell Signaling Technology (Beverly, MA, USA), and anti-NHE1, and anti-NHE2 from Millipore (Temecula, CA, USA)), all of which were detected by using the ECL plus (GE Healthcare, Buckinghamshire, UK). We measured the band densities with Image Lab (BIO-RAD, Hercules, CA, USA) after scanning with Chemidoc XRS Plus system (BIO-RAD).

Statistical analysis
Data are expressed as means ± SEM. Statistical analysis was carried out using Student’s t-test. The differences were considered significant when the P value was less than 0.05.

Results

mRNA transcriptions of NHE isoforms
We first investigated the mRNA transcriptions of NHE isoforms 1 to 4 in MKN28 cells by RT-PCR, and detected mRNA expression of NHE1, NHE2 and NHE4, but not NHE3 in MKN28 cells (Fig. 1A). Then, we performed real-time RT-PCR to determine the mRNA expression levels of NHE1, NHE2, NHE3 and NHE4. NHE1 was the most expressed one among them, and NHE2 was also relatively largely expressed compared with NHE3 and NHE4 (Fig. 1B). We further observed expression of NHE1 and NHE2 proteins (Fig. 1C), although NHE1 protein was much easier to be detected than NHE2 protein (Fig. 1C).

Effects of EIPA on cell proliferation
We studied the effects of a potent NHE inhibitor, EIPA, on the cell proliferation. EIPA is an amiloride-derivative NHE inhibitor. We exposed the cells to EIPA for 48 h and counted the cell numbers. The exposure of the cells to EIPA inhibited the proliferation of MKN28 cells in a dose- and time-dependent manner (Fig. 2A and B). These results suggest that NHE plays an important role in cell proliferation of MKN28 cells.

Effects of EIPA on the cell cycle
We next investigated effects of EIPA on cell cycle using flow cytometry. The cells were exposed to 25 µM EIPA [29, 30]. Cell cycle analysis indicated that the application of EIPA for 48 h significantly increased the G2/M fraction, and reduced the S phase (Fig. 3A). The representative data on cell cycle analysis is shown in Figure 3B. These data suggest that EIPA inhibits the cell proliferation by suppressing the transition from the G1 phase to the S phase.
Effects of EIPA on pH

We further studied if EIPA shows its inhibitory effects on cell growth and cell cycle by changing pH_c. Interestingly, the treatment with EIPA for 48 h did not significantly change pH_c of MKN28 cells (EIPA, 7.41 ± 0.01; control (DMSO alone), 7.43 ± 0.01: Fig. 4A), although EIPA induced a transient decrease in pH_c within 5 min after application of EIPA (data not shown). These data suggest that pH_c of MKN28 cells was regulated not only by NHE but also by other ion transporters compensating for changes in pH_c due to the EIPA-caused inhibition of the NHE-mediated H^+ transport. Therefore, we next studied possible roles of Cl-/HCO_3^- exchangers (anion exchanger (AE, Cl-/HCO_3^- exchanger) and Na^+ -driven Cl-/HCO_3^- exchanger (NDCBE)) in pH_c regulation of MKN28 cells. To clarify roles of Cl-/HCO_3^- exchangers, AE and NDCBE, in pH_c regulation in the presence of EIPA, we applied DIDS, an inhibitor of AE and NDCBE. EIPA co-applied with DIDS significantly decreased pH_c to 7.33 ± 0.02 from 7.41 ± 0.01 (EIPA alone) or 7.41 ± 0.01 (DIDS alone) as shown in Figure 4A. DIDS alone had no significant effects on pH_c (Fig. 4A). These results strongly suggest that AE and/or NDCBE would compensate for a change in pH_c caused by EIPA-induced NHE-inhibition.

Effects of EIPA on [Cl_c]

Based on the result that EIPA co-applied with DIDS, but neither EIPA nor DIDS alone, decreased pH_c, we speculated that AE and/or NDCBE would prevent cells from EIPA-induced accumulation of H^+ in the cytosolic space (a decrease in pH_c) by stimulating uptake of HCO_3^- coupled with release of Cl. If so, [Cl_c] should be decreased due to an elevation of Cl^- release exchanged with HCO_3^- uptake to maintain pH_c normal under the NHE-inhibited condition with EIPA. To study if this speculation is correct, we next measured [Cl_c] using a Cl^- sensitive
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**Fig. 3.** Cell cycle analysis in MKN28 cells exposed to EIPA. A: The percentage of MKN28 cells in each phase of the cell cycle. The population of cells in the G\textsubscript{0}/G\textsubscript{1} phase was increased and that of cells in the S phase was decreased by exposure to EIPA for 48 h in a dose-dependent manner. B: Flow cytometry analysis of MKN28 cells exposed to EIPA or DMSO alone for 48 h. EIPA caused G\textsubscript{0}/G\textsubscript{1} arrest in MKN28 cells. n = 6. *P < 0.05 (compared with DMSO).

**Fig. 4.** Measurements of pH\textsubscript{c} and [Cl\textsuperscript{-}] in MKN28 cells exposed to EIPA. A: pH\textsubscript{c} of MKN28 cells exposed to DMSO, EIPA, DIDS, or EIPA + DIDS. EIPA or DIDS alone caused no significant change in pH\textsubscript{c}, but co-application of EIPA and DIDS significantly decreased pH\textsubscript{c} in MKN28 cells. DMSO, n = 35; EIPA, n = 31; DIDS, n = 27; EIPA + DIDS, n = 27. B: [Cl\textsuperscript{-}] of MKN28 cells exposed to EIPA or DMSO for 48 hrs. [Cl\textsuperscript{-}] was significantly decreased by EIPA (n = 13) compared to DMSO treatment (n = 13). Co-application of EIPA and DIDS or DIDS alone caused no significant change in [Cl\textsuperscript{-}] compared to DMSO treatment. *P < 0.05.

fluorescent dye MQAE. Indeed, as shown in Figure 4B, [Cl\textsuperscript{-}] was lower (28.12 ± 3.03 mM) in EIPA-treated cells than that (42.88 ± 4.98 mM) in control cells. Like the case of pH\textsubscript{c}, we next studied possible roles of Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers (anion exchanger (AE, Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger) and Na\textsuperscript{+}-driven Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger (NDCBE)) in [Cl\textsuperscript{-}] regulation of MKN28 cells. To clarify roles of Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers, AE and/or NDCBE, in regulation of [Cl\textsuperscript{-}], in the presence of EIPA, we applied DIDS, an inhibitor of Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchangers, AE and NDCBE. Co-application of DIDS with EIPA significantly increased [Cl\textsuperscript{-}] from the level induced by EIPA alone (Fig. 4B), while DIDS alone had no effects on [Cl\textsuperscript{-}]. These results strongly suggest that AE or NDCBE alone or together would not contribute to regulation of [Cl\textsuperscript{-}] in the absence of EIPA (i.e., under a condition that NHEs function) but extrude Cl\textsuperscript{-} from the cytosolic space to the extracellular space for uptake of HCO\textsubscript{3}\textsuperscript{-} in order to maintain pH\textsubscript{c} normal under the lowered pH\textsubscript{c} condition caused by EIPA-induced NHE-inhibition.

**Effects of EIPA on the expression of cell cycle-associated proteins, p21, p53 and Rb**

We have previously reported that expression of p21 increases under a low [Cl\textsuperscript{-}] condition in a p53-independent manner, diminishing Rb phosphorylation [31]. Based on this observation, we speculated that EIPA would elevate expression of p21 in a p53-independent manner by lowering [Cl\textsuperscript{-}], resulting in diminution of cell growth. Indeed, p21 expression in EIPA treated cells is 2.5 times larger compared to that in control cells (Fig. 5A and B),...
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while EIPA, having no effects on phosphorylated-p53 (Ser15; an activated form of p53; Fig. 5A and C), caused a decrease in total p53 (Fig. 5A and D). Induction of p21 expression is mediated by p53 including both phosphorylated and dephosphorylated forms [32], although phosphorylation of p53 at Ser15 elongates the life-time of p53 protein contributing to the total amount of p53 protein expression. If the EIPA action on p21 expression would be mediated via a p53-dependent pathway, EIPA should increase the total amount of p53 regulating p21 expression. However, EIPA decreased the total amount of p53 protein expression. This suggests that the stimulatory action of EIPA on p21 protein expression would not be mediated via a p53-dependent pathway, although EIPA had a regulatory (diminishing) action on expression of p53 protein without any effect on the amount of phosphorylated p53 at Ser15. We further studied the action of EIPA on Rb (Fig. 6), indicating that EIPA induced a decrease in the phosphorylated Rb (p-Rb) and total Rb in MKN28 cells exposed to EIPA or DMSO. A: Expression of phosphorylated and total Rb detected by Western Blotting using anti-Rb and anti-phosphorylated-Rb (807/811, 780) antibodies. B: The relative expression level of total Rb to GAPDH. C: The expression level of phosphorylated (807/811) Rb relative to GAPDH. D: The expression level of phosphorylated (780) Rb relative to GAPDH. Application of EIPA significantly decreased the expression of phosphorylated Rb. *P < 0.05.
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in phosphorylated-Rb (Fig. 6A, C and D) without any significant effects on expression of total Rb (Fig. 6A and B).

**Effects of EIPA on the expression and phosphorylation of MAPKs**

We have previously demonstrated that mitogen-activated protein kinases (MAPKs; p38, JNK, and ERK) are phosphorylated (activated) under a lowered [Cl-] condition [31]. Therefore, we performed western blotting to study if EIPA phosphorylates (activates) MAPKs. As shown in Figure 7, EIPA phosphorylated (activated) all of them (p38, JNK, and ERK) without any significant effects on expression of MAPKs. Taken together, the observations in our present and previous [28] studies suggest that in MKN28 cells EIPA treatment inactivated Rb via up-regulation of p21 expression by activating MAPKs, resulting in the inhibition of cell proliferation.

**Discussion**

The present study shows that EIPA, a potent NHE inhibitor, diminished the proliferation of gastric cancer MKN28 cells by decreasing [Cl-], but not affecting pHc. Unlike the microenvironments in normal tissue the extracellular microenvironments of cancer cells are more acidic than their intracellular environments. This pH gradient is created and maintained by the secretion of H+ from cancer cells into their extracellular spaces. NHE1 plays an important role in regulation of pHc and activity of NHE1 is indicated to be co-related to tumor aggressiveness [33]. In the present study, we show that an amiloride-derived potent NHE inhibitor, EIPA, suppressed the proliferation of gastric cancer MKN28 cells in
a dose-dependent manner. Many investigators have previously demonstrated that NHE is a target for regulating cancer proliferation; i.e., NHE inhibitors decrease pH,[18, 20, 34, 35], regulating the expression of checkpoint proteins (i.e., cyclinB1 and cdc2) for cell cycle or signal transductions (i.e., Wee1 kinase)[36]. On the other hand, our previous reports [25, 28, 31, 37, 38] indicate cytosolic Cl plays an important role in cell proliferation. Therefore, we should consider two important factors, 1) pH, and 2) [Cl], regarding regulation of cell proliferation. Putney and Barber [36] have reported that diminution of NHE function induces G2/M arrest in fibroblasts associated with a decrease in pH. On the other hand, our present study showed that EIPA induced G2/M arrest without any effect on pH. Therefore, these apparently different effects of NHE blockade on cell cycle would be caused by different effects on pH; i.e., blockade of NHE induces a decrease in pH, in the study by Putney and Barber [36], but not in our present study. These observations lead us to hypothesize that: 1) in the case that blockade of NHE decreases pH, blockade of NHE diminishes cell proliferation associated with G2/M arrest, and 2) in the case that blockade of NHE decreases [Cl], without any changes in pH, blockade of NHE diminishes cell proliferation associated with G2/M arrest. A study [39] shows a suppressive action of NHE inhibitor on the proliferation and migration of cancer cells without measuring pH, although the suppression mechanism is unclear. L’Allemain et al.[40] have demonstrated that the inhibition of NHE1 has no effects on pH of fibroblasts in a bicarbonate-buffered medium, suggesting roles of HCO3- transporters in keeping pH constant even if NHE is inhibited. However, it is unknown how other transporters act on regulation of pH under NHE-inhibited conditions [23, 41]. Therefore, in the present study we investigated the function of DIDS-sensitive HCO3- transporters such as AE, NDCBE, and Na-HCO3 cotransporter (NBC) in the presence of EIPA. We confirmed that mRNAs of AE isoform 2 (AE2), NDCBE isoforms (slc4A8 and slc4A10), and NBC isoform 2 (NBC2) were expressed in MKN28 cells (data not shown). Therefore, we studied the effect of DIDS on the regulation of pH. DIDS alone did not alter pH, but the co-administration of DIDS and EIPA significantly reduced pH. These observations suggest that pH of MKN28 cells is regulated by not only NHE but also HCO3- transporters, and that HCO3- transporters compensate for pH changes caused by NHE inhibition via HCO3- import into the cytosolic space. Further, our present study indicated that EIPA decreased [Cl], and the EIPA-induced decrease in [Cl] was rescued by co-administration of DIDS and EIPA. This suggests that the compensation of pH changes caused by NHE inhibition via HCO3- import into the cytosolic space is accompanied by a release of Cl via DIDS-sensitive HCO3- transporters. Therefore, we suggest that AE and/or NDCBE (DIDS-sensitive Cl/HCO3- exchangers) but not Cl-independent HCO3- transporters such as NBC would compensate for pH changes caused by NHE inhibition via HCO3- import into the cytosolic space. AE-2 is a Cl/HCO3- exchanger. The extracellular Cl concentration ([Cl]e) was 123 mM, and the [Cl]i was about 40 mM. On the other hand, the extracellular HCO3- concentration ([HCO3]e) was 25 mM, but we had no information on the cytosolic HCO3- concentration ([HCO3]i). In general, if Pco in the cytosolic space would be identical to that in the extracellular space (i.e., 40 mm Hg), [HCO3] should be identical to [HCO3] since pH also was identical to the extracellular pH (pH) (7.4; Fig. 4). Under this condition, Cl should be imported into the cytosolic space from the extracellular space, and HCO3- should be released from the cytosolic space to the extracellular space. If so, AE would not compensate for NHE-inhibition induced decrease in pH; i.e., AE would export HCO3- from the cytosolic space to the extracellular space by using the chemical potential of Cl ([Cl]e = 123 mM; [Cl]i = 40 mM). On the other hand, NDCBE imports HCO3- from the extracellular space to the cytosolic space using the electrochemical potential of Na larger than the chemical potential of Cl. Based on electrochemical potentials of Na, Cl, and HCO3-, the compensation of NHE-inhibition induced decrease in pH would be mediated by NDCBE but not by AE2.

It is obvious that various fundamental cellular functions including cell proliferation depend on pH. Therefore, various ion transporters such as Cl/HCO3 exchangers act together to keep pH constant (normal) associated with changes in other ionic environments such as
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$[\text{Cl}^-]_c$, as shown in the present study. On the other hand, $\text{Cl}^-$ has been shown to play various roles in cellular functions in our previous reports [25, 26, 28, 31, 37, 42-48]. Previously, we reported low $\text{Cl}^-$ condition inhibited proliferation of MKN28 cells via activated stress activated MAPKs (p38 and JNK) and increased p21 expression, and each inhibitor of MAPKs (p38 and JNK) abolished the effects [28]. Our present and previous studies [28, 31] clearly indicates that even if EIPA causes no alteration in $\text{pH}_c$, an EIPA-induced change in $[\text{Cl}^-]_c$ is a large enough signal to affect proliferation of cancer cells by inactivating Rb via up-regulation of p21 expression mediated through activation of MAPKs (p38 and JNK). Observations obtained from our present and previous studies [31] at least suggest that reduction of $[\text{Cl}^-]_c$ activates MAPKs, although we should further clarify the mechanism how reduction of $[\text{Cl}^-]_c$ increases phosphorylation of MAPKs; a possible mechanism is that cytosolic $\text{Cl}^-$ regulates phosphorylation of MAPKs via a change in affinity of MAPKs to MAPK kinase (MAPKK) or MAPKK activity by directly binding to MAPKs or MAPKK like regulation of rhodopsin [49].

To further confirm that the target of EIPA is NHE, we applied siRNA on NHE1 and NHE2. siRNA against NHE1 and NHE2 diminished the mRNA expression of NHE1 and NHE2 to 10% of negative control (data not shown), but decreased the protein expression of NHE1 and NHE2 only to 50% of negative control (data not shown). Namely, we succeed the knock-down of mRNA expression of NHE1 and NHE2 by siRNA; nevertheless, we could not knock down the protein expression of NHE1 and NHE2 using siRNA against NHE1 and NHE2. This means that we could not apply siRNA techniques to confirm the effect of NHE on proliferation of MKN28 cells.

If cancer cells develop much more activity and/or expression of ion transporting systems keeping $\text{pH}_c$ constant (normal) even under high metabolic conditions producing a much larger amount of $\text{H}^+$ compared with normal cells, it is a better idea for us to develop some drugs targeting disturbance of $[\text{Cl}^-]_c$ homeostasis rather than that of $\text{pH}_c$ using blockers of $\text{H}^+$ transporters for novel anti-cancer therapies.

In conclusion, NHE inhibition suppressed the proliferation of gastric cancer MKN28 cells by inactivating Rb via up-regulation of p21 expression mediated via activation of MAPKs (p38 and JNK) through NDCBE-induced reduction of $[\text{Cl}^-]_c$ without any change in $\text{pH}_c$ (Fig. 8). This is the first study revealing that an NHE inhibitor suppresses the proliferation of cancer cells indirectly by regulating $[\text{Cl}^-]_c$. Our observations also suggest that the regulation of $[\text{Cl}^-]_c$ could be an important target for the development of novel cancer therapeutics.
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