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Anion exchanger isoform 2 operates in parallel with Na^+/H^+ exchanger isoform 1 during regulatory volume decrease of human cervical cancer cells

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Abstract Intracellular pH (pH_i) homeostasis was investigated in human cervical cancer SiHa cells undergoing regulatory volume decrease (RVD) to determine which transport systems were involved. Using isoform-specific primers, mRNA transcripts of Na^+/H^+ exchanger isoform 1 (NHE1) and isoform 3 were identified by reverse transcriptase polymerase chain reaction (RT-PCR) and the results confirmed by Western immunoblotting. From anion exchanger isoforms 1–3 (AE1–3), only the mRNA transcript of AE2 was identified by RT-PCR and the identity was confirmed by digestion with a specific restriction endonuclease. SiHa cells loaded with the fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein and resuspended in isotonic media showed a stable pH_i. In contrast, a gradual internal acidification took place following resuspension in hypotonic media. The NHE inhibitors, HOE694 (10 μM) and amiloride (1 mM), showed a similar potency in enhancing the rate and extent of the hypotonicity-induced internal acidification. The absence of extracellular Na^+ also substantially enhanced the acidification during RVD. These results suggest that internal acidification during RVD is mainly compensated by the operation of NHE1. Extracellular Cl^- was critically necessary for the pH_i acidification during RVD. The hypotonicity-induced acidification was significantly attenuated by 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, a concentration inhibiting more than 90% AE activity. This indicates that AE2 mediates a net Cl^- influx with compensating HCO_3^- efflux during RVD. We conclude that AE2 operates in parallel with NHE1 to regulate pH_i during RVD of human cervical cancer cells.

Key words: Na^+/H^+ exchanger; Anion exchanger; Regulatory volume decrease

1. Introduction

The regulation of cell volume and intracellular pH (pH_i) are two fundamental homeostatic mechanisms maintaining cellular function [1,2]. Both processes involve changes in intracellular ionic composition and can interact significantly. Classically, regulatory volume decrease (RVD) involves the loss of K^+, Cl^- and non-electrolytes (e.g. taurine) [3], but will perturb pH_i due to changes in intracellular Na^+, Cl^- and HCO_3^- content [1,2]. Thus, the pH regulation via Na^+/H^+ exchanger (NHE) and HCO_3^-/Cl^- anion exchanger (AE) is affected during RVD, resulting in an acidification of the cells [4]. Surprisingly, these transporters have been considered principally in the context of regulatory volume increase (RVI), where cells gain Na^+ and Cl^- for cell swelling [2]. In the present work, we identify which isoforms of NHE and AE are present in human cervical cancer cells and then assess their involvement in pH homeostasis during RVD.

2. Materials and methods

2.1. Cell culture

SiHa cells, a human cervical cancer cell line, were obtained from the American Type Culture Collection (Rockville, MD, USA). SiHa cells were maintained at 37°C in a 5% CO_2/95% air atmosphere and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Lab., Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 80 IU/ml penicillin and 80 μg/ml streptomycin (Sigma-Aldrich Company Ltd, Dorset, England). For electrophysiological experiments, cells were detached by 0.01% trypsin, seeded on cover slips and kept in culture for 24–36 h before use. Only non-confluent single cells were used in the recordings of membrane current.

2.2. Chemicals and solutions

The primary antibodies for NHE isoforms 1 and 3 (NHE1 and NHE3) were purchased from Chemicon International, Inc. (Temecula, CA, USA). The secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase was obtained from New England Biolabs Inc. (Beverly, MA, USA). Restriction endonuclease *Ban*I was obtained from Promega (Madison, WI, USA). HOE694, a specific NHE1 inhibitor, was a gift from Dr. H.-J. Lang (Aventis Pharmaceuticals, Frankfurt, Germany). All other chemicals were obtained from Sigma-Aldrich Company Ltd. (Dorset, England). The osmolarity of solutions was measured using a vapor pressure osmometer (Wescor 5500, Schlag Instruments, Gladbach, Germany). The isotonic HCO_3^--free medium contained (in mM): NaCl 100, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10 and mannitol 70, titrated to pH 7.4 with NaOH (300 ± 1 mosmol/l, $n = 5$). The isotonic HCO_3^- medium contained (in mM): NaCl 100, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10, $NaHCO_3$ 20 and mannitol 40, titrated to pH 7.4 with NaOH (301 ± 3 mosmol/l, $n = 3$). The components of the hypotonic medium were the same as those of the isotonic medium except mannitol was omitted, resulting in a 22 and 15% hypotonicity for HCO_3^--free and HCO_3^--containing media. Before experiments, the HCO_3^--containing media were adjusted to pH7.4 by continuous bubbling with 5% CO_2/95% air. Inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in all experiments was less than 0.05%. This DMSO concentration has no effect on pH_i measurements.

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNAs were isolated with a RNeasy Mini Kit (Qiagen Inc.,

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Valencia, CA, USA) following the manufacturer's instructions. After annealing 5 μg of total RNA with oligo(dT), cDNA was prepared with Superscript II (Life Technologies Inc., Frederick, MD, USA) following the manufacturer's instructions. Oligonucleotide primers were synthesized over regions specific for the NHE1, NHE3 and β-actin cDNA. For NHE1, forward (5′-TCTGCCGTCTCCAC-TGTCTCCA-3′) and reverse (5′-CCCTTCAGCTCCTCATTCAC-CA-3′) primers were used to amplify a 422 bp fragment from human NHE1 (nt 1838–2259). For NHE3, forward (5′-GGAGCAGC-GACGGCGGAGCAT-3′) and reverse (5′-TGAAATTCTGCGCA-GGGCTCT-3′) primers were used to amplify a 323 bp fragment from human NHE3 (nt 1813–2135). For β-actin, forward (5′-TTCAACTCCATCATGAAGAAGTGTGACGTG-3′) and reverse (5′-CTAAGTCATAGTCCGCCTAGAAGCATT-3′) primers were used to amplify a 312 bp fragment from human β-actin (nt 2595–3016). Three isoforms of AE, termed AE1–3, have been cloned so far. To detect AE, the isoform-independent primers were designed based on consensus nucleotide sequences from transmembrane spanning domain 1, 5′-TTCATCTACTTTGCCGCCCTG-3′ (forward), domain 5, 5′-GTCTCATAGATGAAGATGAG-3′ (reverse), and domain 6, 5′-CGACAGAGGGGCCGTGTTGGG-3′ (reverse). Positive PCR amplification for transmembrane spanning domain 1–5 (AEM1–5) is expected as a 377 bp fragment for AE1–3. For AEM1–6, the positive PCR amplification is expected as a 492, 573 and 549 bp fragment for AE1, AE2 and AE3, respectively. The PCR reaction volume of 50 μl contained 2 μl of template cDNA (or water for negative control), 0.2 μM of forward and reverse primers, 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). After heating the reaction contents for 3 min at 94°C, 30 cycles of PCR were performed (each consisting of incubation of 30 s at 94°C, 30 s at 57°C and 1 min at 72°C). 10 μl of PCR-amplified products were fractionated by 1.5% agarose gel containing ethidium bromide.

2.4. Western immunoblotting

Expression of NHE1 and NHE3 was also determined by immunoblotting, as described previously [5]. In brief, 50 μg protein extract from SiHa cells was resolved by 8% SDS–PAGE and then transferred to polyvinylidene difluoride (PVDF; Stratagene, La Jolla, CA, USA) membranes. Non-specific binding was blocked with 5% (w/v) non-fat dried milk in TBST (20 mM Tris, pH 7.5, 137 mM NaCl, 0.1% Tween 20) overnight at 4°C. The blots were incubated with primary antibodies for NHE1 or NHE3 at 1/2000 dilution in TBST for 2 h at room temperature, washed with TBST four times (10 min/each time), and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase at 1/5000 dilution for 1 h at room temperature. Following washing with TBST for four times, the PVDF membrane was developed with enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech, Bucks, UK).

2.5. Measurement and manipulation of pH_i

Measurements of pH_i were carried out at room temperature using the pH-sensitive fluorescent probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein/acetoxymethyl ester (BCECF/AM) as described in detail elsewhere [6]. Briefly, cells were detached from culture flasks by treatment with phosphate-buffered Ca^{2+}-free saline containing 1 mM EDTA and 0.01% trypsin. To load the cells with the pH-sensitive fluorescent probe BCECF/AM (10 μM), the cell suspension of 10^6 cells/ml was incubated in DMEM culture medium at room temperature for 40 min. After loading, cells were washed twice with phosphate-buffered saline and resuspended in various solutions as required. Dye fluorescence was measured in a fluorimeter (F-2000, spectrophotometer, Hitachi, Tokyo, Japan). Excitation wavelength was alternated between 490 and 439 nm and fluorescence intensity was monitored at 535 nm. For each experiment, the calibration of the signal of 490/439 nm ratio was performed using the high K^+-nigericin method [7]. Preliminary experiments to measure the fluorescence of supernatant from dye-loaded cells indicated that, over the time course of recording employed in the present study, the leakage of BCECF from cervical cancer cells is minimal. The ammonium rebound acid-loading technique [8] was used to acidify the cells. Cells were incubated in isotonic solution containing 20 mM NH_4Cl for 10 min. The pH_i recovery was recorded following acidification upon removal of external NH_4Cl, and resuspension in isotonic solution.

2.6. Electrophysiological measurements

The whole-cell mode of the patch-clamp technique was used to measure membrane currents at room temperature (22–25°C) as previously described [9,10]. When the pipettes were connected to the input stage of an Axopatch-200A amplifier (Axon Instruments, Burlingame, CA, USA), their DC resistance varied between 3 and 5 MΩ. A Ag–AgCl wire was used as reference electrode. To measure the activity of the volume-sensitive Cl^- channel, the KCl was replaced by CsCl in the media and the pipette solutions contained (in mM): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, $CaCl_2$ 1.93, EGTA 5, ATP 2, GTP 0.5, HEPES 5. The pipette solution was adjusted to pH 7.2 with CsOH and the osmolarity was 295 ± 1 mosmol/l ($n = 3$). The current–voltage relationship and time course of swelling-activated Cl^- current were obtained from a ramp protocol. The ramp protocol consisted of: a step to −80 mV for 0.4 s, followed by a 1.3 s linear voltage ramp to +80 mV, after which the potential was stepped back to the holding potential of −20 mV. This voltage protocol was repeated every 15 s from a holding potential of −20 mV. Currents were sampled at 2 ms intervals (1024 points per record, filtered at 200 Hz). Current densities were determined by normalizing the whole-cell current to the membrane capacitance. Data from electrophysiological experiments were digitized and analyzed using pCLAMP software (Version 6.0.3, Axon Co., Foster City, CA, USA).

2.7. Statistics

All values in the present study were reported as mean ± S.E.M. (standard error of the mean). ANOVA, Student's paired or unpaired *t*-test was used for statistical analyses. Differences between values were considered significant when $P < 0.05$.

3. Results

3.1. Molecular identification of NHE and AE

NHE and AE are thought to contribute to the housekeeping function of pH_i regulation in most cell types [1,2,11]. The molecular identification of members of the NHE and AE family is an important initial step towards studying their function and regulation in human cervical cancer cells. SiHa cells present at least two isoforms of the NHE family, identified by molecular evidence. Using isoform-specific primers, mRNA transcripts of NHE1 and NHE3 were detected by RT-PCR.

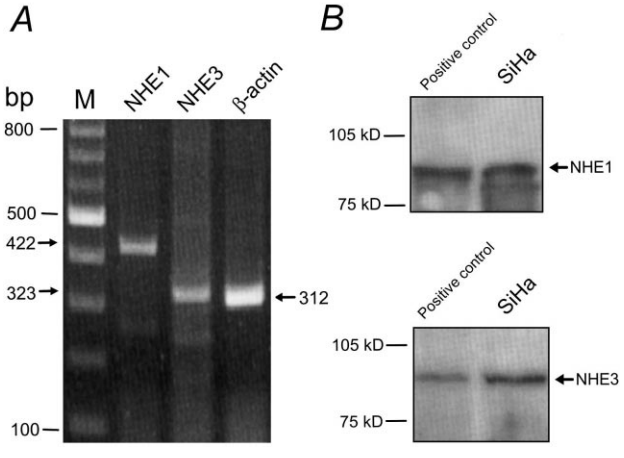

Fig. 1. Molecular identification of NHE family in human cervical cancer SiHa cells. A: mRNA transcripts of NHE1 and NHE3 were detected by RT-PCR. The primer pairs produced PCR products of the predicted sizes of 422, 323 and 312 bp for NHE1, NHE3 and β-actin, respectively. M: DNA molecular mass marker. B: Western immunoblotting for NHE1 and NHE3. Whole-cell extracts were separated by 8% SDS–PAGE (50 μg/lane), transferred to PVDF membrane, and immunoblotted with anti-NHE1 or anti-NHE3. Positive control was the protein from bovine articular chondrocytes [29].

The primer pairs produced PCR products of the predicted sizes of 422, 323 and 312 bp for NHE1, NHE3 and β-actin, respectively (Fig. 1A). Consistent results were demonstrated in Western immunoblotting. Fig. 1B suggests that the molecular mass of NHE proteins in SiHa cells is approximately 91 kDa for NHE1 and 95 kDa for NHE3.

To identify the presence of AE, PCR primers were designed based on consensus nucleotide sequences of AE from AEM1–5 or AEM1–6. Using the primer pair to amplify AEM1–5, the size of the PCR product was 377 bp, which was consistent with the predicted size for AE1–3 (Fig. 2A). Furthermore, using the primer pair to amplify AEM1–6, the PCR product showed a strong signal close to 573 bp, which was the product of AE2 (Fig. 2A). The identity of this PCR product was confirmed by digestion with a specific restriction endonuclease (Fig. 2B). For the PCR product of AE2, restriction endonuclease *Ban*I has a cutting site 453 bp from the 5′ end, whereas this enzyme dose not have any cutting site in the product for either AE1 or AE3. Following digestion with *Ban*I at 50°C for 3 h, the agarose gel analysis indicated that the PCR product of AEM1–6 (573 bp) cleaved into two segments of 453 and 120 bp. This indicated that the PCR product was the amplification of AEM1–6 of AE2.

3.2. Functional demonstration of NHE

To study the functional profile of NHE, cells were acidified using the NH_4^+ prepulse method [6] and the recovery from the acid load was evaluated in nominally HCO_3^--free media (Fig. 3). In the presence of extracellular Na^+, SiHa cells displayed a rapid recovery from the acid load that was abolished by the non-specific NHE inhibitor 1 mM amiloride and the selective NHE1 inhibitor, 10 μM HOE694 (Fig. 3A). The presence of extracellular Na^+ was also necessary for the recovery from the acid load (Fig. 3B). Thus, two of the characteristic properties of NHE1 were demonstrated, namely, (i) regulating pH_i in a Na^+-dependent manner; (ii) sensitivity to amiloride and HOE694. The steady-state pH_i was insensitive to HOE694

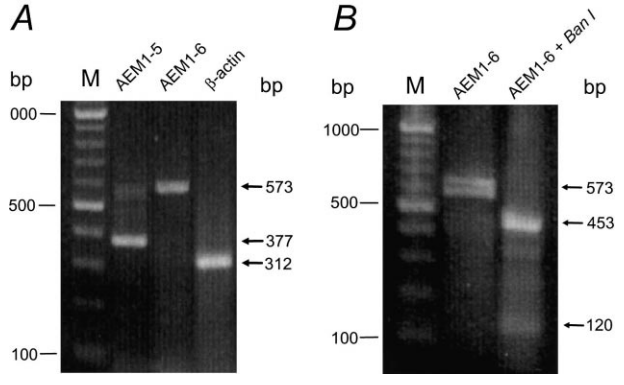

Fig. 2. Molecular identification of AE2 in human cervical cancer SiHa cells. A: mRNA transcripts of AE transmembrane spanning (see Section 2) were detected by RT-PCR. The primer pairs produced PCR products of 377, 573 and 312 bp for AEM1–5, AEM1–6, and β-actin, respectively. B: The identity of PCR product was confirmed by digestion with specific restriction endonuclease. For the PCR product of AEM1–6, restriction endonuclease *Ban*I has a cutting site 453 bp from the 5′ end for AE2. Following digestion with *Ban*I at 50°C for 3 h, the PCR product of AEM1–6 (573 bp) cleaved into two segments of 453 and 120 bp. This indicated that the PCR product was the amplification of transmembrane segment from AE2. M: DNA molecular mass marker.

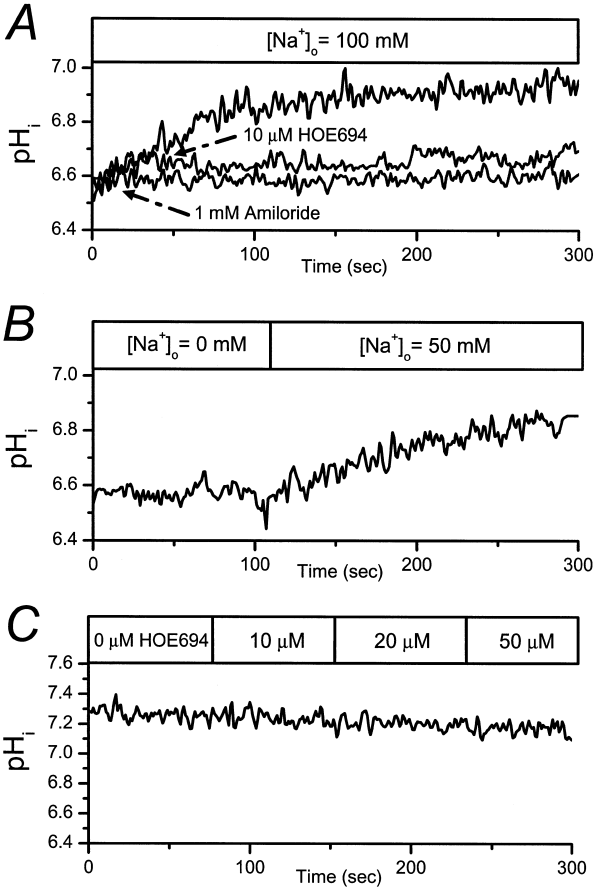

Fig. 3. Functional characteristics of NHE in SiHa cells. A: NHE activity is sensitive to amiloride and HOE694. Representative pH_i recordings from six individual experiments following NH_4^+-induced acidification. Cells were acidified by exposure to 20 mM NH_4^+ for 10 min, and the recovery of pH_i recorded in nominally HCO_3^--free isotonic Na^+-containing media. The arrow indicates the time point of addition of NHE inhibitor, 10 μM HOE694 or 1 mM amiloride. B: Extracellular Na^+ is necessary for NHE activity. Representative pH_i recordings from six individual experiments following NH_4^+-induced acidification. Cells were acidified by exposure to 20 mM NH_4^+ for 10 min. The recovery of pH_i was initially measured in nominally HCO_3^--free isotonic Na^+-free media and subsequently in nominally HCO_3^--free isotonic Na^+-containing media. NMDG was used as a substitute for Na^+. C: The steady-state pH_i is insensitive to HOE694, indicating that NHE1 activity is nearly quiescent under isotonic conditions. The pH_i recordings were performed in nominally HCO_3^--free isotonic Na^+-containing media. $[Na^+]_o$: extracellular Na^+ concentration.

(Fig. 3C), indicating that the NHE1 activity was nearly quiescent under isotonic conditions.

3.3. Intracellular acidification during RVD

The change of pH_i during RVD in either HEPES- or CO_2/HCO_3^--buffered media was subsequently studied. As shown in Fig. 4, SiHa cells loaded with the fluorescent dye and resuspended in isotonic media showed nearly no change in pH_i. In contrast, a gradual cytoplasmic acidification (-0.11 ± 0.01 pH unit, $n = 10$) took place following resuspension in hypotonic HEPES-buffered, HCO_3^--free medium. The hypotonicity-induced cytoplasmic acidification was attenuated by 100 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS; Fig. 4), a concentration inhibiting more than 90% AE activity [12,13]. However, DIDS at this concentration had only a mi-

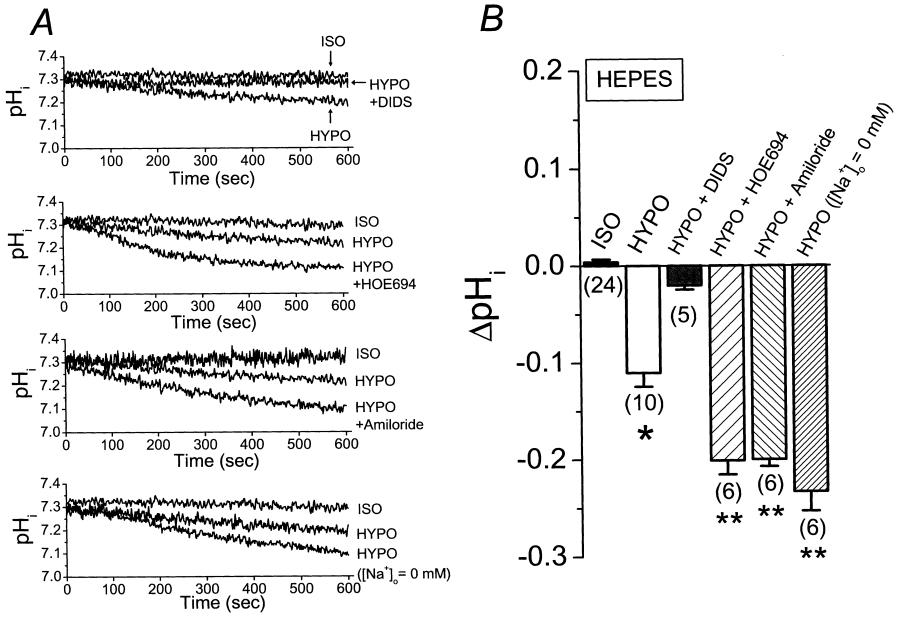

Fig. 4. Hypotonicity-induced pH_i acidification measured in HEPES-buffered media. A: Representative pH_i recordings of SiHa cells in isotonic HEPES-buffered medium, hypotonic HEPES-buffered medium, hypotonic HEPES-buffered medium containing 100 μM DIDS or NHE inhibitor (10 μM HOE694 or 1 mM amiloride), or hypotonic HEPES-buffered, Na^+-free medium. NMDG was used as a substitute for Na^+. B: Summary of pH_i change (ΔpH_i) under various conditions. Each bar represents mean ± S.E.M. and the number of experiments is indicated in parentheses beside each bar. ISO: isotonic; HYPO: hypotonic; $[Na^+]_o$: extracellular Na^+ concentration. $^*P < 0.001$, $^{**}P < 0.0001$, compared with isotonic condition. There was no significant difference in the extent of enhancement of acidification between amiloride-treated, HOE694-treated and extracellular Na^+-free group.

nor effect on the volume-sensitive Cl^- current of SiHa cells. As shown in Fig. 5A,B, the membrane currents under isotonic conditions recorded during the ramp protocol were small. Application of hypotonic solutions induced cell swelling, detected by visual inspection, which was accompanied by an activation of large outwardly rectifying currents. DIDS (100 μM) only inhibited the volume-sensitive Cl^- current by 5–15% ($n = 8$), measured at +80 mV (Fig. 5).

The extent of the acidification was significantly enhanced in the presence of 10 μM HOE694 (-0.20 ± 0.007 pH unit, $n = 6$) or 1 mM amiloride (-0.19 ± 0.008 pH unit, $n = 6$). A similar pattern of enhanced cytoplasmic acidification was also noted where extracellular Na^+ was replaced by *N*-methyl-D-glucamine (NMDG) (Fig. 4C,D). There was however no significant difference in the extent of enhancement of acidification between amiloride-treated, HOE694-treated and extracellular Na^+-free groups, suggesting that swelling-induced acidification is mainly compensated by the operation of NHE1 in the nominally HCO_3^--free media.

Switching from HEPES- to CO_2/HCO_3^--buffered solution caused the steady-state pH_i to increase by a mean of 0.09 in pH units (7.29 ± 0.01, $n = 24$ vs. 7.38 ± 0.01, $n = 18$; $P < 0.001$). This suggests that HCO_3^--dependent acid extrusion systems are also operating in CO_2/HCO_3^--buffered media.

In CO_2/HCO_3^--buffered media, the hypotonicity-induced cytoplasmic acidification was markedly attenuated by 100 μM DIDS (Fig. 6). In contrast, the rates and extent of the acidification were significantly enhanced in the presence of 10 μM HOE694 or 1 mM amiloride (Fig. 6). These results suggest that both HCO_3^-- and NHE-dependent mechanisms were involved in the pH_i regulation during RVD. However, compared to HOE694- or amiloride-treated cells, the rate and extent of the acidification were considerably enhanced by the absence of extracellular Na^+ (Fig. 6). This implies that in CO_2/HCO_3^--buffered media, in addition to NHE1, another Na^+-dependent acid extrusion system is likely to be involved in pH_i regulation during RVD.

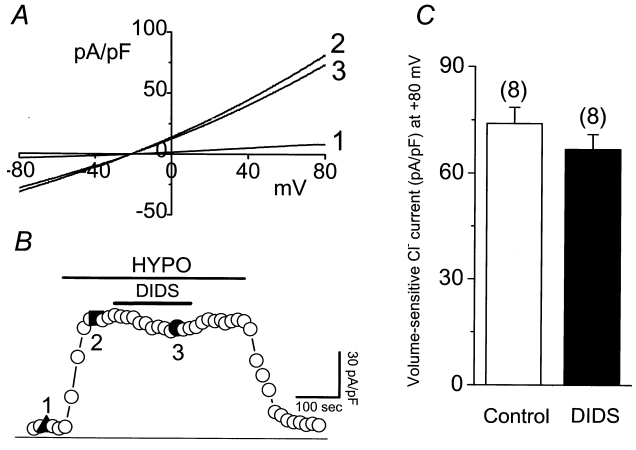

Fig. 5. DIDS has only a minor effect on the volume-sensitive Cl^- channel in SiHa cells. A: Representative recordings of volume-sensitive Cl^- currents from ramp protocol. Trace 1: Isotonic membrane current; trace 2: hypotonic membrane current; trace 3: currents recorded after perfusion with hypotonic solution in the presence of 100 μM DIDS. B: Time course of membrane currents activated at membrane potential of +80 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. The labelled points correspond to the current traces recorded in (A). Thick horizontal bar: Application of hypotonic solution or DIDS; thin horizontal line: zero current level. C: Summary of volume-sensitive Cl^- current measured at +80 mV in the absence (control group) or presence of 100 μM DIDS. Each bar represents mean ± S.E.M. and the number of experiments is indicated in parentheses beside each bar.

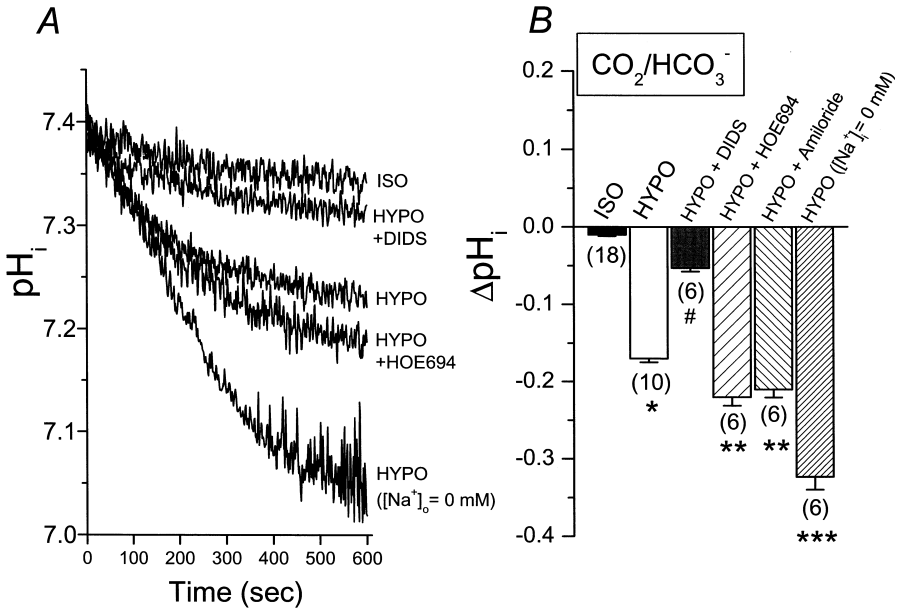

Fig. 6. Hypotonicity-induced pH_i acidification measured in CO_2/HCO_3^--buffered media. A: Representative pH_i recordings of SiHa cells in CO_2/HCO_3^--buffered media. B: Summary of pH_i change (ΔpH_i) under various conditions. Each bar represents mean ± S.E.M. and the number of experiments is indicated in parentheses beside each bar. ISO: Isotonic CO_2/HCO_3^--buffered medium; HYPO: hypotonic CO_2/HCO_3^--buffered medium; HYPO+DIDS: hypotonic CO_2/HCO_3^--buffered medium containing 100 μM DIDS; HYPO+HOE694: hypotonic CO_2/HCO_3^--buffered medium containing 10 μM HOE694; HYPO+amiloride: hypotonic CO_2/HCO_3^--buffered medium containing 1 mM amiloride; HYPO (extracellular Na^+ concentration = 0 mM): CO_2/HCO_3^--buffered extracellular Na^+ concentration-free medium. NMDG was used as a substitute for Na^+. $[Na^+]_o$: extracellular Na^+ concentration. $\#P < 0.05$, $*P < 0.001$, $**P < 0.0001$, $***P < 0.00001$, compared with isotonic condition.

3.4. Intracellular acidification during RVD requires extracellular Cl^-

Cell swelling leads to cytoplasmic acidification, which might represent the exit of HCO_3^- through anion channels, by release of H^+ from acidic intracellular compartments [2] or by the enhancement of Cl^-/HCO_3^- exchange due to decreasing intracellular Cl^- activity [4]. In Figs. 4 and 6, 100 μM DIDS almost completely abolished the hypotonicity-induced intracellular acidification. At this concentration, DIDS can inhibit more than 90% of AE activity [12,13], but has only a minor

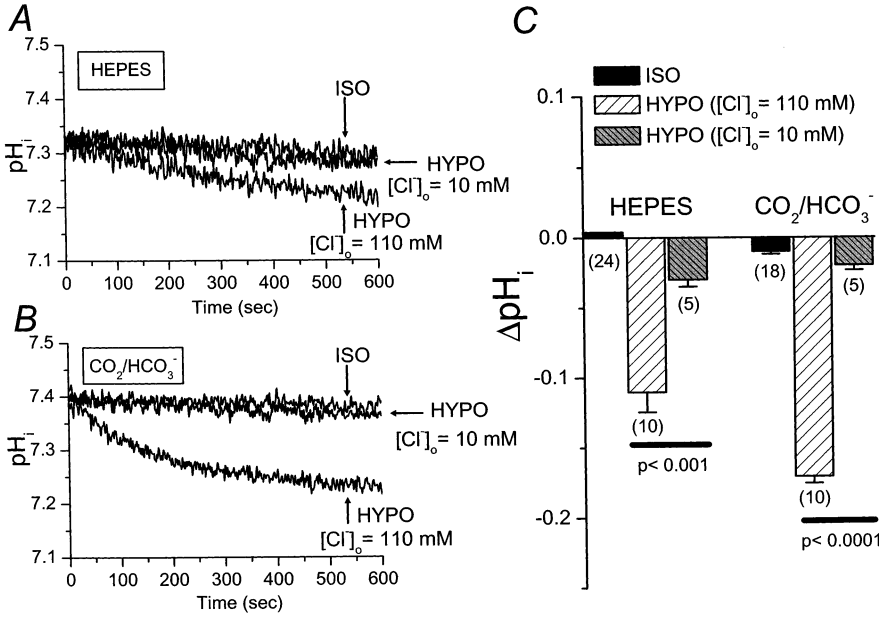

Fig. 7. Intracellular acidification during RVD requires extracellular Cl^-. A,B: Representative pH_i recordings of SiHa cells in HEPES- or CO_2/HCO_3^--buffered media containing different Cl^- concentrations. C: Summary of pH_i change (ΔpH_i) under various conditions. Each bar represents mean ± S.E.M. and the number of experiments is indicated in parentheses beside each bar. ISO: Isotonic HEPES- or CO_2/HCO_3^--buffered medium containing 110 mM Cl^-; HYPO: hypotonic HEPES- or CO_2/HCO_3^--buffered medium containing 10 or 110 mM Cl^-. Gluconate was used as a substitute for Cl^-. $[Cl^-]_o$: extracellular Cl^- concentration.

effect on the volume-sensitive Cl^- channel in human cervical cancer cells (Fig. 5). To investigate the proposition that the source of pH_i acidification is the exchange of extracellular Cl^- for intracellular HCO_3^- via the AE, we performed experiments at varying extracellular Cl^- concentration and followed changes in pH_i. Removal of extracellular Cl^- by replacement with gluconate did not affect the steady-state pH_i either in HEPES- or CO_2/HCO_3^--buffered media (data not shown). However, hypotonic shock induced only a slight acidification in nominally CO_2/HCO_3^--free media when the extracellular Cl^- concentration was reduced from 110 to 10 mM by the replacement with equimolar amounts of gluconate (Fig. 7). Likewise under the same condition, hypotonic shock did not result in acidification for cells suspended in CO_2/HCO_3^--buffered media (Fig. 7).

4. Discussion

This study presents molecular and functional evidence to demonstrate that human cervical cancer cells undergoing RVD show an altered cytoplasmic pH which results from the parallel activation of AE2 and NHE1. Although some studies have shown that hypotonic shock induces cytoplasmic acidification in some cell types [4,12], this is the first report to specify that AE2 and NHE1 are two of the major pH_i regulatory mechanisms during RVD.

In swollen cervical cancer cells, K^+ and Cl^- can cross the membrane either via separate K^+ and anion channels, or via the electroneutral KCl cotransporter. The volume-sensitive anion channel shows a broad sensitivity for different anions and organic osmolytes. For example, the anion permeability sequence is $I^- > Br^- > Cl^-$ in human cervical cancer cells [9]. It is a general characteristic of RVD in various cell types that K^+ loss exceeds Cl^- loss [1,14]. In our unpublished observations, cervical cancer cells also presented the same phenomena during RVD. Thus, a loss of other anions and/or titrations of negative, fixed charges has to accompany cellular cation exit. Besides the release of amino acids that can contribute to the anion loss, an exit of HCO_3^- generated within the cells is possible. An exit of cellular HCO_3^- in response to hypotonicity can explain the pH_i acidification during RVD and has been demonstrated in renal cells [15,16], Ehrlich ascites tumor cells [4] and human promyelocytic leukemic HL-60 cells [12]. There are two possible routes for the exit of intracellular HCO_3^-: (i) through the volume-sensitive anion channel with a permeability ratio (HCO_3^-/Cl^-) of 0.6 [17]; (ii) via an AE. The latter mechanism was supported by our results with the inhibition of the exchanger and cellular acidosis by a stilbene inhibitor or by the removal of extracellular Cl^-. We demonstrated that 100 μM DIDS abolished pH_i acidification during RVD. Because 100 μM DIDS inhibits more than 90% AE activity [12,13] but has only a minor effect on the volume-sensitive anion channel, the main pathway for the exit of cellular HCO_3^- during RVD is concluded to be AE rather than an anion channel. This is further supported by the evidence that the hypotonicity-induced pH_i acidification totally depends on extracellular Cl^-. This is similar to the previous findings in Ehrlich cells [4]. In addition, the uptake of extracellular Cl^- in exchange for intracellularly-generated HCO_3^- via AE2 can partly explain why K^+ loss exceeds Cl^- loss during RVD.

The AE genes (AE1–3) encode a family of transport proteins responsible for the electroneutral exchange HCO_3^- and Cl^- across membranes. These transporters are important in processes such as pH_i regulation, volume regulation and HCO_3^- metabolism. AE1, the best-described AE isoform, is essential for maintaining a stable erythrocyte cell shape and flexibility via functional interaction with membrane cytoskeleton [18]. Thus, organisms with reduced or disrupted AE1 gene expression showed major erythrocyte instabilities and defective anion exchange capacity and acidosis [18]. AE2, abundant in the gastrointestinal tract [19,20], is thought to encode the parietal cell basolateral Cl^-/HCO_3^- exchanger [21], responsible for the uptake of Cl^- destined for HCl secretion and extrusion of HCO_3^-, which are generated intracellularly at a very high rate during acid secretion [22]. AE3 is prominently expressed in brain and heart. Disturbances in AE3 expression are proposed with some human neurological diseases [23,24] and may provide a potential pharmacological target for antiarrhythmic and cardioprotective drugs [25]. The present work demonstrates that AE2 is the only AE isoform present in human cervical cancer cells.

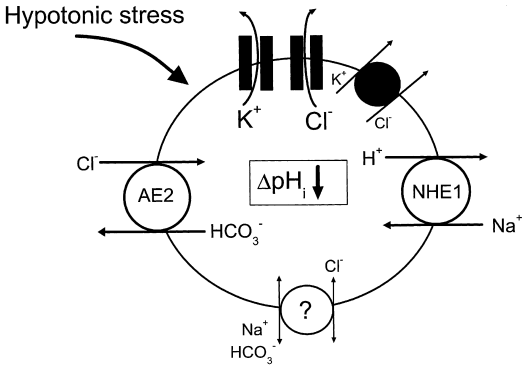

Fig. 8. Summary of the pH_i regulatory mechanisms during RVD of human cervical cancer SiHa cells. Hypotonic stress induces K^+ and Cl^- efflux through separate ion channels, or via the electroneural KCl cotransporter. AE2 operates to uptake extracellular Cl^- in exchange for cellular HCO_3^-. The operation of AE2 induced pH_i acidification which in turn activates NHE1 for acid extrusion. In addition to NHE1, other Na^+- and HCO_3^--dependent acid extrusion systems (indicated by ?) play a supportive role in the pH_i regulation during RVD.

Like AE, NHE is involved in both pH_i regulation and volume regulation [11]. So far, seven mammalian isoforms of NHE have been cloned [26,27]. The molecular mass of NHE proteins in cervical cancer SiHa cells is approximately 91 kDa for NHE1 and 95 kDa for NHE3. There is some variability in NHE isoform molecular weight, depending on species and the degree of glycosylation of the protein [27,28]. The main role of NHE in cell volume regulation has largely focused on RVI [1,2,6,11,29]. During RVI, the Na^+, K^+, $2Cl^-$ cotransporter operates in parallel with NHE1, resulting in net uptake of NaCl leading to an increase in cell volume [1,2]. Here, we provide both molecular and functional evidence that NHE1 is present in SiHa cells. The pharmacological evidence further indicates that swelling-induced acidification is to a large extent offset by the operation of NHE1. The present model for cervical cancer cells is identical to that presented previously for Ehrlich cells [4], although the molecular identity of the exchangers involved was not established at that time. It is likely to be a general phenomenon, and highlights

the potential for conflicting roles of NHE in volume and pH regulation.

Considering the results overall, the pH_i regulatory mechanisms during RVD in human cervical cancer cells can be summarized as follows (Fig. 8). Osmotic swelling induces K^+ and Cl^- efflux through separate ion channels, or via the electroneutral KCl cotransporter [5,9,30]. The K^+ loss during RVD exceeding the Cl^- loss is partly due to extracellular Cl^- exchange for intracellular HCO_3^- via AE2. The operation of AE2 induced pH_i acidification which in turn activated NHE1 activity for acid extrusion. In addition to the important role of NHE1, other Na^+- and HCO_3^--dependent acid extrusion systems may play a supportive role in the pH_i regulation during RVD (question mark in Fig. 8), but presently they await characterization.

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