ClC-3 is a candidate of the channel proteins mediating acid-activated chloride currents in nasopharyngeal carcinoma cells

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Wang L, Ma W, Zhu L, Ye D, Li Y, Liu S, Li H, Zuo W, Li B, Ye W, Chen L. ClC-3 is a candidate of the channel proteins mediating acid-activated chloride currents in nasopharyngeal carcinoma cells. Am J Physiol Cell Physiol 303: C14–C23, 2012. First published April 11, 2012; doi:10.1152/ajpcell.00145.2011.—Acid-activated chloride currents have been reported in several cell types and may play important roles in regulation of cell function. However, the molecular identities of the channels that mediate the currents are not defined. In this study, activation of the acid-induced chloride current and the possible candidates of the acid-activated chloride channel were investigated in human nasopharyngeal carcinoma cells (CNE-2Z). A chloride current was activated when extracellular pH was reduced to 6.6 from 7.4. However, a further decrease of extracellular pH to 5.8 inhibited the current. The current was weakly outward-rectified and was suppressed by hypertonicity-induced cell shrinkage and by the chloride channel blockers 5-nitro-2-3-phenylpropylamino benzoic acid (NPPB), tamoxifen, and 4,4′-disothiocyanostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS). The permeability sequence of the channel to anions was I− > Br− > Cl− > gluconate−. Among the ClC chloride channels, ClC-3 and ClC-7 were strongly expressed in CNE-2Z cells. Knockdown of ClC-3 expression with CIC-3 small interfering (si)RNA prevented the activation of the acid-induced current, but silence of ClC-3 expression with ClC-7 siRNA did not significantly affect the current. The results suggest that the chloride channel mediating the acid-induced chloride current was volume sensitive. ClC-3 is a candidate of the channel proteins that mediate or regulate the acid-activated chloride current in nasopharyngeal carcinoma cells.

acidification; chloride channels; tumor cells; extracellular pH

THE EXTRACELLULAR pH value presents a property of weak alkalinity about 7.4 in physiologic conditions. The environmental acidity or pH of living cells is one of the major factors that influence molecular processes involved in cell cycle progression, cell proliferation, and differentiation (27). Changes of extracellular pH may modulate the activities of ion channels. It has been reported that extracellular acidification inhibited inwardly rectifying potassium channels (11, 15, 30), mechanosensitive potassium channel (or tandem pore domain potassium channel) TREK1 (25), intracellular calcium-activated potassium channel KCa2.2 and KCa2.3 (24), the voltage-gated potassium channel Kv1.5 (14), human ether-à-go-go potassium channel (6) and voltage-dependent Ca2+ channel (1), but activated mechanosensitive potassium channel TREK2 (25) and modulated the activities of ATP-sensitive potassium channel (12). The effects of pH changes on ion channels may be related to the protonation of histidine residues in the outer pore regions of channels, which have been reported to be proton interaction sites that regulate channel availability and inactivation (14, 24, 25).

The influence of extracellular acidification on Cl− channels has been investigated in several types of normal cells, and discrepant results were reported. Chloride channels were activated by extracellular acidification in human skeletal muscle cells (13), rat carotid body cells (21), and rat Sertoli cells (2) but were inhibited in Drosophila melanogaster (26) and Xenopus oocytes (8). Nobles and colleagues (20) showed that though some differences existed, many properties of acid-activated Cl− current are similar to those of the swelling-activated Cl− current, implying that the two currents are related and that extracellular acidification reflects a novel stimulus for volume-activated Cl− current. However, most of the investigations were carried out in normal cells. As for the tumor cells that live in an acidic microenvironment, the influence of extracellular acidification on chloride channels is far from clear.

The molecules that are responsible for the activation or modulation of acid-sensitive chloride current are not defined. In mammals, ClC proteins show differential tissue distribution and perform different functions. Some reports show that ClC-7 and ClC-4 may mediate the acid-induced Cl− current (5, 13), but the results presented in other reports do not support the hypothesis (2, 8), suggesting that different cells may express different isoforms of Cl− channels or channel regulators which are responsible for the activation of the acid-induced chloride current. Analysis of current properties indicates that there are some similarities between the acid-induced Cl− current and the volume-sensitive Cl− current. ClC-3 has been proposed by others and us to be the molecular candidate involved in the activation or regulation of volume-sensitive Cl− current (7, 9, 18, 28). The role of ClC-3 proteins in activation of acid-induced Cl− current is not clear.

In this study, the activation and properties of the chloride current induced by extracellular acidification were investigated in human nasopharyngeal carcinoma cells. The molecular nature responsible for the activation of the acid-sensitive chloride channel was identified.

MATERIALS AND METHODS

Cell Culture

The poorly differentiated human nasopharyngeal carcinoma CNE-2Z cells were routinely grown in 25-cm2 plastic tissue culture flasks in RPMI 1640 medium with 10% newborn calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. The cells were subcultured every 2 days (29).
Solutions and Chemicals

The pipette solution contained (in mM) 70 N-methyl-\(\beta\)-glucamine chloride (NMDG-Cl), 1.2 MgCl\(_2\), 10 HEPES, 1 EGTA, 140 \(\tau\)-mannitol, and 2 ATP. The isotonic bath solution contained (in mM) 70 NaCl, 0.5 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES, and 140 \(\tau\)-mannitol. Solution osmolarity was measured with a freezing-point osmometer (OSMOMAT 030; Gonotec). The pH of the pipette solutions was adjusted to 7.25 with Tris base. The osmolarity of pipette and isotonic bath solutions was adjusted to 300 mosmol/l with \(\tau\)-mannitol. The hypotonic and hypertonic bath solutions were obtained by depletion or addition of \(\tau\)-mannitol from or into the isotonic bath solution, giving the osmolarity of 160 mosM/l for 47% hypotonic solution and 440 mosM/l for 47% hypertonic solution. In anion substitution experiments, 70 mM NaCl in the bath solution was replaced by equimolar of NaI, NaBr, or sodium gluconate. The pH value of bath solutions was adjusted to 5.8, 6.6, and 7.4 with Tris-base. The chloride channel blockers 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO, for DIDS and NPPB at 100 mM) or in methanol (for tamoxifen at 40 mM), and diluted to the indicated final concentrations with the isotonic bath solution (pH 6.6).

Preparation of Cells for Current Recordings

Cells cultured in the flasks were trypsinized, centrifuged, and resuspended in RPMI 1640 medium with 10% newborn calf serum, 100 IU/ml penicillin and 100 \(\mu\)g/ml streptomycin. Cell suspension was plated on round coverslips of 22 mm diameter (150 l/coverslip), which were located in 35-mm tissue culture dishes, and were then incubated at 37°C for 2–3 h before current recordings were taken (4).

Whole Cell Current Recordings

Whole cell currents were measured with the patch-clamp technique as described previously (4) using a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The patch-clamp pipettes were made from standard wall borosilicate glass capillaries with an inner filament on a two-stage vertical puller and gave a resistance of 4–6 M\(\Omega\) when filled with the pipette solution. Electrode and whole cell capacitance was determined by adjusting and minimizing capacity transients in response to a 20-mV voltage step using the amplifier functions, following instructions of the amplifier manual. Once the whole cell configuration was established, cells were held at the chloride equilibrium potential (0 mV), and then were stepped repeatedly to the 200-ms pulses of 0, 40, and 80 mV, with 4-s interval between steps. Command voltages and whole cell currents were recorded simultaneously by a computer via a laboratory interface (CED 1401, Cambridge, UK) with a sampling rate of 3 kHz. Voltage pulse generation, data collection, and current analysis were performed by the computer using the EPC software package (CED). In analysis of data collected, all current measurements were made at 10 ms after onset of each voltage step. Experiments were carried out at room temperature (20–24°C). The inhibition of Cl\(^-\) currents was calculated using the following equation: Inhibition (%) = [(current\(_{test}\) – current\(_{inhibit}\) – current\(_{ctrl}\))/(current\(_{test}\) – current\(_{ctrl}\)] \times 100%, where current\(_{test}\) and current\(_{inhibit}\) are the currents recorded before and after inhibitory treatments, respectively, and current\(_{ctrl}\) is the background current under isotonic conditions.

Evaluation of Anion Permeability Ratios

The permeability ratios (\(P_X/P_Cl\)) of various anions (\(X^-\)) relative to that of Cl\(^-\) were calculated using the following modified Goldman-Hodgkin-Katz equation (10):

\[
P_X/P_Cl = \frac{[Cl^-]_n \exp(-\Delta V_{rev} V /F/RT)}{[X^-]_n \exp(-\Delta V_{rev} V /F/RT)}
\]

where \([Cl^-]_n\) and \([X^-]_n\) are the Cl\(^-\) concentrations in the normal and the substituted bath solutions, \(\Delta V_{rev}\) is the concentration of the substituted anion, \(\Delta V_{rev}\) is the difference of the reversal potentials for Cl\(^-\) and \(X^-\), \(F\) is the Faraday constant, \(R\) is the gas constant, and \(T\) is absolute temperature. In anion substitution experiments, an agar bridge was used to connect the reference electrode (AgCl wire) to the bath solution to minimize the baseline drift caused by anion substitution.

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted with the reagents from Invitrogen (Carlsbad, CA), following the manufacturer’s instructions, resuspended in 30 \(\mu\)l RNase free water, examined by agarose gel electrophoresis, and quantitated by spectrophotometry (absorbance at 260 nm). All primers were synthesized by GenePharma (GenePharma, Shanghai, China) and are shown in Table 1. RT-PCR was performed with the Qiagen RT-PCR kit (Qiagen, Valencia, CA) using a thermocycler (Biometre, Gottingen, Germany), according to the following parameters: 1) reverse transcription at 50°C for 30 min; 2) PCR activation at 95°C for 15 min; 3) 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min; and 4) final extension at 72°C for 10 min. Aliquots of each PCR product were separated on a 2% agarose gel, stained with ethidium bromide, and analyzed by illumination with ultraviolet and visible light (Multi Genius, Syngene).

Small interfering RNA Treatments

The sequences of ClC-3 small interfering (si)RNA duplexes were as follows: sense, 5'-CAAGGUCAATCGGAGCCAAATT-3'; antisense, 5'-AUAGCAGAGAAAACUUUGGTAAG-3' (31). Three ClC-7 siRNA duplexes were tested. The sequences of ClC-7 siRNA-1 were the following: sense, 5'-GCAAUCCCACCGAGCAATT-3'; antisense, 5'-AGUGGGGCGTCTAATG-3'. ClC-7 siRNA-2 was as follows: sense, 5'-GCGGUUGACGCCGAAATT-3'; antisense, 5'-UUCAGGAGCACCGTCTAAG-3'. Three ClC-7 siRNA-3 was as follows: sense, 5'-GCCGCGGAGAAGCTCTAG-3'. Sequences of the negative control siRNA duplex was as follows: sense, 5'-UUCAGGAGCACCGTCTAAG-3'; antisense, 5'-UUGAGGAGACCTATG-3'. siRNAs were synthesized and labeled with FAM carboxyfluorescein by GenePharma (GenePharma, Shanghai, China) and stored at -20°C. CNE-2Z cells were incubated in RPMI 1640 medium without antibi-

Table 1. Primers used in RT-PCR analysis

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<th>Gene</th>
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<th>Reverse Sequence (5'-3')</th>
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CIC, chloride channel.
otics in 24-well culture plates for 24 h and reached 30–50% confluence. The cells were transfected with siRNA (100 nM) or negative control siRNA plus lipofectamine 2000 (1 μl in 500 μl medium, Invitrogen) in serum and antibiotics-free culture medium for 6 h and in normal RPMI 1640 medium containing serum for 48 h. Electrophysiological recordings were carried out 48 h after transfection.

Western Blot Analysis

CNE-2Z cells were lysed using the buffer containing Tris-Cl (50 mM), NaCl (150 mM), NaN3 (0.02%), Nonidet P-40 (1%), SDS (0.1%), sodium deoxycholate (0.5%), leupeptin (5 μg/ml), and aprotinin (1 μg/ml). Coomassie brilliant blue was used to quantify the protein content of cell lysates. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), which were blocked at room temperature (24°C to 26°C) for 1 h in a solution containing (in mM) 130 NaCl, 2.5 KCl, 10 Na2HPO4, 1.5 KH2PO4, 0.1% Tween 20, and 5% BSA (pH 7.4). The membranes were incubated overnight at 4°C with primary antibodies (rabbit anti-ClC-3 antibody, 1:300, Alomone Laboratories; rabbit anti-ClC-7 antibody and rabbit anti-ClC-5 antibody, 1:1,000, Abcam, Cambridge, UK; mouse anti-actin antibody, 1:500, Boster Bio-technology, Wuhan, China) and then with the horseradish peroxidase-linked secondary antibodies (goat anti-rabbit IgG, 1:1,500; goat anti-mouse IgG, 1:1,000; ProteinTech Group, Chicago, IL) for 1 h at room temperature. Final detection was accomplished with Western blot luminol reagents (ProteinTech Group, Chicago, IL).

Statistical Analysis

Values are expressed as means ± SE (n, number of observations) and, where appropriate, have been analyzed using ANOVA. P < 0.05 was considered to be significant. All experiments were repeated at least three times.

RESULTS

Activation of Chloride Currents by Extracellular Acidification

A weak background current was recorded with the whole cell recording technique in the normal pH value (pH 7.4, Fig. 1, A and B): typical time courses of recordings. C–E: typical current traces recorded in the control bath solution (pH 7.4) and after extracellular acidification to pH 6.6 and 5.8. F: current-voltage relationships at different pH (means ± SE; n = 8). **P < 0.01 (vs. pH 7.4 and pH 5.8). ISO, isotonic bath solution. HYPO, 47% hypotonic bath solution.
The density of the current was $5.58 \pm 0.96 \text{ pA/pF}$ at $+80$ mV and $-3.90 \pm 0.59 \text{ pA/pF}$ at $-80$ mV ($n = 8$). Decreasing the pH value from 7.4 to 6.6 induced a current within 1–2 min, which reached the peak within 20 min (Fig. 1, A and D). The current showed a weak outward rectification, with mean current density of $62.56 \pm 5.75 \text{ pA/pF}$ at $+80$ mV ($n = 8$) and $-43.48 \pm 4.70 \text{ pA/pF}$ at $-80$ mV. The difference between outward and inward currents was significant ($P < 0.01$). The current-voltage relationship demonstrated that the current reversed at a voltage close to the calculated $E_{\text{Cl}}$ ($-0.9 \text{ mV}$), with a mean value of $-6.45 \pm 0.78 \text{ mV}$ ($n = 8$, Fig. 1F). Moreover, the current was inactivated gradually at $+80$ mV (Fig. 1D).

The current activated at pH 6.6 was inhibited significantly by the decrease of pH to 5.8 (Fig. 1, A and E), with mean inhibition of $97.12 \pm 6.37\%$ at $+80$ mV ($n = 4$) and $96.48 \pm 5.29\%$ at $-80$ mV. Furthermore, no significant activation of Cl$^-$ currents was observed when pH was reduced directly from 7.4 to 5.8 ($n = 3$) (Fig. 1B). The difference between the current recorded at normal pH (7.4) and that at pH 5.8 was not significant ($P > 0.05$). The results suggest that the acid-induced chloride current occurs only in weak acid environment in CNE-2Z cells.

**Properties of Acid-Induced Cl$^-$ Currents**

**Volume sensitivity.** When the Cl$^-$ current induced by pH 6.6 was activated and reached the peak (Fig. 2, A–C), the isotonic bath solution was replaced with 47% hypertonic bath solution (Fig. 2, A and D). The result showed that the current was inhibited rapidly and completely by the hypertonic treatment, with mean inhibition of $103.04 \pm 3.51\%$ for the outward current at $+80$ mV and $100.10 \pm 6.40\%$ for the inward current at $-80$ mV ($n = 5$, $P < 0.01$). The difference of inhibition between outward current and inward current was not significant ($P > 0.05$).

**Effects of Cl$^-$ channel blockers.** It was demonstrated by us previously that the Cl$^-$ channel blockers NPPB and tamoxifen inhibited the volume-activated Cl$^-$ currents ($I_{\text{Cl,vol}}$) (3). Here, our results showed a rapidly blocking effect of the Cl$^-$ channel blockers NPPB, tamoxifen, and DIDS on the acid-induced Cl$^-$ currents (Fig. 3). When the currents were activated by the decrease of pH from 7.4 to 6.6 and reached the peak, the isotonic bath solution (pH 6.6) was replaced by the isotonic bath solution containing 100 $\mu$M of NPPB (pH 6.6), 20 $\mu$M tamoxifen (pH 6.6), or (100 $\mu$M) DIDS (pH 6.6), respectively.

As shown in Fig. 3A, extracellular applications of 100 $\mu$M NPPB inhibited significantly the acid-activated chloride currents by $88.13 \pm 4.82\%$ at $+80$ mV and by $87.91 \pm 3.78\%$ at $-80$ mV ($n = 5$, $P < 0.01$). Similar to the effect of NPPB, extracellular applications of 20 $\mu$M tamoxifen attenuated the acid-activated chloride currents (Fig. 3B) by $89.83 \pm 5.22\%$ at $+80$ mV and by $92.81 \pm 5.28\%$ at $-80$ mV ($n = 6$, $P < 0.01$). The difference between the inhibitions of the outward and inward currents by NPPB and tamoxifen was not significant ($P > 0.05$). DIDS also suppressed the acid-activated chloride currents, but the outward current was more sensitive to DIDS than the inward current (Fig. 3C). The inhibition (by $69.43 \pm 3.02\%$) of the outward current at $+80$ mV was stronger than that (by $42.91 \pm 3.48\%$) of inward current at $-80$ mV ($n = 6$, $P < 0.01$). Further experiments indicated that the inhibitory effects of NPPB, tamoxifen, and DIDS were partly reversible (data not shown).

**Permeability for various anions.** When the acid-induced Cl$^-$ current was activated and reached the peak, the isotonic solution (pH 6.6) containing 70 mM of Cl$^-$ was replaced with the solution containing equimolar $\Gamma^-$, Br$^-$, or gluconate$^-$. The anion substitution shifted the reversal potential. The permeability ratios of $P_{\text{Cl}}/P_{\text{Br}}$, $P_{\text{Cl}}/P_{\text{gluconate}}$, and $P_{\text{gluconate}}/P_{\text{Cl}}$, calculated from the shifts in reversal potential by using the modified

![Fig. 2. Inhibition of acid-induced Cl$^-$ current by 47% hypertonic bath solution. Cells were bathed in the control isotonic solution of pH 7.4, the isotonic solution of pH 6.6, and the 47% hypertonic solution (HYPER) of pH 6.6. A: typical time course of activation and inhibition of the acid-induced Cl$^-$ current. B–D: typical current traces recorded under the different bath conditions.](http://ajpcell.physiology.org/)}
Goldman-Hodgkin-Katz equation, were $1.14 \pm 0.03$ ($P < 0.05$, $n = 4$), $1.03 \pm 0.03$ ($P > 0.05$, $n = 4$), and $0.28 \pm 0.05$ ($P < 0.01$, $n = 4$), respectively, resulting in the sequence of anion permeability of $\Gamma^- > Br^- > Cl^- > gluconate^-$ (Fig. 4).

**Expression of mRNA and Protein of CIC Chloride Channel Families in CNE-2Z Cells**

The above results indicate that $Cl^-$ currents were activated by extracellular acidification. Expression of mRNAs of CIC chloride channel genes in human nasopharyngeal carcinoma cells was examined by RT-PCR. As shown in Fig. 5A, a strong specific band for CIC-3 or 7 was observed, but no specific bands for CIC-1, 2, 4, and 6 were identified. As for CIC-5, a weak band was presented. To study further, the endogenous expression of CIC-3, CIC-5, and CIC-7 protein was detected by Western blot technique. The results of the Western blot analysis showed that CIC-3 and CIC-7 proteins were expressed at a high level, while the CIC-5 showed only a very weak

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**Fig. 3. Inhibition of acid-induced chloride currents by the chloride channel blockers 5-nitro-2-3-phenylpropylamino benzoic acid (NPPB), tamoxifen, and 4,4'-disothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS). Activation of the acid-induced chloride currents and inhibition of the currents by the chloride channel blockers NPPB (100 μM), tamoxifen (20 μM), and DIDS (100 μM) are shown in A, B, and C, respectively. Typical time courses are shown in a. Representative current traces recorded in the acidic bath solution (pH 6.6), in the absence or presence of the indicated blockers, are presented in b and c. Current-voltage relationships are shown in d (means ± SE, $n = 5$, 6, and 6 in Ad, Bd, and Cd, respectively). *$P < 0.05$; **$P < 0.01$ (vs. pH 7.4).

**Fig. 4. Anion permeability of the acid-activated Cl$^-$ channel.** When the acid-induced current reached a peak and leveled off, the isotonic solution containing 70 mM Cl$^-$ (pH 6.6) was substituted by the isotonic solution containing equimolar $\Gamma^-$, Br$^-$, or gluconate$^-$ (pH 6.6). The permeability ratios ($P_{\chi}/P_{Cl}$) of various anions ($\chi^-$) relative to that of Cl$^-$ were calculated using the modified Goldman-Hodgkin-Katz equation. A: typical time course of the currents at 0, ±40, and ±80 mV after application of the isotonic solution (pH 6.6) containing different anions. B: statistical values of the permeability for different anions (means ± SE, $n = 4$). *$P < 0.05$; **$P < 0.01$ (vs. Cl$^-$).
expression (Fig. 5B). Densitometric analysis indicated that the relative intensity of ClC-3, ClC-5, and ClC-7 were 0.19 ± 0.02, 0.02 ± 0.04, and 0.22 ± 0.03, respectively (n = 3, Fig. 5C).

**Suppression of ClC-3 Protein Expression and Depletion of Acid-Induced Cl\(^{-}\) Currents by ClC-3 siRNA Treatments**

The above data indicate that the properties of acid-activated currents were similar to those of the volume-sensitive Cl\(^{-}\) currents. ClC-3 was expressed in CNE-2Z cells and has been proposed by others and us to be the molecular candidate involved in the activation or regulation of volume-sensitive Cl\(^{-}\) currents (7, 9, 18, 28, 31). To investigate the role of ClC-3 in the activation of the acid-activated chloride currents, the effect of knockdown of ClC-3 expression by ClC-3 siRNA was tested. The knockdown of ClC-3 protein expression was confirmed by Western blotting (Fig. 6, A and B). The expression of ClC-3 proteins was inhibited significantly by the ClC-3 siRNA treatments (100 nM) in the presence of lipofectamine 2000 (1 μl/500 μl). Transfection of cells with the negative control siRNA (100 nM) plus lipofectamine 2000 (1 μl/500 μl) or with lipofectamine 2000 alone did not significantly alter ClC-3 expression (Fig. 6, A and B).

In the patch-clamp experiments, CNE-2Z cells were treated with FAM carboxyfluorescein-labeled ClC-3 siRNA (100 nM) and lipofectamine 2000 (1 μl in 500 μl medium) for 48 h before current measurements. Cell fluorescence, indicating successful transfection of ClC-3 siRNA, was monitored with a fluorescent microscope, and the cells with strong fluorescence were recorded. The results showed that ClC-3 siRNA treatments prevented the activation of the acid-induced chloride currents (Fig. 6, C–H). In the control cells treated with the negative control siRNA, decreasing the extracellular pH from 7.4 to 6.6 activated a chloride current (Fig. 6, C–F). However, the acid-induced Cl\(^{-}\) current could not be activated by extracellular acidification (pH 6.6) in the cells treated with ClC-3 siRNA (Fig. 6, C, D, G, and H). The mean current density was only 8.03 ± 1.85 pA/pF at +80 mV (n = 5) in ClC-3 siRNA-transfected cells. Compared with the acid-induced current in the negative siRNA control cells (55.79 ± 4.53 pA/pF at +80 mV, n = 6), the acid-induced current was almost completely depleted by ClC-3 siRNA treatments. The results indicate that the acid-induced chloride current is associated with the expression of ClC-3 proteins.

**Effects of ClC-7 siRNA on Acid-Induced Cl\(^{-}\) Currents**

The above results also indicated that ClC-7 was strongly expressed in CNE-2Z cells. To investigate the role of ClC-7 in activation of acid-activated chloride currents, the effect of knockdown of ClC-7 expression by ClC-7 siRNA was tested. Three interfering sequences were designed to knock down the expression of ClC-7. As shown in Fig. 7, semiquantitative analysis showed that the expression of ClC-7 mRNA was inhibited significantly by ClC-7 siRNA-1 and ClC-7 siRNA-2 (100 nM) in the presence of lipofectamine 2000 (1 μl/500 μl) (n = 3, P < 0.01). ClC-7 siRNA-3 showed only a relatively weak effect in knocking down the ClC-7 mRNA expression (n = 3, P > 0.05). Transfection of cells with the negative control siRNA (100 nM) plus lipofectamine 2000 (1 μl/500 μl) or with lipofectamine 2000 alone did not significantly alter ClC-7 mRNA expression (n = 3, P > 0.05). The ClC-7 siRNA was used to silence the ClC-7 mRNA expression and to confirm the knockdown effect on protein expression by the Western blotting. As shown in Fig. 7, B and C, the expression of ClC-7 protein was significantly inhibited by the ClC-7 siRNA-1 treatment (n = 3, P < 0.01).

In the patch-clamp experiments, ClC-7 siRNA-1 was used. CNE-2Z cells were treated with FAM carboxyfluorescein-labeled ClC-7 siRNA-1 (100 nM) and lipofectamine 2000 (1 μl in 500 μl medium) for 48 h before current measurements. Only the cells with strong fluorescence (indicating successful transfection of ClC-7 siRNA) were recorded. As shown in Fig. 7, D–I, the acid-induced Cl\(^{-}\) currents could still be activated by extracellular acidification (pH 6.6) in the cells treated with ClC-7 siRNA, with mean current density of 59.29 ± 5.90 pA/pF at +80 mV (n = 7), which was not significantly different from that recorded in the cells treated with the negative siRNA (64.19 ± 2.72 pA/pF, n = 4, P > 0.05). The results indicate that the activation of acid-induced chloride currents is not dependent on the expression of ClC-7 proteins.

**DISCUSSION**

In this study, it has been demonstrated that a chloride current was activated by extracellular acidification in nasopharyngeal carcinoma cells. The current was weakly outward rectified and volume sensitive and was inhibited by extracellular applications of the chloride channel blockers, NPPB, tamoxifen, and DIDS. The permeability for various anions was different, with
a sequence of $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{gluconate}^-$. These properties of the acid-induced currents were to some extent similar to those of the volume-activated $\text{Cl}^-$ current reported previously (3, 19, 20).

Extracellular acidification has been reported to activate chloride channels in other cell types. Extracellular acidification induced a strong outward-rectified $\text{Cl}^-$ current in human skeletal muscle cells, with a permeability sequence of $\text{I}^- > \text{Cl}^- > \text{F}^-$ (13). In rat Sertoli cells, an acid-induced $\text{Cl}^-$ current with strong outward rectification and a fast time-dependent onset was recorded and the current was not inactivated at the pH 5.5 (2). The weak outward rectification shown in our results in nasopharyngeal carcinoma cells was not in accord with the strong outward rectification of normal cells reported by others (2, 13). This discrepancy may result from the different sensitivities of the channels to extracellular acidification in different cell types, from different recording conditions, or even from the difference of channels in molecular nature. It was shown that an inward-rectified $\text{Cl}^-$ current was induced by extracellular acidification in rat cortical astrocytes (16). Furthermore, Schnizler and colleagues (26) found that there was a novel member of ligand-gated ion channel subunits, pHCl, expressed in Drosophila; pHCl mediated a chloride current that was inhibited by extracellular protons and activated by avermectins.
in a pH-dependent manner, with a linear current-voltage relationship.

What is the molecule that is responsible for the activation of the acid-activated chloride current? It was shown that injection of ClC-7-cRNA to oocytes of *Xenopus laevis* promoted the expression of ClC-7 and induced an acid-activated Cl\(^{-}\)/H\(^{+}\) current with strong outward rectification (5). Auzanneau and colleagues (2) found that four isoforms of chloride channels, including rClC-2, rClC-3, rClC-6, and rClC-7, were expressed in rat Sertoli cells, but these isoforms were all not acid-activated Cl\(^{-}\) channels (2). ClC-4 was reported to be activated by extracellular acidification in human skeletal muscles (13), but Friedrich and colleagues (8) demonstrated that the currents mediated by ClC-4 and ClC-5 were inhibited by extracellular acidification. In this study, we have examined the expression of ClC chloride channels in human nasopharyngeal carcinoma cells. ClC-3 and ClC-7 mRNAs and proteins were strongly expressed while ClC-1, ClC-2, ClC-4, ClC-5, and ClC-6 were not observed or were weakly expressed in CNE-2Z cells. ClC-7 has been supposed to mediate the acid-activated Cl\(^{-}\)/H\(^{+}\) current in *Xenopus laevis*. However, in this study, silence of ClC-7 expression by ClC-7 siRNA did not significantly affect Fig. 7. Effects of ClC-7 siRNA treatment on ClC-7 expression and acid-induced chloride currents. CNE-2Z cells were incubated in normal medium (control) or in medium containing lipofectamine 2000 (Lipofectamine, 1 µL/500 µl), or transfected with 100 nM negative control siRNA (negative siRNA) or 100 nM ClC-7 siRNA (ClC-7 siRNA-1, 2, and 3) in the presence of lipofectamine 2000 (1 µL/500 µl) for 48 h. A: typical RT-PCR of ClC-7 mRNA expression under different treatments. The expression of the ClC-7 mRNA was significantly knocked down by ClC-7 siRNA-1 and 2, and with a weak inhibition by ClC-7 siRNA-3. In protein expression and current recording experiments, ClC-7 siRNA-1 was used to silence ClC-7 expression. B: typical results of Western blot analysis. C: relative intensity of ClC-7 protein expression under different treatments (means ± SE, n = 3). The expression of the ClC-7 protein was significantly knocked down by ClC-7 siRNA-1 (n = 3, P < 0.01). D: typical time course of the currents at +80 mV and −80 mV in the cells treated with ClC-7 siRNA-1 or negative control siRNA in the presence of lipofectamine 2000. E: current-voltage relationship under different treatments (means ± SE; n = 7 for ClC-7 siRNA-1 and 4 for negative siRNA). F–I: representative current traces at 0, ±40, and ±80 mV under different treatments. **P < 0.01 (vs. negative siRNA).
the activation of the acid-activated Cl⁻ currents. The results indicate that the activation of the acid-activated Cl⁻ currents in CNE-2Z cells may not be dependent on the expression of CIC-7 channels. Our further study demonstrated that knockdown of CIC-3 expression by CIC-3 siRNA prevented the activation of the chloride currents induced by the decrease of extracellular pH. The result suggests that CIC-3 protein plays an important role in the activation of the acid-induced Cl⁻ currents in CNE-2Z cells. CIC-3 protein may be the chloride channel or the channel regulator that is responsible for the activation the acid-induced currents. These results imply the diversity of the proteins responsible for the activation of the acid-induced chloride currents. Different cells may express different isoforms of Cl⁻ channels or channel regulators.

As shown above, the properties of the acid-induced currents were to some extent similar to those of the volume-activated Cl⁻ current reported previously (3, 19, 20). What is the relationship between the two currents? We found in this study that the acid-activated chloride currents were volume sensitive. Cell shrinkage induced by hypertonic challenges inhibited the currents. Furthermore, both the acid-induced Cl⁻ currents and the volume-activated Cl⁻ currents were attenuated by CIC-3 siRNA treatments (31). These results suggest that the acid-induced chloride channel is a volume-sensitive chloride channel. CIC-3 is a protein that regulates or mediates the acid-induced chloride currents and the volume-sensitive chloride currents. However, the results do not exclude the possibility that other CIC proteins may also be involved in the activation or modulation of the two currents. To clarify the role of different CIC proteins in activation of the two currents, more work needs to be done.

What are the roles of the acid-induced chloride currents? In many abnormal conditions, such as ischemia and hypoxidosis, more acidic metabolites will be produced and the stabilization of extracellular pH may be broken. Furthermore, the metabolites may increase the intracellular osmolality and swell the cells. Activation of the chloride channels by acidification and cell swelling may increase the ability of cells to regulate their volume, and therefore to increase the viability. It is well known that the microenvironment of tumor cells is more acidic than that of normal cells, and extracellular acid in tumors plays important roles in metastasis and resistance to anticancer drugs (22, 23). Acid-induced channels may be involved in tumor growth by mediation of the chloride currents activated by acidity. It was demonstrated by us previously that chloride channels are associated with the regulation of the cell cycle, cell proliferation, and migration in nasopharyngeal carcinoma cells (4, 17). The roles of the acid-activated chloride currents in regulation of these cell functions need to be clarified in future studies.

In conclusion, this study demonstrates that human nasopharyngeal carcinoma cells express a chloride channels that can be activated by extracellular acidification and is sensitive to cell volume changes. CIC-3 is involved in the activation of the channel.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

W.M., L.Z., W.Y., L.W., and L.C. conception and design of the research; W.M., D.Y., Y.L., S.L., H.L., and B.L. performed the experiments; W.M. and W.Z. prepared the figures; W.M. and L.Z. drafted the manuscript; L.Z. and W.Z. analyzed the data; L.Z., L.W., and L.C. interpreted the results of the experiments; L.Z. edited and revised the manuscript; L.W. and L.C. approved the final version of the manuscript.

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