Regulation of Intracellular Cl⁻ Concentration through Volume-regulated CIC-3 Chloride Channels in A10 Vascular Smooth Muscle Cells*

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We previously found that antisense oligonucleotide specific to CIC-3 (CIC-3 antisense) prevented rat aortic smooth muscle cell proliferation, which was related to cell volume regulation. In the present study, we further characterized the regulation of intracellular Cl⁻ concentrations ([Cl⁻]i) via volume-regulated CIC-3 Cl⁻ channels in an embryo rat aortic vascular smooth muscle cell line (A10 cell) and CIC-3 cDNA-transfected A10 cells (CIC-3-A10) using multiple approaches including [Cl⁻]i measurement, whole cell patch clamp, and application of CIC-3 antisense and intracellular dialysis of an anti-CIC-3 antibody. We found that hypotonic solution decreased [Cl⁻]i and evoked a native I_{Cl,vol} in A10 cells. The responses of [Cl⁻]i and I_{Cl,vol} to hypotonic challenge were enhanced by expression of CIC-3, and inhibited by CIC-3 antisense. The currents in A10 (I_{Cl,vol}) and in CIC-3-A10 cells (I_{Cl,CIC-3}) were remarkably inhibited by intracellular dialysis of anti-CIC-3 antibody. Reduction in [Cl⁻]i and activation of I_{Cl,vol} and I_{Cl,CIC-3} in A10 and CIC-3-A10 cells, respectively, were significantly inhibited by activation of protein kinase C (PKC) by phorbol-12,13-dibutyrate (PDBu) and inhibition of tyrosine protein kinase by genistein. Sodium orthovanadate (vanadate), a protein-tyrosine phosphatase inhibitor, however, enhanced the cell swelling-induced reduction in [Cl⁻]i, accompanied by the activation of I_{Cl,vol} and I_{Cl,CIC-3} in a voltage-independent manner. Our results suggest that the volume-regulated CIC-3 Cl⁻ channels play important role in the regulation of [Cl⁻]i and cell proliferation of vascular smooth muscle cells.

Cell swelling occurs in many physiological responses and pathological processes. In most cells, the cell swelling occurs in the early phase of cell proliferation probably caused by water influx that accompanies changes in cell metabolism (such as obligatory uptake of amino acids) in the cell cycle (1). An increase in cell volume usually evokes regulatory volume decrease (RVD) process through activation of various transporters and ion (K⁺ and Cl⁻) channels, which induces the effluxes of K⁺, Cl⁻, and H₂O, and returns the cell volume to normal size. It is generally understood that RVD is mainly mediated by Cl⁻ efflux through a volume-regulated Cl⁻ channel (VRC). Therefore, VRC may play an essential role in cell proliferation through regulation of cell volume. In our previous study, we determined effects of different kinds of Cl⁻ channel blockers on endothelin-1-induced proliferation in cultured rat aortic vascular smooth muscle cells. It was found that the aortic vascular smooth muscle cell proliferation was only inhibited by DIDS (2). Furthermore, CIC-3 antisense inhibited the functional expression of CIC-3 and endothelin-1-induced proliferation in cultured rat aortic vascular smooth muscle cells (3). These results provide evidence that CIC-3 may be the gene responsible for I_{Cl,vol} and mediate volume regulation in vascular smooth muscle cells.

Although it has been suggested that the CIC-3 gene may encode VRC and mediate the volume regulation process in guinea pig ventricular myocytes (4), canine pulmonary smooth muscle cells (5), bovine non-pigmented ciliary epithelial cells (6), HeLa cells, and Xenopus laevis oocytes (7), others have presented results against the CIC-3 hypothesis (8, 9, 20–22). Therefore, molecular identification of VRC has not been determined. In the present study, we further determined the relationship between volume-regulated Cl⁻ and CIC-3 channels in A10 and CIC-3-A10 vascular smooth muscle cells by continuously monitoring the change in [Cl⁻]i, whole cell patch clamp, CIC-3 antisense, and intracellular dialysis of an anti-CIC-3 antibody techniques. Our results strongly suggest that the CIC-3 channel is responsible for swelling-induced Cl⁻ current and Cl⁻ movement and mediates volume regulation in A10 vascular smooth muscle cell.

MATERIALS AND METHODS

Cell Culture—A10 vascular smooth muscle cells from the American Type Culture Collection. A10 cells were grown in Dulbecco’s modified Eagle’s medium/F12 medium with 10% fetal calf serum, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cultures were maintained at 37 °C in a humidified incubator in a 95% O₂ plus 5% CO₂ atmosphere. For electrophysiological experiments, the cells were subcultured in coverslips for 1–2 days.

gpCIC-3 cDNA Transfection—Cells were plated in 24-well Corning tissue culture plates. Twenty-four hours later, cells were transfected with 1 µg/ml gpCIC-3/pc DNA 3.1 plasmid, which contains a full-length gpCIC-3 cDNA, and pcDNA3.1 vector (gpCIC-3/pcDNA3.1 was kindly provided by Dr. J. R. Hume, University of Nevada School of Medicine, Reno, Nevada). The vector contains a genetin-resistant marker.

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§ The abbreviations used are: VRC, volume-regulated Cl⁻ channel; DIDS, 4,4'-diisothiocyanato stilbene-2,2'-disulfonic acid; 4e-PMA, 4-ethyl-12-myristate 13-acetate; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; VSOAC, volume-sensitive osmolyte and anion channels; MEQ, 6-methoxy-N-ethylquinoline iodide; F, farad; gp, glycoprotein.
Transfection was performed with Lipofectamine2000 reagent according to the manufacturer (Invitrogen, Life Technologies, Inc.). Stably transfected clonal cell lines were selected using Geneticin (G418) at 400 μg/ml. All oligonucleotides were labeled with fluorescein as described under “Material and Methods.” There was no fluorescence detectable in A10 cells before transfection. The fluorescence can be detected in these cells after 6 h of incubation with oligonucleotides labeled with fluorescein, indicating oligonucleotides have been taken by A10 cells. Panel a, untransfected A10 cells; panel b, cells transfected with 100 μg/ml missense oligonucleotide; panel c, cells transfected with 100 μg/ml sense oligonucleotide; panel d, cells transfected with 100 μg/ml antisense oligonucleotide. B, densitometric analysis shows that 100 μg/ml CIS-3 antisense oligonucleotide decreased CIS-3 expression in a concentration-dependent manner, whereas the transfections of sense, missense, and Lipofectamine2000 failed to change CIS-3 expression (n = 6); **, p < 0.01 versus control. Representative Western blots for CIS-3 protein expression are shown at the bottom.

Cl– currents were recorded with an Axopatch 200B Amplifier (Axon Instrument, Foster City, CA) using conventional whole cell recording technique. Patch pipettes were made from borosilicate glass using a two-stage puller (pp-83, Narishige, Tokyo, Japan) and had the resistances of 3–5 MΩ when the pipettes were filled with the pipette solution. A 3 mV KCl-agar salt bridge between the bath and the Ag–AgCl reference electrode was used to minimize the changes of liquid junction potentials. To determine the whole cell current-voltage curve, the cell

**Fig. 1. Effect of CIS-3 cDNA transfection on CIS-3 protein expression in A10 cells.** A, densitometric analysis shows a significant increase in CIS-3 protein expression induced by CIS-3 cDNA transfection (n = 6; **, p < 0.01 versus control), whereas pcDNA and Lipofectamine2000 (lipo) did not significantly alter the endogenous CIS-3 protein expression. B, representative Western blots are shown.

**Fig. 2. Effect of CIS-3 antisense oligonucleotide transfection on CIS-3 protein expression in A10 cells.** A, representative images of fluorescence in A10 cells (×400). All oligonucleotides were labeled with fluorescein as described under “Material and Methods.” There was no fluorescence detectable in A10 cells before transfection. The fluorescence can be detected in these cells after 6 h of incubation with oligonucleotides labeled with fluorescein, indicating oligonucleotides have been taken by A10 cells. Panel a, untransfected A10 cells; panel b, cells transfected with 100 μg/ml missense oligonucleotide; panel c, cells transfected with 100 μg/ml sense oligonucleotide; panel d, cells transfected with 100 μg/ml antisense oligonucleotide. B, densitometric analysis shows that 100 μg/ml CIS-3 antisense oligonucleotide decreased CIS-3 protein expression in a time-dependent manner. After 48 h of CIS-3 antisense treatment, the decrease in CIS-3 protein expression reached maximum. C, densitometric analysis shows that 25–200 μg/ml CIS-3 antisense oligonucleotide decreased CIS-3 expression in a concentration-dependent manner, whereas the transfections of sense, missense, and Lipofectamine2000 failed to change CIS-3 expression (n = 6); **, p < 0.01 versus control. Representative Western blots for CIS-3 protein expression are shown at the bottom.

**Measurement of Cl–** 6-Methoxy-N-ethylquinolinium iodide (MEQ) was reduced to its cell-permeable derivative 6-methoxy-N-ethyl-1,2-dihydroquinoline (dihydro-MEQ) as described previously (10). The MEQ reduction was carried out by adding of 32 μl sodium borohydride to MEQ solution at room temperature under flowing nitrogen in the dark for 30 min. Dihydro-MEQ was freshly prepared before experiment.

Cells were incubated with 100–150 μM diH-MEQ in a Ringer’s buffer solution containing (mM): 119 NaCl, 2.5 KCl, 1.0 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 26 NaHCO3, and 11 glucose, pH 7.4 at room temperature in the dark for 20 min. In cytoplasm, dihydro-MEQ is quickly oxidized to MEQ, which is sensitive to Cl–. Fluorescence quenching induced by Cl– was monitored by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) with 350-nm excitation wavelength and 435-nm emission wavelength.

**Electrophysiological Experiments**—Cells in a chamber of 500 μl in volume were continuously superfused at the rate of 2 ml per min. The
Fig. 3. The characteristics of Cl⁻ currents induced by hypotonic solution in A10, ClC-3-A10, and ClC-3 antisense-transfected A10 cells. A, representative traces of Cl⁻ currents induced by isotonic, hypotonic, and hypertonic solutions in A10 cell (top), ClC-3-A10 cell (middle), and ClC-3 antisense-transfected A10 cells (bottom). B, mean current densities measured at -80 mV (downward bars) and +80 mV (upward bars) in A10 cells (n = 12), ClC-3-A10 cells (n = 19) and ClC-3 antisense-transfected A10 cells (n = 13). **, p < 0.01 versus A10 cells. C, representative I_{Cl,vol} (top) and I_{Cl,ClC-3} (bottom) recorded in isotonic solution and hypotonic solution containing [Cl⁻] of 116 mM and 39 mM. Changing the [Cl⁻] from 116 to 39 mM in hypotonic solution remarkably reduced both I_{Cl,vol} and I_{Cl,ClC-3}. D, I-V curves for I_{Cl,vol} and I_{Cl,ClC-3} in hypotonic solutions with [Cl⁻] of 116 mM and 39 mM. In hypotonic solution with [Cl⁻] of 116 mM, the reverse potentials of I_{Cl,vol} and I_{Cl,ClC-3} were -1.6 ± 2.0 and -2.3 ± 1.7 mV (p < 0.05), respectively. The alteration of [Cl⁻] from 116 to 39 mM shifted the I-V curves of I_{Cl,vol} (n = 6) and I_{Cl,ClC-3} (n = 6) to the right, and the reverse potentials were shifted to 27.9 ± 2.6 and 26.6 ± 1.9 mV (p < 0.05) respectively. [Cl⁻] was kept in 116 mM during the experiments. The calculated Cl⁻ equilibrium potential (E_{Cl}) was 0 mV in 116 mM [Cl⁻], and 28.4 mV in 39 mM [Cl⁻]. The reverse potentials were similar to E_{Cl}. E, representative traces of I_{Cl,vol} (top) and I_{Cl,ClC-3} (bottom) in isotonic, hypotonic, and hypertonic plus 100 μM DIDS solutions. DIDS remarkably inhibited I_{Cl,vol} and I_{Cl,ClC-3}. F, inhibitory effects of DIDS on I_{Cl,vol} (n = 7) and I_{Cl,ClC-3} (n = 8) at +80 mV and -80 mV. The effects of DIDS at +80 mV were significantly higher than that at -80 mV. This suggests that the effect of DIDS is voltage-dependent. **, p < 0.01 versus -80 mV.

was held at -40 mV, and test potentials were applied from -100 mV to +120 mV for 400 ms in 20 mV increments at an interval of 5 s. To obtain time-dependent changes in current amplitude, cells were clamped from a holding potential of -40 mV to a hyperpolarizing potential of -80 mV for 250 ms, then back to +40 mV for 25 ms, and then to a depolarizing potential of +100 mV for 250 ms. This protocol was repeated every 30 s. Currents were filtered at a frequency of 2 kHz and digitized at 5 kHz using pCLAMP8.0 software (Axon Instruments). The data were directly entered into the hard drive of a PC-compatible computer. All experiments were performed at room temperature (25°C).

The hypotonic bath solution contained (mM): 107 N-methyl-d-glucamine chloride (NMDG-Cl), 1.5 MgCl₂, 2.5 MnCl₂, 0.5 CaCl₂, 0.05 CdCl₂, 10 glucose, and 10 Hepes, pH 7.4 adjusted with NMDG. This solution osmolarity measured by a freezing point depression osmometer (OSMOMAT300, Germany) was 220 mOsmol/kg H₂O. A 300 mOsmol/kg H₂O isotonic solution was prepared by adding 70 mM n-mannitol to the hypotonic solution. A 370 mOsmol/kg H₂O hypertonic bath solution was prepared by adding 140 mM n-mannitol to the hypotonic solution. In the experiment for Cl⁻ dependence, Cl⁻ in the hypotonic medium was replaced by equimolar aspartate⁻ to obtain the hypotonic solution containing 39 mM [Cl⁻]. In the experiment for anion selectivity, the hypotonic bath solution contained (mM): 115 NaX, 10 glucose, and 10 Hepes, pH 7.4 adjusted by NaOH. X⁻ denotes I⁻, Cl⁻ or aspartate⁻. The pipette solution contained (mM): 95 CsCl, 20 TEACl, 5 ATP-Mg, 5 EGTA, 5 Hepes, and 80 n-mannitol, pH 7.2 adjusted by CsOH. The osmolarity of this solution was 300 mOsmol/kg H₂O. In the intracellular dialysis experiments, anti-CIC-3 antibody was diluted to 300 μg/ml by distilled water, and added to the pipette solution (final concentration was 5 μg/ml). For preabsorbed anti-CIC-3 antibody, antibody and antigen were dissolved by distilled water separately, mixed in a ratio of 1:10, then stored at 4 °C overnight. The mixed solution was added to the pipette solution. Finally, the pipette solution contained 5 μg/ml antibody and 50 μg/ml antigen. The osmolarity of the pipette solutions was not significantly altered by adding antibody alone or antigen-presorbed antibody.

Based on the shift of the reversal potential (ΔE_rev), the permeability ratios (P_x/P_Cl) were calculated by the modified Goldman-Hodgkin-Katz equation shown in Equation 1,

$$P_x/P_{Cl} = [Cl^-]_{exp}/[Cl^-]_{rev}[X^-]/[Cl^-]_{exp}$$ (Eq. 1)

where [Cl⁻]_{exp} and [Cl⁻]_{rev} are the Cl⁻ concentrations in the normal and substituted extracellular solutions. [X⁻] is the concentration of the substituting anion. F is the Faraday constant, R is the gas constant, and T is absolute temperature.

Statistical Analysis—All data are expressed as mean ± S.E. Statistical analyses were performed using the Student’s t test or analysis of variance. Values of p < 0.05 were considered significant.

Anti-CIC-3 antibody and antigen were supplied by Alomone Labs (Jerusalem, Israel). Trypsin, CsCl, CsOH, L-aspartate acid, EGTA, TEAC, Hepes, ATP-Mg, DIDS, 4-α-phorbol 12-myristate 13-acetate (4α-PMA), PD Bu, genistein, vanadate, Tris, MgSO₄, and Dulbecco’s modified Eagle’s medium/F12 were obtained from Sigma.

RESULTS

Expression of CIC-3 cDNA in A10 Cells—Expression of CIC-3 protein in A10 cells was determined by Western blot with the
use of a polyclonal antibody directed against CIC-3. The anti-CIC-3 antibody recognized a major band at 80–90 kDa. The CIC-3 protein expression was significantly increased in CIC-3-A10 cells (Fig. 1).

Effect of CIC-3 Antisense on Expression of CIC-3 Protein—To determine the uptake of oligonucleotide by A10 cell, the oligonucleotides were labeled with fluorescence. As shown in Fig. 2A, under resting conditions the fluorescence in the cells was negligible, but the fluorescence in cells treated with antisense, sense, or missense were greatly increased, which confirmed the uptake of oligonucleotides by these cells. Fig. 2B shows that 100 μg/ml CIC-3 antisense oligonucleotide decreased CIC-3 protein expression in a time-dependent manner. After 48 h of CIC-3 antisense treatment, the decrease in CIC-3 protein expression reached a maximum. Fig. 2C shows that the cells in the quiescent state were transfected with oligonucleotides by incubation for 48 h with Lipofectamine,

The Characteristics of Cl⁻ Currents—In A10 cells, the patch clamp whole cell currents were very small when the cells were exposed in the isotonic solution. If the bath solution was changed from isotonic solution to hypotonic solution, it evoked the large outward rectifying currents (I_{Cl,vol}) with a reverse potential of −2.0 ± 1.5 mV. In CIC-3-A10 cells, hypotonic solution also evoked an outward rectifying current (I_{Cl,CIC-3}) with a reverse potential of −2.6 ± 1.2 mV. Compared with I_{Cl,vol}, I_{Cl,CIC-3} was larger. The current densities of I_{Cl,vol} (n = 12) and I_{Cl,CIC-3} (n = 19) were −23.5 ± 1.5 and −55.9 ± 2.7 pA/pF (p < 0.05 versus I_{Cl,vol}) at −80 mV, respectively, and 41.8 ± 2.8 and 100.6 ± 5.6 pA/pF (p < 0.01 versus I_{Cl,vol}) at +80 mV, respectively (Fig. 3, A and B). When the [Cl⁻] in the bath solution was changed from 116 to 39 mM, the current was significantly decreased, with a change in the reverse potential from −1.6 ± 2.0 to 27.9 ± 2.6 mV in A10 cells (n = 6) and from −2.3 ± 1.7 to 26.8 ± 1.9 mV in CIC-3-A10 cells (Fig. 3, C and D). Both I_{Cl,vol} and I_{Cl,CIC-3} were inhibited by DIDS. After exposure to DIDS, the current densities of I_{Cl,vol} (n = 8) and I_{Cl,CIC-3} (n = 7) were reduced from −21.8 ± 2.4 to −14.3 ± 1.6 pA/pF and −56.0 ± 4.1 to −34.1 ± 2.5 pA/pF at −80 mV, respectively, and reduced from 36.4 ± 3.0 to 16.4 ± 1.6 pA/pF and 101.1 ± 9.1 to 41.1 ± 4.9 pA/pF at +80 mV, respectively. DIDS had the same inhibition in I_{Cl,vol} and I_{Cl,CIC-3}. However, DIDS had more inhibition on currents at +80 mV than that at −80 mV (Fig. 3, E and F). This indicates that the effects of DIDS on I_{Cl,vol} and I_{Cl,CIC-3} were voltage-dependent.

100 μg/ml CIC-3 antisense remarkably decreased I_{Cl,vol} by 54.3 ± 4.6% at +80 mV and 56.0 ± 5.8% at −80 mV, which were similar to the decreased magnitude of CIC-3 protein expression induced by 100 μg/ml CIC-3 antisense, whereas sense, missense, and Lipofectamine failed to inhibit this current. Based on results from the CIC-3 antisense experiments, we further examined the inhibitory effects of intracellular dialysis of an anti-CIC-3 antibody on I_{Cl,vol} and I_{Cl,CIC-3}. Fig. 4 shows that intracellular dialysis of an anti-CIC-3 antibody could completely block the I_{Cl,vol} (Fig. 4, A and B) and I_{Cl,CIC-3} (Fig. 4, C and D). To exclude nonspecificity of the anti-CIC-3 antibody,
the effect of preabsorbed anti-CIC-3 antibody on the current was determined. It was found that intracellular dialysis of preabsorbed anti-CIC-3 antibody did not inhibit \( \text{ICl}_{\text{vol}} \) and \( \text{ICl}_{\text{ClC-3}} \) (Fig. 4, A and B).

Table I illustrates the anion selectivity of both the \( \text{ICl}_{\text{vol}} \) channel in A10 cell and \( \text{ICl}_{\text{ClC-3}} \) channel in CIC-3-A10 cell. The reverse potential induced by \( I^- \) was more minus than that by \( \text{Cl}^- \), whereas aspartate produced a positive reverse potential. The anion selectivity order of these channels was as follows: \( I^- > \text{Cl}^- > \text{aspartate} \). There was no significant difference in reverse potentials induced by anions and the anion permeability between \( \text{ICl}_{\text{vol}} \) and \( \text{ICl}_{\text{ClC-3}} \).

The Change in \([\text{Cl}^-]\), Induced by Hypotonic Solution—In \([\text{Cl}^-]\), measuring experiments, change of the medium from isotonic solution to hypotonic solution significantly decreased \([\text{Cl}^-]\) in A10 from 31.1 ± 2.1 to 24.9 ± 1.2 mM (\( n = 30; p < 0.01 \) versus isotonic solution), whereas in CIC-3-A10 cells, the hypotonic solution induced more of a decrease in \([\text{Cl}^-]\) than that in A10 cells. The \([\text{Cl}^-]\) in CIC-3-A10 cells was reduced from 30.6 ± 1.1 to 19.9 ± 1.0 mM (\( n = 30; p < 0.05 \) versus A10 cell in hypotonic solution). In contrast, 100 \( \mu \text{g} / \text{mL} \) CIC-3 antisense significantly inhibited the decrease in \([\text{Cl}^-]\), induced by hypotonic solution, and made \([\text{Cl}^-]\), have a lower response to hypotonic challenge. \([\text{Cl}^-]\) was diminished from 31.1 ± 2.1 to 28.5 ± 1.9 mM (\( n = 30; p < 0.05 \) versus A10 cell in hypotonic solution; Fig. 5).

Effects of PDBu, Genistein, and Vanadate on \( \text{ICl}_{\text{vol}}, \text{ICl}_{\text{ClC-3}}, \) and \([\text{Cl}^-]\)]——Fig. 6A illustrates that 100 \( \mu \text{M} \) PDBu completely blocked \( \text{ICl}_{\text{vol}} \) (A, top trace in a) and \( \text{ICl}_{\text{ClC-3}} \) (A, bottom trace in a). The decrease in \([\text{Cl}^-]\), induced by hypotonic solution in A10 and CIC-3-A10 cells was completely reverted to normal levels by PDBu (Fig. 6D), whereas, 4a-PMA (negative control for phorbol ester activation of PKC) had no significant effect on \( \text{ICl}_{\text{vol}} \) (A, top trace in b), \( \text{ICl}_{\text{ClC-3}} \) (A, bottom trace in b), and \([\text{Cl}^-]\), induced by hypotonic solution in A10 and CIC-3-A10 cells (Fig. 6D).

Fig. 6B shows that 30 \( \mu \text{M} \) genistein completely inhibited \( \text{ICl}_{\text{vol}} \) (top trace) and \( \text{ICl}_{\text{ClC-3}} \) (bottom trace). The levels of \([\text{Cl}^-]\), induced by hypotonic solution in A10 and CIC-3-A10 cells were almost elevated to the normal level by genistein (Fig. 6E). Whereas, the protein-tyrosine phosphatase inhibitor, sodium orthovanadate, potentiated \( \text{ICl}_{\text{vol}} \) (Fig. 6C, top trace) and \( \text{ICl}_{\text{ClC-3}} \) (Fig. 6C, bottom trace) in a voltage-independent manner. The densities of currents for \( \text{ICl}_{\text{vol}} \) before and after exposure to 500 \( \mu \text{M} \) vanadate were -24.1 ± 2.3 and -34.2 ± 3.1 \( \text{pA} / \text{pF} \) at -80 mV (\( n = 8 \)), respectively, and 40.2 ± 2.9 and 55.6 ± 2.7 \( \text{pA} / \text{pF} \) at +80 mV (\( n = 8 \)), respectively. For \( \text{ICl}_{\text{ClC-3}} \), the densities of currents were -63.5 ± 5.2 and -94.7 ± 5.0 \( \text{pA} / \text{pF} \) at -80 mV (\( n = 8 \)), respectively, and 96.7 ± 5.4 and 141.4 ± 6.5 \( \text{pA} / \text{pF} \) at +80 mV (\( n = 8 \)), respectively. The increases in \( \text{ICl}_{\text{vol}} \) and \( \text{ICl}_{\text{ClC-3}} \) induced by vanadate were 45.7 ± 4.4 and 48.5 ± 5.0% at -80 mV, and 47.1 ± 4.8 and 49.3 ± 4.7% at +80 mV, respectively. This was consistent with the effect of vanadate on \([\text{Cl}^-]\). This protein-tyrosine phosphatase inhibitor further significantly reduced \([\text{Cl}^-]\) level, which was in lower level induced by hypotonic solution, from 24.8 ± 1.6 to 21.2 ± 1.4 mM (\( n = 30; p < 0.05 \)) in A10 cells, and from 20.9 ± 1.3 to 16.2 ± 1.1 mM (\( n = 30; p < 0.05 \)) in CIC-3-A10 cells (Fig. 6F).

**DISCUSSION**

In the present study, we first demonstrated that hypotonic solution significantly reduced \([\text{Cl}^-]\), and simultaneously activated an outward rectifying \( \text{ICl}_{\text{vol}} \) in A10 cells. Both CIC-3 antisense and intracellular dialysis of anti-CIC-3 antibody significantly inhibited the hypotonicity-induced decrease in \([\text{Cl}^-]\), and activation of \( \text{ICl}_{\text{vol}} \). We then found that overexpression of CIC-3 in A10 cells (CIC-3-A10 cells) yielded a larger \( \text{ICl}_{\text{vol}} \) with the same reverse potential, sensitivity to voltage-dependent inhibition by DIDS, and anion selectivity (\( I^- > \text{Cl}^- > \text{aspartate}^- \)) as that of endogenous \( \text{ICl}_{\text{vol}} \) in A10 cells under hypotonic conditions. Furthermore, we showed that the hypotonic cell swelling induced more decrease in \([\text{Cl}^-]\), in CIC-3-A10 cells, and activation of \( \text{ICl}_{\text{ClC-3}} \) also was remarkably inhibited by intracellular dialysis of anti-CIC-3 antibody. Finally, we found that hypotonic cell swelling-induced changes in \([\text{Cl}^-]\), are not only intimately linked to the action of \( \text{ICl}_{\text{vol}} \) and \( \text{ICl}_{\text{ClC-3}} \) but

| n | A10 | CIC-3-A10 |
|---|-----|-----------|
| Cl⁻ | 6   | -1.0 ± 0.9 | 7 | -2.0 ± 0.9 |
| I⁻ | 6   | -7.5 ± 0.5 | 7 | -7.9 ± 0.8 |
| Asp⁻ | 6 | 36.8 ± 2.8 | 7 | 37.6 ± 2.1 |
| \( P/P_{Cl} \) | 6 | 1.29 ± 0.06 | 7 | 1.26 ± 0.08 |
| \( P_{\text{aspiration}}/P_{Cl} \) | 6 | 0.21 ± 0.05 | 7 | 0.22 ± 0.05 |

**Fig. 5.** The responses of \([\text{Cl}^-]\), to hypotonic challenge in A10, CIC-3-A10, and CIC-3 antisense-transfected A10 cells. A, representative traces of \([\text{Cl}^-]\), levels induced by hypotonic solution in CIC-3 antisense-transfected A10 cell (green line), A10 cells (red line), and CIC-3-A10 cells (purple line). B, in isotonic solution, the \([\text{Cl}^-]\), levels in these cells were similar (\( n = 30; p > 0.05 \)). However, changes in medium from isotonic to hypotonic solutions, \([\text{Cl}^-]\), in CIC-3-A10 cells (\( n = 30 \)) was lower than that in A10 cells (\( n = 30 \)), whereas CIC-3 antisense (\( n = 30 \)) inhibited the response of \([\text{Cl}^-]\), to hypotonic solution. * \( p < 0.05 \); ** \( p < 0.01 \) versus A10 cells.
also, as having been reported for $I_{\text{Cl,vol}}$ and $I_{\text{Cl,ClC-3}}$ in other cells (16–18), well controlled by endogenous PKC and protein-tyrosine phosphorylation. These data provide compelling evidence that ClC-3 channels are responsible for the Cl$^{-}$ transport and $I_{\text{Cl,vol}}$ during hypotonic perturbations in A10 cells.

The molecular identification of the protein responsible for $I_{\text{Cl,vol}}$ has been particularly difficult to resolve. The main rea-
son for this difficulty is that endogenous I_{Cl,vol} is expressed practically in all types of cells, which can be always superimposed with membrane currents because of transgenic expression of candidate genes. Several molecular candidates responsible for I_{Cl,vol} were previously proposed: 1) the multidrug transporter, P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) family of transporters (11), 2) pICln, which encodes a small 235 amino acid protein with little homology to any known anion channel structure (12, 3) CIC-2, a member of the large CIC superfamily of voltage-dependent Cl− channels (13), and 4) CIC-3, also a member of the CIC superfamily (4). Although both P-gp and plCln expression appeared to yield chloride currents with many of the properties of native I_{Cl,vol} it now seems clear that the currents observed in these studies were not due to expression of either P-gp or plCln but likely due to contamination by endogenous I_{Cl,vol} (14). Although CIC-2 represents a bona fide anion channel regulated by cell volume, expressed CIC-2 currents differ significantly in voltage sensitivity, anion selectivity, and pharmacology from conventional, outwardly rectifying I_{Cl,vol} found in most cells (15).

The CIC-3 hypothesis was based on the fact that stable or transient transfection of a full-length CIC-3 cDNA cloned from guinea pig ventricle (gpCIC-3) into NIH/3T3 cells yielded a basally active chloride conductance that was strongly modulated by cell volume (4). Many properties of the expressed I_{gpCIC-3} resemble those reported for native I_{Cl,vol} in heart and other tissues, including an outwardly rectifying unitary slope conductance of 40 pS, an anion selectivity of Γ− > Cl− > Asp−, inactivation at positive potentials, increase by extracellular hypotonicity, and inhibition by hypertonicity, by extracellular nucleotides, by phorbol esters, by stilbene derivatives, and by tamoxifen. Furthermore, site-directed mutagenesis of an asparagine near the end of the transmembrane-spanning domains (N579K) altered rectification and anion selectivity of the expressed I_{gpCIC-3}. These observations were initially confirmed by other independent studies of several groups (16–18). Thus, CIC-3 represented a viable molecular candidate responsible for native I_{Cl,vol} in heart or any other mammalian cell type (19).

Several reports (20–22), however, failed to support a role for exogenously expressed CIC-3 as a viable candidate for native I_{Cl,vol}. The magnitude of swelling-activated Cl− current is not significantly different between nontransfected and human CIC-3 cDNA-transfected HEK293 cells (22). Overexpression of CIC-3 in HEK293 cells (22) and X. oocytes (23, 24) did not produce outward rectifying I_{Cl,vol}. In CIC-3 cDNA-transfected CHO-K1 cells, the CIC-3 channel current was not identified in the endogenous swelling-activated channel current, and not activated by cell swelling (20). Some of these inconsistencies might be attributed to failure to successfully express functional CIC-3 protein and/or difficulties in effectively separating transgenic currents from endogenously expressed Cl− currents present in expression cell systems such as X. oocytes and some mammalian cells (25).

Furthermore, the controversy surrounding the actual physiological role of CIC-3 Cl− channels was additionally fueled by the report that disruption of the Clec3 gene did not change the volume-regulated chloride current in hepatocytes, pancreatic acinar cells (26), and salivary acinar cells (27) from Clec3−/− mice. In these and another studies (28), CIC-3 was primarily localized to intracellular membranes where it was proposed to function primarily in vesicular acidification, although other studies have clearly demonstrated plasma membrane localization of heterologously expressed CIC-3 (22, 29–31) and endogenous CIC-3 (32, 33) in various cell types.

Recently, it has been shown that volume-sensitive osmolyte and anion channels (VSOACs) currents activated by hypertonic medium in atrial myocytes and pulmonary arterial smooth muscle cells from Clec3−/− and Clec3+/− mice were remarkably similar in activation kinetics, steady-state current densities, slight outward rectification, and anion selectivity. However, there also are significant differences in sensitivity to PKC regulation, inhibition by intracellular dialysis with a new anti-CIC-3 antibody, ATP, depletion, and high free [Mg2+]i between them (34). These authors have suggested that in response to Clec3 gene deletion, there may be compensatory changes in expression of other proteins that alter VSOAC channel subunit composition or associated regulatory subunits that give rise to VSOACs with different properties. It appears that differential sensitivity of native VSOACs to anti-CIC3 antibody and phorbol esters in different cell types in normal animals is consistent with the possible expression of distinct VSOACs subtypes. In fact, the responses of volume-regulated chloride current to PKC regulation in vascular smooth muscle cells from different blood vessels are not the same. Activation of PKC by PDBu increased the amplitude of swelling-activated chloride current in rabbit portal vein (35), whereas PKC activation inhibited I_{Cl,vol} in mouse pulmonary arterial smooth muscle cells (34). As shown in this study, PDBu significantly inhibited I_{Cl,vol} and increased [Cl−], under hypertonic condition. Our results provide evidence to support that CIC-3 channels are responsible for volume-regulated Cl− transportation and Cl− currents and may play an important role in volume regulation in A10 vascular smooth muscle cells.

Alternatively, it is possible that contradictory data about CIC-3 in mediating cell volume regulation are caused by differences in [Cl−], and CIC-3 expression levels in different types of cells. In vascular smooth muscle cells, [Cl−], is generally higher than other tissues, and the Cl− equilibrium potential (E_{Cl}) is approximately −20 mV, which is more positive than the resting membrane potential. Therefore, activation of the Cl− channels will lead to a net Cl− efflux followed by the membrane depolarization (36, 37). It is currently not known why [Cl−] is higher in vascular smooth muscle. In sharp contrast to vascular smooth muscle cells, some neurons have passively distributed intracellular Cl−, and it sets the resting membrane potential closer to E_{Cl} (38). It is interesting that the CIC-3 expression level in vascular smooth muscle cells, including aorta smooth muscle cells is very high (39), whereas only a small fraction of CIC-3 is expressed on the surface membrane in hepatocytes CHO-K1 cells and human hepatoma cell line Huh-7 (28). Our results may, therefore, shed a new light on understanding the regulation of intracellular Cl− concentrations in different cell types.

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