ClC-2 chloride channels contribute to HTC cell volume homeostasis

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Membrane Cl− channels are major contributors to cellular volume homeostasis. There are three families of voltage-gated chloride channels: ClC-1, ClC-2, and ClC-3. Heterologous expression of ClC-2 channel cDNA leads to the appearance of volume-sensitive Cl− currents in HEK-293 cells, consistent with a role in cell volume regulation. Since channel properties in heterologous models are potentially modified by cellular background, we evaluated whether endogenous ClC-2 proteins are functionally important in cell volume regulation. As shown by whole cell patch clamp techniques in rat HTC hepatoma cells, cell volume increases stimulated inwardly rectifying Cl− currents when non-CIC-2 currents were blocked by DIDS (100 μM). A cDNA closely homologous with rat brain CIC-2 was isolated from HTC cells; identical sequence was demonstrated for CIC-2 cDNAs in primary rat hepatocytes and cholangiocytes. CIC-2 mRNA and membrane protein expression was demonstrated by in situ hybridization, immunocytochemistry, and Western blot. Intracellular delivery of antibodies to an essential regulatory domain of CIC-2 decreased CIC-2-dependent currents expressed in HEK-293 cells. In HTC cells, the same antibodies prevented activation of endogenous Cl− currents by cell volume increases or exposure to the purinergic receptor agonist ATP and delayed HTC cell volume recovery from swelling. These studies provide further evidence that mammalian CIC-2 channel proteins are functional and suggest that in HTC cells they contribute to physiological changes in membrane Cl− permeability and cell volume homeostasis.

CELL VOLUME IS A DYNAMIC PARAMETER that is closely coupled to physiological changes in solute transport, intracellular metabolism, and membrane ion permeability. In most cells, increases in volume are followed after a delay by increases in membrane K+ and Cl− permeability of 20-fold or more. The resulting efflux of ions represents a critical adaptive response that favors passive water loss and restoration of cell volume toward basal values (1). Volume recovery is usually incomplete, however, and emerging evidence suggests that small residual differences from baseline act as a signal that directly influences a broad range of cellular processes, including gene expression, kinase activation, metabolism, and membrane transport (15). Consequently, molecular identification of the channels involved represents an important focus for defining the mechanisms that link changes in cell function to hormonal and other pathways that alter the cellular hydration state.

Recently, complementary DNAs encoding multiple members of the CIC family of voltage-gated Cl− channels have been identified. CIC-2 transcripts are distributed broadly in most mammalian tissues, including secretory epithelia such as lung, kidney, and liver (20, 21, 35). Membrane hyperpolarization, hypotonic exposure, and extracellular acidity have been shown to activate inwardly rectifying Cl−-selective currents following expression of CIC-2 proteins in different model systems (2, 12, 14, 18, 27, 32, 40). Although heterologous expression of CIC-2 enhances cell volume recovery from swelling (10, 40), the biophysical properties of these currents are distinct from the outwardly rectifying, volume-sensitive currents typical of most mammalian cells (8, 25, 31, 33, 37, 39). Thus the role of native CIC-2 channels as endogenous regulators of cell volume has not been firmly established.

In liver epithelial cells, Cl− current activation during volume increases is regulated by a sensitive autocrine signaling pathway involving release of the purinergic agonist ATP into the extracellular space and subsequent stimulation of P2 receptors coupled to membrane Cl− channels (23, 38). To assess whether native expressed Cl− channels encoded by CIC-2 contribute to volume-sensitive changes in membrane Cl− permeability in liver epithelia, rat liver CIC-2 cDNA, mRNA, and membrane-associated protein were identified. Selective inhibition of CIC-2 proteins by intracellular delivery with antibodies to an essential regulatory domain 1) decreased heterologous CIC-2 currents in HEK-293 cells, 2) inhibited activation of native currents in HTC hepatoma cells during in-
creases in cell volume and P2 receptor stimulation, and 3) delayed HTC cell volume recovery from swelling. These findings are consistent with the concept that endogenous CIC-2 channels are functionally active and contribute to volume-sensitive changes in membrane Cl− permeability.

MATERIALS AND METHODS

Cells and solutions. Most studies were performed using HTC rat hepatoma cells, which possess metabolic pathways similar to those found in primary hepatocytes. Previous studies indicate that recovery from HTC cell swelling depends on activation of separate K+− and Cl−-selective whole cell currents (3). HTC cells were grown at 37°C in 5% CO2-95% air atmosphere in MEM containing 5% fetal calf serum, 2 mM l-Gln, 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). For physiological studies, culture medium was replaced with a standard isotonic extracellular buffer, which contained (in mM) 140 NaCl, 4 KCl, 1 KH2PO4, 2 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4 with NaOH).

CIC-2 cDNA isolation. An amplified HTC cell cDNA library (Superscript Lambda system, GIBCO BRL; provided by N. Lomri, University of California at San Francisco) was screened with a 477-bp oligonucleotide cDNA probe spanning the D12-D13 loop of rat brain CIC-2 (gift of J. Cuppoletti and D. Malinowska, University of Cincinnati). In addition, cDNAs were synthesized from HTC cell, primary rat hepatocyte, and normal rat cholangiocyte mRNA using the high-fidelity Pfu DNA polymerase (Stratagene). cDNAs were sequenced by the chain termination method using [α-35S]dATP and T7 DNA polymerase (Sequenase version 2.0; Amersham Life Science), and by the 373A DNA Sequencer (Taq DyeDeoxy terminator cycle sequencing kit; Applied Biosystems) using the AmpliTaq polymerase.

In situ hybridization. For both in situ hybridization and immunocytochemistry, HTC cells on plastic coverslips were fixed with 4% paraformaldehyde for 15 min. The pellet was resuspended in 65% ethanol and 30% acetic acid. In brief, cells were grown at 37°C and 5% CO2-95% air on coverslips in multiwell (no. 24) plates containing DMEM supplemented with fetal bovine serum (1:10), ampicillin (0.1 mg/ml), and gentamicin (0.04 μg/ml). Precipitated DNA (500 ng/well) was added to achieve a final concentration of 2–5 ng/μl. Cotransfection was accomplished by adding equal amounts of the CIC-2 and green fluorescent protein (GFP) plasmid DNA for a total of 500 ng/well. For GFP-only controls, equal amounts of GFP and nonspecific salmon sperm DNA was added to achieve the same relative DNA ratios. The plasmid (pAC-CMV) containing the full-length coding region for CIC-2 or a construct containing the coding sequence for GFP (pEGFP-N1; Clontech), both under the control of the cytomegalovirus immediate-early promoter (CMV1p), were used in these experiments. Cells were allowed to grow overnight, after which the culture medium was replaced with standard supplemented DMEM (see above) and then grown for another 24–48 h before experimentation. Cells expressing GFP were assumed to have been transfected with CIC-2. GFP expression was observed within 12–16 h of transfection and appeared stable in culture for as long as 4 days. Individual GFP-expressing cells were identified for patch clamp analysis.

Analysis of Cl− currents: whole cell patch clamp recording. Whole cell currents were measured using patch clamp recording techniques as previously described (38). Cell volume increases were induced by exposure to a buffer containing 20% less NaCl or by addition of 50 mM sucrose to the pipette solution as indicated. The standard pipette (intracellular) solution contained (in mM) 130 KCl, 10 NaCl, 2 MgCl2, 10 HEPES/KOH (pH ~ 7.2), and free Ca2+ adjusted to ~100 nM (0.5 CaCl2, 1 EGTA) with a total Cl− concentration of 145 mM. Non-CIC-2 currents were inhibited by addition of DIDS (final concentration 100 μM; Calbiochem). Membrane Cl− currents during cytosolic dialysis with CIC-2 antibodies (0.005 μg/ml final concentration in the pipette solution) were compared with control currents using 1) standard pipette solutions devoid of antibodies, 2) heat-inactivated CIC-2 antibodies (100°C for 30 min, 0.005 μg/ml), 3) polyclonal rabbit antibodies to β-galactosidase (5 μg/ml, 5 Prime–3 Prime), and 4) antibody buffer without CIC-2 antibodies containing 0.0001% sodium azide. Recordings were made beginning ~15 min after achieving the whole cell configuration using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Current-voltage relationships were measured by 400-ms
steps to test potentials between −120 mV and +100 mV in 20-mV increments of other protocols as indicated. In the whole cell configuration, Vp corresponds to the membrane potential, and upward deflections of the current trace indicate outward membrane current. Whole cell currents (pA) and current density (pA/pF) refer to measurements at Vp −80 mV (E Pe) to minimize any contribution of K+ currents, as previously described (38).

Measurement of cell volume. Microinjection of individual HTC cells was performed using previously described methods (30). Ten percent of the volume of the injection solution (48 mM K2HPO4, 14 mM NH4H2PO4, and 45 mM KH2PO4) was replaced with 0.1 mg/ml of either ClC-2 or polyclonal rabbit glial fibrillary acidic protein antibodies (DAKO) in PBS. Rhodamine dextran (Molecular Probes) was included at a final concentration of 1 mg/ml so that injected cells could be identified and imaged. Injection pressures were adjusted to produce a minimal detectable change in cellular refringence, and any cells demonstrating irregular profiles suggestive of cell damage or fluorescence localized to nucleus were excluded from analysis. After injection, cells were allowed to recover for 45 min in L-15 media (Life Technologies) at room temperature. Perfusion studies were performed in a chamber (500-μl volume) at a flow rate of 4–5 ml/min; solution was removed across the width of the chamber by capillary action to facilitate laminar flow. Images were captured using a Leica DMRB inverted microscope equipped with epifluorescence and a Cohu charge-coupled device interfaced to a Power Macintosh 7100. In individual experiments, five measurements per cell were made at each time point and subsequently averaged, and injected cells and noninjected (control) cells were measured within the same microscopic field to accurately represent cells under identical conditions within the culture dish. Cell swelling was induced by switching the perfusate to hypotonic buffer (25% less osmolarity by reducing NaCl) to block non-ClC-2 currents (29). An observer blinded to experimental subsets made all measurements, and findings were confirmed by analyzing multiple microscopic fields.

Statistics. Pooled data are presented as means ± SE, where n represents the number of cells for patch clamp and video planimetry studies. Statistical comparisons of patch clamp measurements were made using the paired or unpaired t-test and of cell area measurements using ANOVA with Fischer’s post hoc analysis where appropriate. P < 0.05 was considered significant.

RESULTS

Isolation of a rat liver ClC-2 cDNA. Whole cell patch clamp studies indicate that both primary liver epithelial cells and cell lines exhibit volume-sensitive Cl− currents (I Cl,swell) where increases in cell volume enhance Cl− permeability 30-fold or more (3, 16, 19). They do not, however, exhibit the voltage-activated, inwardly rectifying currents typical of ClC-2 present in heterologous models. To assess whether ClC-2 is expressed in liver cells, ClC-2 channel transcripts were identified in a representative cell model, HTC rat hepatoma cells, by RT-PCR (data not shown). Subsequently, screening of an amplified HTC cDNA library with a 477-bp oligonucleotide probe corresponding to the D12–D13 region of ClC-2 led to the isolation of seven identical ClC-2 cDNAs, each 2,987 bp in length. Sequence analysis of both strands indicates that the liver ClC-2 (λRR) cDNA is identical to bp 172–3206 of the ClC-2 cDNA previously isolated from rat brain, starting just after the first putative initiation codon, except for a single substitution of G to A at position 3202 within the 3′-untranslated region (35). Primary cDNA isolates lacked the bp 1693–1740 segment within the D11 domain of the brain homologue. This in-frame deletion was determined to be a library artifact since PCR of HTC cell cDNA using primers flanking this region always produced the undeleted but not the deleted product (>80 reactions) with an identical sequence to that of rat brain ClC-2. In addition, RT-PCR of the 5′-untranslated region demonstrated identity to bp 148–171 of the rat brain cDNA, including the putative initiation codon at 168–171.

RT-PCR was used to determine the sequence of ClC-2 cDNAs expressed in primary rat hepatocytes and normal rat cholangiocytes, which represent the principal liver cell epithelial cell types. For both, ClC-2 cDNA sequences that include the open reading frame were identical to the rat brain homologue (bp 123–2906 of the rat brain homologue). Thus the protein coding region of the liver epithelial ClC-2 is completely homologous to the previously cloned and studied brain ClC-2 cDNA, predicting a putative 907-amino acid protein.

Detection of ClC-2 transcripts and protein in liver epithelial cells. The cellular expression of ClC-2 mRNA was evaluated. With the use of in situ hybridization, RNA signals were readily apparent in individual HTC cells exposed to antisense, but not sense, amino terminal-specific riboprobes, indicating the presence of ClC-2 mRNA (Fig. 1, A and B). To assess the cellular expression of ClC-2 channel proteins, immunocytochemistry was performed using affinity-purified polyclonal rabbit antibodies directed against a unique cytoplasmic amino terminal region of ClC-2. This sequence is not present in other ClC family members. As shown in Fig. 1C, ClC-2 proteins were present and signal was detected both internally and in the periphery of each cell. In control studies, staining was specifically blocked by coinubation with GST-ClC-2 fusion proteins but not by GST alone (Fig. 1D), indicating selective recognition of ClC-2 proteins.

Endogenous ClC-2 is distributed in the plasma membrane. Western blotting was used to characterize the cellular distribution of ClC-2 proteins. ClC-2 antibodies recognized ~100 kDa proteins in HTC cells (data not shown). Subsequently, plasma membrane distribution was determined by cellular fractionation and plasma membrane isolation. Initial fractionation showed that ClC-2 was almost exclusively present within the membrane fraction, with little or no ClC-2 found within the cytosolic fraction (Fig. 2). After further enrichment of the plasma membrane by sucrose density separation, ClC-2 and Na+-K+-ATPase were both present in the plasma membrane fraction (Fig. 2). These results suggest that endogenous ClC-2 is expressed in the plasma membrane of HTC cells.

Identification of DIDS-resistant currents in HTC cells. Since the properties of ClC-2 channels are different from those of swelling-activated currents in HTC cells, additional studies were performed in the presence of DIDS (100 μM) to block non-ClC-2 currents
Cell volume increases were produced by addition of sucrose (50 mM) to the pipette solution to ensure a standard transmembrane osmolar gradient, and currents were measured at test potentials between $-2160$ mV and $+160$ mV or $-2120$ mV to $+1100$ mV in 20-mV increments as indicated. Under control conditions, swelling-activated currents reversed at $-2164$ mV, values not different from the Cl$^{-}$ reversal potential of 0 mV, and were outwardly rectifying ($n = 9$), as described previously (3). In the presence of DIDS, a residual current was still detectable (Fig. 3). Currents at $-80$ mV decreased from $-544 \pm 122$ pA ($n = 9$) to $-341 \pm 15$ pA ($n = 3$, not significant) in the absence vs. presence of DIDS, and currents at $+80$ mV decreased from $897 \pm 212$ pA to $238 \pm 25$ pA ($P < 0.01$) in the absence vs. presence of DIDS. The DIDS-resistant current was inwardly rectifying and accounted for $\approx 60\%$ of total current over the physiological range of liver cell potentials between $-220$ and $-60$ mV. These findings suggest that a portion of the swelling-activated Cl$^{-}$ conductance in HTC cells has properties consistent with the conductance associated with ClC-2 channels.

Expression of ClC-2 in HEK-293 cells. The polyclonal rabbit antibodies were developed to recognize a target peptide antigen corresponding to amino acids 20–68 of the translated rat brain ClC-2 protein. Since this region has been shown to contribute directly to channel gating and is not found in other members in the ClC family (12, 32), binding would be anticipated to alter ClC-2 channel function in a selective and specific manner. To determine whether these antibodies inhibit channel function, ClC-2 was expressed in HEK-293 cells, which do not have endogenous ClC-2. Cells were transfected with ClC-2-containing plasmids in conjunction with plasmids containing GFP using standard

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**Fig. 1.** CIC-2 mRNA and protein are expressed in HTC cells. HTC cells were fixed and processed as described in MATERIALS AND METHODS. A and B: expression of CIC-2 mRNA was detected using in situ hybridization with digoxigenin-labeled antisense (A) or sense (B) strand probes. Detection of hybridization was performed with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Boehringer Mannheim), which allows colorimetric detection in cells containing CIC-2 mRNA. As shown, signal was detected with the antisense probe (A) but not with the sense strand control probe (B). C and D: detection of protein used an affinity-purified antibody at a concentration of 1 $\mu$g/ml. Antibody incubations were performed in the presence of 100 $\mu$g/ml of the control fusion protein glutathione-S-transferase (GST) (C) or in the presence of the blocking reagent GST-CIC-2 containing the amino terminal domain of CIC-2 fused to the carboxy terminus of GST (D). These results demonstrate the specificity of the antibody blocking by excess antigen. A significant amount of protein is localized to the membrane in addition to the cell interior, as shown by the higher-power inset in C (magnified ×3). Bar = 100 $\mu$m.

**Fig. 2.** Detection of membrane CIC-2 proteins. Membrane fractionation and Western blotting demonstrated that CIC-2 is expressed in plasma membranes. In native HTC cells (left), separation of the total membrane (particulate (P)) and cytosolic fractions (soluble (S)) showed that endogenous ClC-2 is distributed primarily within the particulate fraction-containing membranes. After further separation of HTC cell membranes (right), both Na$^{+}$-K$^{+}$-ATPase (NKA), a known plasma membrane protein, and CIC-2 protein were detected in the same plasma membrane-enriched fraction (pm).
Intracellular delivery of CIC-2 antibodies inhibits swelling- and ATP-dependent Cl⁻ currents. These findings suggest that inhibition of currents by intracellular delivery of these antibodies might provide insights into the function of endogenous CIC-2 channels. Consequently, the effects of intracellular dialysis with CIC-2 antibodies on basal, swelling-activated, and ATP-sensitive whole cell Cl⁻ currents in HTC cells were assessed (Fig. 5). Intracellular delivery of CIC-2 antibodies had no effect on basal currents but markedly prevented activation of I_{Cl,Swell} during exposure to hypotonic buffer (20% less NaCl) in all studies (n = 6, P < 0.01). In contrast, the amplitude of swelling-activated currents in cells perfused with heat-inactivated CIC-2 antibodies (n = 8), a similar dilution of the CIC-2 antibody buffer (n = 4), and polyclonal rabbit antibodies to an unrelated protein β-galactosidase (n = 6) was not different from untreated controls. In other studies, addition of the P2 receptor agonist ATP (10 µM) to isotonic bath solutions, which reproducibly stimulates Cl⁻ currents in HTC cells (9) and is essential for cell volume recovery from swelling (38), failed to induce significant currents in cells perfused with CIC-2 antibodies (n = 3, P < 0.02). These findings indicate that antibodies, which selectively target the cytoplasmic amino terminus of CIC-2 channels, inhibit volume- and ATP-dependent channel opening in rat hepatoma cells.

Functional CIC-2 channels contribute to cell volume homeostasis. In epithelial cells, outward movement of Cl⁻, K⁺, and other organic osmolytes, including amino acids, polyols, and/or methylamines, contributes to recovery of cell volume following swelling (1, 3, 16, 34). To evaluate the relative contribution of CIC-2 vs. other channel types to HTC cell volume homeostasis, intact individual cells were microinjected with CIC-2 antibodies to specifically inhibit native channel function (Fig. 6). In control experiments, hypotonic exposure (25% less osmolarity) induced a rapid increase in the relative area of noninjected cells (10.3 ± 1.0% increase at 1 min compared with basal values; n = 8). Subsequently, relative area decreased, reaching basal values by 8 min despite the continued presence of hypotonic buffer. Neither the magnitude of cell swelling nor recovery of cell area were different in cells within the same field injected with control antibodies (to glial fibrillary acidic protein, 0.1 µg/ml; n = 8), indicating that injection alone did not alter cellular ability to recover during hypotonic exposure. In contrast, injection of cells with CIC-2 antibodies markedly attenuated volume regulation. As shown in Fig. 6, swelling was more rapid in CIC-2 antibody-injected cells (0.1 µg/ml) by 30 s of hypotonic challenge (% area increase 7.6 ± 1.2 and 3.7 ± 0.7 for injected and noninjected cells, respectively; n = 8 for each, P = 0.02). Although noninjected cell area recovered rapidly toward baseline by 8 min, there was no significant recovery in cells injected with antibodies to CIC-2 over the period analyzed (% area increase at 8 min: 9.2 ± 1.8 and 0.2 ± 1.0 for injected and noninjected cells, respectively; P = 0.0006). Thus selective inhibition of CIC-2 channel function prevents recovery of cell volume during hypotonic exposure.

calcium phosphate transfection methods. Subsequently, individual fluorescent cells were selected for patch clamp studies, and the results are shown in Fig. 4. Compared with control cells expressing GFP alone, expression of CIC-2/GFP was associated with a large increase in anion permeability. Current amplitudes measured in CIC-2/GFP-transfected cells was -1,320 ± 230 pA at -80 mV and -3,540 ± 600 pA at -160 mV (n = 7), which is >300- and 70-fold greater than -4 ± 10 and -50 ± 20 pA measured in GFP controls (n = 5, P < 0.001). CIC-2 currents were characterized by inward rectification and activation at hyperpolarizing potentials. As shown in Fig. 4, intracellular dialysis with CIC-2 antibodies (final concentration 0.005 µg/ml added to pipette solution) inhibited currents in CIC-2-expressing cells by ~75%. Currents at -160 mV decreased from -3,539 ± 595 pA in the absence of antibody (n = 7) to -884 ± 195 pA (n = 6, P < 0.01) in the presence of antibody. In contrast, cytoplasmic delivery of antibodies in a similar fashion had no effect on currents in GFP-expressing control cells lacking CIC-2 (-48 ± 19 pA at -160 mV, n = 3). Thus CIC-2 amino terminus-specific antibodies effectively inhibit CIC-2 protein function. These findings are consistent with recent methods applied using CIC-2 antibodies directed against a similar peptide regions to inhibit CIC-2 channels expressed in Sf9 insect cells (40).
suggesting that endogenous ClC-2 channels represent an important volume-sensitive anion efflux pathway in liver epithelial cells.

**DISCUSSION**

In mammalian tissues, cell volume is maintained within a narrow physiological range by adaptive mechanisms that permit rapid and precise changes in membrane K⁺, Cl⁻, and organic osmolyte permeability. The present studies of HTC cells provide evidence that ClC-2 Cl⁻ channels are functionally important in this process and therefore represent a potential site for modulation of volume-sensitive changes in cell transport and metabolism.

ClC-2 transcripts are nearly ubiquitous, and functional channels have been expressed and characterized in multiple model systems, including *Xenopus* oocytes, Sf9 insect cells, and mammalian cells (2, 10, 27, 32, 35, 40). In concert with original observations in oocytes, heterologously expressed ClC-2 currents are activated by membrane hyperpolarization and increases in cell volume and are resistant to the anion channel blocker DIDS (35). In neuronal models, a role for ClC-2 channels in preventing neuronal excitability and paradoxical GABAA-mediated excitation has also been proposed (32). In addition, ClC-2 currents expressed in *Xenopus* oocytes and airway epithelial cells respond to extracellular acidity (14, 27). Despite these different stimuli, ClC-2 currents in these different models are characterized by typical inward rectification, activation at depolarizing potentials, and resistance to DIDS. Although ClC-2 channels expressed in *Xenopus* oocytes and Sf9
insect cells have been shown to contribute to regulatory volume decrease (10, 40), the biophysical properties of these currents are different from swelling-activated anion currents described in most epithelial cells.

Liver cells are subject to substantial volume stresses as a result of high transport and metabolic capacities and exposure to large changes in the solute and hormonal composition of portal blood (11). HTC cells derived from rat hepatoma regulate their volume during osmolar challenges by activation of outwardly rectifying anion-selective currents that are characteristic of most mammalian cells. Despite the dissimilarities between volume-activated currents in native cells and ClC-2 currents in heterologous models, three complementary observations support a role for ClC-2 channels in this process.

First, both molecular and immunocytochemical evidence indicate that ClC-2 channel mRNA and protein are expressed in liver epithelia. The cDNA cloned from an HTC cell library encodes a full-length protein that is identical to the rat brain ClC-2 channel previously characterized. The coding region of the HTC ClC-2 cDNA is completely homologous to cDNAs synthesized from primary rat hepatocytes and cholangiocytes and thus represents a rat liver epithelial ClC-2 channel.

![Graph of current vs voltage](http://hwimage.org/image.png) Fig. 5. Intracellular delivery of ClC-2 antibodies inhibits volume- and ATP-activated Cl currents in HTC cells. A: average current-voltage relations of whole cell Cl currents measured in HTC cells after exposure to hypotonic buffer (I_{Cl-Swell}) are depicted. Control cells were dialyzed with heat-inactivated ClC-2 antibodies (○, Control, 0.005 μg/ml, n = 8), and current values were similar to studies using antibody-free solutions. Swelling-activated currents displayed typical outward rectification and reversal near 0 mV. Intracellular dialysis with ClC-2 antibodies (●, ClC-2 Ab, 0.005 μg/ml, n = 6) markedly inhibited current activation; these values were not significantly different from basal currents in isotonic buffer. B: in a representative recording, exposure to hypotonic buffer (as indicated by the box) caused a reversible increase in membrane ion permeability at test potentials of +0 mV and −80 mV at 10-s intervals. Inward currents at −80 mV, as shown by the downward deflection of the current trace, correspond to I_{Cl-Swell} (Control, top). After recovery in isotonic buffer, currents were also activated in the same cell after addition of extracellular ATP (10 μM, top). In contrast, intracellular dialysis with ClC-2 antibodies (ClC-2 Ab, 0.005 μg/ml, bottom) significantly inhibited Cl currents during exposure to hypotonic buffer and ATP (10 μM). C: compared with basal anion currents (−80 mV) in isotonic buffer (Basal, n = 14), large currents were activated after exposure to hypotonic buffer in cells dialyzed with heat-inactivated ClC-2 antibodies (Hypo + HI Ab, 0.005 μg/ml, n = 8). In contrast, swelling-activated currents were minimal in cells dialyzed with non-heat-inactivated ClC-2 antibodies (Hypo + Ab, 0.005 μg/ml, n = 6). Intracellular delivery of unrelated antibodies to β-galactosidase (Hypo + B-gal Ab, 5 μg/ml, n = 6) or ClC-2 antibody buffer (Hypo + Buf, n = 4) did not inhibit volume-activated currents. Extracellular ATP stimulated a large current response (ATP, 10 μM, n = 3). ATP-induced currents were also markedly inhibited by intracellular dialysis with ClC-2 antibodies (ATP + Ab, n = 3). Current is expressed as current density (pA/pF) to normalize for cell size. For A and C, values represent means ± SE.

![Graph of change in area](http://example.com/graph.png) Fig. 6. Cytoplasmic injection of ClC-2 antibodies inhibits HTC cell volume recovery. Changes in the 2-dimensional profile of individual cells were recorded during perfusion with a 25% hypotonic solution and are expressed as % change in area relative to prestimulus basal values (means ± SE). A representative experiment is shown comparing noninjected cells (Control, hatched bars, n = 8) to cells injected with amino-specific ClC-2 antibodies (ClC-2 Ab inj, black bars, n = 8). Injection of ClC-2 antibodies resulted in an enhanced cell swelling response by 30 s compared with noninjected controls (p_{top IC}<0.02), with both injected and noninjected cells reaching similar volume peaks by 1 min. Subsequently, the relative area of noninjected cells decreased towards basal values by 8 min, and values for these cells were significantly lower than ClC-2-injected cells by 2 min (p_{2min} = 0.01). In contrast to noninjected cells, there was no significant decrease in relative area for ClC-2-injected cells over the period analyzed (p_{min} = 0.03, p_{0min} = 0.0006).

![Image of a graph](http://example.com/image.png)
HTC cells, RNA transcripts were readily detectable by in situ hybridization, and immunocytochemical staining using antibodies to a unique amino terminal domain demonstrated cellular ClC-2 protein expression. With the use of these same antibodies, ClC-2 proteins of ~100 kDa were detected by Western blot in HTC cell membranes. In addition, ClC-2 proteins are detected in the same membrane fraction as Na\(^+\)-K\(^-\)-ATPase, consistent with the plasma membrane localization anticipated for a contributing role in the regulation of membrane Cl\(^-\) permeability.

Second, in the presence of DIDS to block non-CIC-2 channels (7), volume-activated Cl\(^-\) currents are still detectable in HTC cells, accounting for ~60% of current at physiological potentials. The inward rectification and DIDS resistance are consistent with the properties anticipated for functional CIC-2 channels.

Third, intracellular dialysis with antibodies raised against a unique sequence in the essential regulatory amino terminus of ClC-2 results in inhibition of volume-activated currents in HTC cells. The efficacy of these antibodies was first demonstrated by their inhibition of currents associated with ClC-2 expression in HEK-293 cells. Consequently, the same antibodies were used as a tool to determine the potential contribution of CIC-2 channels to whole cell currents in HTC cells. Intracellular delivery of ClC-2 antibodies produced an inhibition of Cl\(^-\) current activation during both cell volume increases and purinergic stimulation by extracellular ATP. These effects are likely to reflect selective inhibition of native CIC-2 channel proteins since 1) ClC-2 antibodies at similar concentrations were effective inhibitors of CIC-2 currents overexpressed in HEK-293 cells; 2) intracellular dialysis with heat-inactivated ClC-2 antibodies, unrelated antibodies, and buffer solutions did not inhibit current activation; and 3) ClC-2 antibodies recognize appropriate proteins in plasma membrane fractions, and immunostaining is specifically blocked by coinubcation with the peptide antigen.

This approach is similar to that of Xiong et al. (40), in which cytosolic perfusion with antibodies to the same regulatory region inhibited CIC-2 currents expressed in Sf9 insect cells. The strategy is based on extensive characterization of the molecular structure and function of the CIC-2 proteins by Jentsch and coworkers (12, 14). Mutations within a putative “essential” amino terminal region, which extends for ~18 amino acids starting at leucine 21, and within the cytoplasmic D7-D8 loop, induce constitutively open channels that are volume insensitive. Whether these domains regulate channel gating by a ball-and-receptor model analogous to that proposed for some K\(^+\) channels or by interaction with other proteins is still speculative. Since the antibodies used in the present studies were targeted to the amino-terminal region of interest, they may prevent the conformational changes in the channel protein associated with cell volume increases that are necessary for pore accessibility. Although further studies are required, our results are compatible with previous experimental evidence that the cytoplasmic amino-terminal domain is directly involved in regulation of channel gating.

The degree of inhibition caused by these antibodies was surprising. Although HTC cells express CIC-2-like, DIDS-resistant currents, these currents account for only a portion of the volume-sensitive conductance. Moreover, the outwardly rectifying, volume-sensitive currents observed in the absence of blockers differ from currents associated with heterologous expression of CIC-2 channels. The explanation for these discrepancies is not readily apparent. However, the differences are not likely to be a reflection of alterations in pore or gating regions intrinsic to CIC-2 since the predicted channel proteins are structurally identical.

Another possibility is that the volume-sensitive currents result from an association of ClC-2 with other as yet unidentified pore-forming or channel-associated proteins. Indeed, ClC channels, such as ClC-1 and ClC-2, are capable of forming heteromultimers with novel biophysical properties (17). Transcripts of other ClC channels are detectable in liver epithelial cells (CIC-3, -6, and -7; data not shown) (24). Expression of ClC-3 in NIH3T3 cells induces outwardly rectified volume-sensitive anion currents, (7) but coassociation with CIC-2 proteins as functional channels has not been demonstrated. Moreover, studies by Shimada et al. (28) indicate that native ClC-3 proteins in hepatocytes are primarily intracellular or localized to the smaller apical (canalicular) domain, consistent with a role in secretion but not cell volume regulation. The methods used for these studies cannot resolve these issues. However, our approach is the first designed to specifically target endogenous CIC-2 proteins in the absence of overexpression, the latter of which would be expected to favor the formation of ClC-2 homomultimers. Thus determination of the significance of these findings will require application of similar techniques to other model systems.

Despite these biophysical limitations, a strong point in favor of a functional role for ClC-2 proteins is that microinjection of intact cells with ClC-2 antibodies prevented cell volume recovery from swelling. Thus, in addition to directly modulating membrane Cl\(^-\) permeability, endogenous ClC-2 channels are likely to play a specific role in cell volume regulation. These findings are compatible with previous observations that overexpression of ClC-2 in Xenopus oocytes and Sf9 insect cells enhances regulatory volume decrease. The repertoire of channels that regulate cell volume is likely to vary from tissue to tissue, with channels unrelated to ClC-2 contributing to volume-sensitive Cl\(^-\) efflux in other cell types. CIC-3 channels, for example, represent viable candidates for I\(_{\text{Cl-Swell}}\) (7). Evidence that pI\(_{\text{Cl}}\) and the P-glycoprotein products of mdr genes modulate volume-sensitive Cl\(^-\) currents in some cell models implies that another level of regulation may involve tissue-specific expression of different channels or channel-associated proteins (22, 36).

In summary, these findings in a model mammalian cell expressing native CIC-2 proteins support a role for ClC-2 channels as an effector of volume-sensitive
changes in membrane Cl⁻ permeability and an important contributor to cell volume homeostasis. CIC-2 channel opening is also coupled to purinergic receptor stimulation by extracellular ATP, which represents a novel mechanism for channel activation. Consequently, modulation of CIC-2 channel expression or gating represents a potential target for the regulation of volume-dependent cellular transport and metabolism.

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