ClC-2 chloride channels contribute to HTC cell volume homeostasis

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Received 11 August 1999; accepted in final form 9 October 2000

ClC-2 chloride channels contribute to HTC cell volume homeostasis. Am J Physiol Gastrointest Liver Physiol 280: G344–G353, 2001.—Membrane Cl− channels play an important role in cell volume homeostasis and regulation of volume-sensitive cell transport and metabolism. Heterologous expression of ClC-2 channel cDNA leads to the appearance of swelling-activated Cl− currents, 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-ClC-2 currents were blocked by DIDS (100 μM). A cDNA closely homologous with rat brain ClC-2 was isolated from HTC cells; identical sequence was demonstrated for ClC-2 cDNAs in primary rat hepatocytes and cholangiocytes. ClC-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 ClC-2 decreased heterologous ClC-2-dependent currents expressed 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-ClC-2 currents were blocked by DIDS (100 μM). A cDNA closely homologous with rat brain ClC-2 was isolated from HTC cells; identical sequence was demonstrated for ClC-2 cDNAs in primary rat hepatocytes and cholangiocytes. ClC-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 ClC-2 decreased ClC-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 ClC-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. ClC-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 ClC-2 proteins in different model systems (2, 12, 14, 18, 27, 32, 40). Although heterologous expression of ClC-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 ClC-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 natively expressed Cl− channels encoded by ClC-2 contribute to volume-sensitive changes in membrane Cl− permeability in liver epithelia, rat liver ClC-2 cDNA, mRNA, and membrane-associated protein were identified. Selective inhibition of ClC-2 proteins by intracellular delivery with antibodies to an essential regulatory domain 1) decreased heterologous ClC-2 currents in HEK-293 cells, 2) inhibited activation of native currents in HTC hepatoma cells during in-

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increases 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 K2HPO4, 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 (Starstedt) were fixed with 4% paraformaldehyde for 12 h. In situ hybridization was performed using digoxigenin-labeled sense and antisense riboprobes directed against the amino terminal domain of CIC-2 as previously described (29).

CIC-2 antibody development and immunocytochemistry. Rabbits were immunized by standard protocols with a purified glutathione-S-transferase (GST)-CIC-2 fusion protein containing amino acids 20–68 in frame with pGEX-2T (Pharmacia). The peptide target region includes the putative essential and modulatory domains previously characterized; deletions within this region have been shown to induce open phenotypes that are either insensitive (essential domain) or sensitive (modulatory domain) to volume changes (12). Serum was depleted of GST, and antibodies were purified by affinity chromatography using a cyanoan bromide-activated Sepharose 4b affinity matrix. Antibody staining was detected by the avidin-biotin-peroxidase method as previously described (32). For control studies, cells were concomitantly with antibodies and either purified GST or GST-CIC-2 at 100 μg/ml. These antibodies were used for in situ, Western blot, whole cell patch clamp, and cell volume experiments.

Western blot analysis. Total membrane fractions were separated from the cytosolic fraction by subjecting the cell lysate to high-speed centrifugation. Briefly, cells were disrupted by agitating the cells in ice-cold 1 mM NaHCO3 for 30 min. After light homogenization, the homogenate was cleared of nuclei and large debris by centrifuging at 10,000 g for 15 min. The supernatant was subjected to centrifugation at 190,000 g for 60 min. The pellet was solubilized in 5x PAGE buffer (5% SDS, 25% sucrose, 50 mM Tris, 5 mM EDTA, 5% β-mercaptoethanol, and protease inhibitor cocktail). The supernatant was lyophilized and solubilized in an equal volume to the pellet sample. Equivolume membrane and cytosol samples were assayed for CIC-2 content by Western blotting, as previously described (6); antibody dilutions were 1:500 (primary) and 1:20,000 (secondary). Samples enriched in plasma membranes were isolated by sucrose gradient centrifugation (5).

Briefly, HTC cells were disrupted and homogenized as described above. The homogenate was then centrifuged at 27,000 g for 15 min. The pellet was resuspended in 65% sucrose and adjusted to 44% using a refractometer. This sample was overlaid with 41% sucrose and centrifuged at 190,000 g for 70 min, and the plasma membrane fraction was recovered from the pellet sample. The recovered plasma membrane fraction was then solubilized in 5x PAGE buffer and used for immunoblotting for CIC-2 and Na−-K−-ATPase.

Heterologous expression of CIC-2 in HEK-293 cells. Transfection of immortalized human embryonic kidney cells (HEK-293) grown at low density was accomplished by standard calcium phosphate precipitation methods with some modification (13, 26). In brief, cells were grown at 37°C and 5% CO2 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 (CMVIE), 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− of 145 mM (4). 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 ID 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 otherwise protocols as indicated. In the whole cell configuration, \( V_p \) 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 \( V_p = -80 \text{ mV} (E_C) \) 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 K\(_2\)HPO\(_4\), 14 mM NH\(_4\)PO\(_4\), and 45 mM KH\(_2\)PO\(_4\)) 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 DMRIRB 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 facilitate laminar flow. Images were captured using a Leica DMRIRB 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 facilitate laminar flow. Images were captured using a Leica DMRIRB 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 facilitate laminar flow.

**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 (\( I_{\text{Cl,swell}} \)) 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 (\( \lambda \text{RR} \)) 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 HTC and brain ClC-2 (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 coincubation 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.
(35). 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 160 mV and 160 mV or 120 mV to 110 mV in 20-mV increments as indicated. Under control conditions, swelling-activated currents reversed at 164 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 ± 122 pA (n = 9) to 341 ± 15 pA (n = 3, not significant) in the absence vs. presence of DIDS, and currents at 80 mV decreased from 97 ± 212 pA to 38 ± 25 pA (P < 0.01) in the absence vs. presence of DIDS. The DIDS-resistant current was inwardly rectifying and accounted for ~60% of total current over the physiological range of liver cell potentials between −20 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 of 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
Intracellular delivery of ClC-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 ClC-2 channels. Consequently, the effects of intracellular dialysis with ClC-2 antibodies on basal, swelling-activated, and ATP-sensitive whole cell Cl− currents in HTC cells were assessed (Fig. 5). Intracellular delivery of ClC-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 ClC-2 antibodies (n = 8), a similar dilution of the ClC-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 ClC-2 antibodies (n = 3, P < 0.02). These findings indicate that antibodies, which selectively target the cytoplasmic amino terminus of ClC-2 channels, inhibit volume- and ATP-dependent channel opening in rat hepatoma cells.

**Functional ClC-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 ClC-2 vs. other channel types to HTC cell volume homeostasis, intact individual cells were microinjected with ClC-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 ClC-2 antibodies markedly attenuated volume regulation. As shown in Fig. 6, swelling was more rapid in ClC-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 ClC-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 ClC-2 channel function prevents recovery of cell volume during hypotonic exposure,
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 GABA₄-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. In
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 ClC-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 ClC-2 channel proteins since 1) ClC-2 antibodies at similar concentrations were effective inhibitors of ClC-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 ClC-2 currents expressed in Sf9 insect cells. The strategy is based on extensive characterization of the molecular structure and function of the ClC-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 ClC-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 ClC-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 ClC-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 (ClC-3, -6, and -7; data not shown) (24). Expression of ClC-3 in NIH/3T3 cells induces outwardly rectified volume-sensitive anion currents, (7) but coassociation with ClC-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 ClC-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. ClC-3 channels, for example, represent viable candidates for $I_{\text{Cl-Swell}}$ (7). Evidence that pI Cln 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 ClC-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.

We would like to acknowledge J. Cuppoletti and D. Malinowska (University of Cincinnati) for their gracious help in the initiation of the liver CIC-2 cloning project and J. Schaack (UCHSC) for his advice and assistance with cell transfection experiments.

This work was supported by National Institute of Diabetes and Kidney Diseases Grants DK-46082, DK-43278, and K08-DK-02539–01 and by the Waterman Fund for Liver Research.

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