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ClC-2 Activation Modulates Regulatory Volume Decrease

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Abstract. ClC-2 belongs to a large family of chloride channels and its expression in certain cell types is associated with the appearance of swelling-activated chloride (Cl^-) currents. In the present report, we examined the hypothesis that ClC-2 plays a role in regulatory volume decrease by expressing ClC-2 in Sf9 cells using the baculovirus system. First, we showed that ClC-2 protein expression is associated with appearance of a Cl^- conductance which is activated by hypo-osmotic shock and can be distinguished from swelling-activated chloride currents endogenous to Sf9 cells on the basis of its pharmacology and specific inhibition by an anti-ClC-2 antibody. Second, we show that the rate of regulatory volume decrease is significantly enhanced in Sf9 cells expressing ClC-2 protein. Hence, our data support the hypothesis that ClC-2 is capable of mediating regulatory volume decrease.

Key words: ClC-2 — Chloride channels — Patch-clamp electrophysiology — Baculovirus expression — Anti-ClC-2 antibody — Regulatory volume decrease

Introduction

Most cells are capable of cell volume recovery following swelling induced by hypotonic shock. Regulatory volume decrease (RVD) is achieved primarily by the electrogenic efflux of cellular potassium and chloride through distinct conductance paths [15, 17]. The molecular basis for regulatory volume decrease is largely unknown. While many candidates have been proposed for the chloride conductance path which mediate RVD, there remains considerable controversy in the field [15, 17]. Further, it is conceivable that different chloride ion channels may mediate RVD in different cell types.

Two members of the ClC family of chloride channels, ClC-2 and ClC-3 have been reported to be sensitive to activation by cell swelling [4, 8, 10, 11, 18]. Whilst the structural basis for ClC-2 channel activation by hypotonic shock has been well studied, the biological role for ClC-2 activation in regulatory volume decrease has not been clearly defined. In the model proposed by Jordt et al. [11], interaction of an ''essential-region'' within the ''N''-terminus of ClC-2 and a docking site on the intracellular loop between the putative transmembrane segments 7 and 8 maintains the ClC-2 channel closed. Further, swelling may activate ClC-2 by causing dissociation of these two structures. However, it is not clear if the activation of ClC-2 by swelling then acts to regulate cell size. In the present studies, we sought to determine the role for ClC-2 activation in RVD in ClC-2-transfected Sf9 cells. As untransfected Sf9 cells can volume regulate after swelling, our task was to distinguish the specific role of ClC-2 chloride channel activity in this vital cellular function.

Materials and Methods

Production and Purification of Anti-ClC-2 Antibodies

GST-fusion proteins were used as antigens for obtaining polyclonal antibodies to ClC-2. A nucleotide sequence corresponding to amino acids 31–74 (N-terminal) was amplified from clone B12-2 (from T.J. Jentsch), ligated into pGEX-2T (Pharmacia) and sequenced. GST fusion protein expression was induced in DH5α host bacteria and purified using glutathione sepharose beads according to the protocol provided by Pharmacia. The fusion protein was then used to immunize rabbits in order to generate anti-ClC-2 antisera.

Preimmune sera was purified using a protein A column according to the manufacturers procedures (Bio-rad). Anti-ClC-2 sera was affin-

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ity purified on a column with immobilized GST-N-terminus fusion protein. This antibody, in the concentration of 1 μg/ml, could detect recombinant ClC-2 protein in 7.5 μg of total Sf9 membrane on Western analysis.

Construction and Isolation of Recombinant Baculovirus

Clone B12-2, encoding the full length cDNA of rat ClC-2 was received from Dr. Thomas Jentsch. The open reading frame (ORF) was amplified using the oligonucleotides 5′-GCTAGGATCCGAGATGGCGGC-CGCAAC-3′ and 5′-GGAATTCACTGGCACTTGTCATCA-3′, which overlap the start and stop codon respectively, of rat ClC-2 and contain BamHI and EcoRI sites respectively, for cloning purposes. The resulting amplified fragment was digested with BamHI and EcoRI, and ligated into the BglII and EcoRI sites of the baculoviral transfer vector pBlueBac4 (Invitrogen). The bulk of the ORF was then replaced with unamplified DNA from the original B12-2 clone, using the restriction sites NotI and BspEI. Regions not replaced by this procedure were sequenced to ensure that no errors were introduced during amplification. The resulting clone encodes an unambiguous ORF representing rat ClC-2 driven from the baculovirus polyhedrin promoter.

Sf9 Cell Culture

Recombinant baculovirus was produced and titred in Sf9 cells grown in Grace's insect cell culture medium as previously described [1, 12], with the following modification: Sf9 cells were cotransfected with pBlueBac4, a new generation of baculoviral transfer vector, encoding ClC-2 cDNA and linear wild type viral DNA (Bac-N-Blue DNA, Invitrogen) to produce recombinant ClC-2 containing virus.

Patch Clamp Studies of Sf9 Cells

Sf9 cell membrane currents were measured using conventional whole cell patch clamp techniques [9]. Patch-clamp electrodes were constructed from borosilicate glass capillaries (O.D. 1.5 mm, I.D. 1.18 mm) with an inner filament (WPI, Sarasota, FL) on a Narishige PP-83 patch electrode puller using the standard two-pull technique. The tip resistance was 4–10 MΩ when filled with pipette solution (*see* below for composition). Whole-cell currents were measured with the use of an Axopatch-200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and were filtered at 100 Hz with a 6-pore Bessel Filter. Sampling rate was 3.03 kHz for most data. Junction potentials were corrected, and cell capacitance and series resistance (typically 4–5 MΩ) remained uncompensated in most cells. Voltage protocols were generated using pCLAMP software (version 6.0.3, Axon Instruments) controlled by a 486 IBM PC computer via a TL-1 DMA interface (Axon Instruments) and the same software package was used for acquisition and subsequent analysis of the data. Current-voltage (*I-V*) relationships were determined in a stepwise clamp protocol. From a holding potential of −60 mV, voltage pulses of 2.98 sec were stepped from −120 to +40 mV in a 20 mV steps in most of experiments. In some experiments, a holding potential of 0 mV was employed. Bath and pipette solutions were designed to enhance the recording of Cl^- currents. In most cases, the pipette solution contained (in mM): N-Methyl-D-Glucamine (NMDG) chloride: 140, EGTA: 2, MOPS: 10, Mg-ATP: 4, GTP: 0.5 at pH 6.8 and 295 mOsm kg^{-1}. This pH, (pH = 6.8), mimics that of Grace's Sf9 cell culture medium [12]. In experiments in which the voltage activation of ClC-2 was assessed, the bath solution contained (mM): N-Methyl-D-glucamine (NMDG) chloride: 140, $CaCl_2$: 2 and MOPS: 10 at pH 6.8. For experiments in which the swelling activation was studied, the isotonic (290–295 mOsm) bath solution contained (in mM): NMDG-Cl: 70, $CaCl_2$: 2, $MgCl_2$: 2, MOPS: 10, sucrose: 100 and the pH was adjusted to pH = 6.8. Hypotonic bath solution (145–150 mOsm) was made by removing sucrose from the above bath solution while maintaining equal ionic strength and pH. Sf9 cells were subjected to hypotonic shock by perfusion of the hypotonic solution into the recording chamber with the use of a syringe pump (sp120p, WPI). The working volume of the recording chamber was set to 1.5 ml and the rate of perfusion was 1.0 ml/min. The reference electrode was a Ag-AgCl plug connected to a 2 M KCl agar bridge.

Light Scattering Assay

A 1 ml volume of Sf9 cell suspension culture containing 1×10^6 cells was centrifuged and the pelleted cells were resuspended in a cuvette with 2 ml of a sodium and potassium-free buffer containing (in mM): 140 NMDG-Cl, 10 glucose, 10 MOPS and 0.5 $CaCl_2$ at pH 6.8 (295 mOsm kg^{-1}). Mannitol was used to adjust the osmolarity so that the sodium and potassium-free buffer was identical to the cell culture medium. Osmolarity was determined with a vapor pressure osmometer (Wescor 5500, Logan, UT). The cuvette was stirred continuously and maintained at 27°C. Right angle light scattering was measured with emission and excitation wavelengths of 400 nm using a F-4000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). In most experiments, the cells were pretreated with 10 μg/ml gramicidin for 5 min prior to suspension in the NMDG-Cl containing buffer. Under these conditions, there is a chemical gradient for K^+ efflux and the addition of gramicidin ensures that anion permeability, rather than cation permeability will be limiting to the loss of salt from the cell and to cell shrinkage. To stimulate swelling-activated chloride channels, the cells were subjected to 67% hypotonic shock by rapid dilution with double-distilled, deionized water. One ml of water was added to the 2 ml of cells suspended in isotonic buffer. Then, 1 ml of the final cell suspension was removed. The initial deflection in light scattering primarily reflects a change in cell number. The subsequent gradual increase in 90° light scattering reflects cell swelling as this change is not observed if 1 ml of isotonic buffer is added instead of 1 ml water. Maximum light scattering is obtained in approximately 1 min after hypotonic shock and the rate of regulatory volume decrease assessed as the time required for 50% volume recovery to a steady baseline value.

Data Analysis

Whole cell currents were analyzed with the Clampan program in the pCLAMP package. Statistical analyses were performed using the Student's *t* test (two-tailed). Differences between two groups were considered significant with P values <0.05.

Results

Detection of ClC-2 Protein by Immunoblotting

Expression of ClC-2 in Sf9 cells, forty-eight hours after infection with the recombinant baculovirus, was detected by immunoblotting using an affinity-purified polyclonal rabbit antibody generated against a GST-fusion peptide corresponding to N-terminal (31–74) amino acids of rat

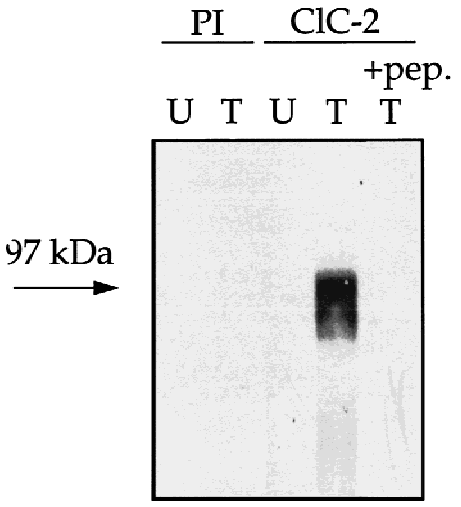

Fig. 1. Detection of ClC-2 expression in Sf9 cells by Western Blot Analysis. Expression of ClC-2 in Sf9 cells was detected by immunoblotting using affinity-purified polyclonal antibody generated against GST-fusion peptide corresponding to N-terminal amino acids (31–74) of ClC-2. The ClC-2 antibody (ClC-2) recognized a polypeptide with a molecular mass of approximately 97kD in ClC-2-transfected Sf9 cells (T), not untransfected cells (U). This signal was not recognized by preimmune IgG (PI). The specificity of the ClC-2 antibody for ClC-2 was confirmed in studies wherein the ClC-2 immunoreactivity was competed by purified peptide corresponding to the N-terminal amino acids of ClC-2 (+pep).

ClC-2 (Fig. 1). This antibody specifically recognizes ClC-2 as it detected a single band in ClC-2 transfected Sf9 cells corresponding to a molecular mass of approximately 97 kD, a value close to that predicted from the sequence for ClC-2. No signal was detected in untransfected Sf9 cells. Nonspecific IgG antibodies purified from preimmune serum failed to detect the ClC-2 band. Specificity of this antibody for ClC-2 was confirmed in peptide competition studies wherein the signal detected in Sf9 cells transfected with ClC-2 could be effectively competed with the N-terminus ClC-2 peptide used to generate the antibody.

Functional Expression of ClC-2 in Sf9 Cells

Our electrophysiological studies indicate that expression of ClC-2 in Sf9 cells confers a conductance with properties similar to those previously described in other expression systems [6, 8, 10, 16, 18]. In Fig. 2, we show the currents evoked in Sf9 cells studied in the whole cell configuration with symmetrical NMDG-Cl solutions (140 mM). A voltage-step protocol, fashioned after other studies of ClC-2, was used to assess voltage dependent currents. The cell was initially clamped at a resting potential of 0 mV and then stepped from −140 to +40 mV through 20 mV increments. Uninfected Sf9 cells (n = 13) or Sf9 cells infected with control, wild-type baculovirus (not containing ClC-2 cDNA, n = 23, *data not shown*) exhibited small, voltage-independent currents in whole-cell patch clamp studies. On the other hand, six of eight Sf9 cells infected with baculovirus containing recombinant ClC-2 cDNA for 15–20 hr, exhibited hyperpolarization-activated currents. The conductance conferred with ClC-2 expression, possessed an inwardly rectifying, current-voltage (*I-V*) relationship with a reversal potential close to zero, −5 ± 4 mV (n = 6), in symmetrical NMDGCl solutions (Fig. 2).

As detected in other expression systems, ClC-2 expressed in Sf9 cells can also be activated by cell swelling (Fig. 3). According to Thiemann et al. [18], swelling-activated anion currents conferred with ClC-2 expression in *Xenopus* oocytes are inhibited by the chloride channel blocker, 5-nitro-2,3-(phenylpropylamino)-benzoic acid (NPPB), but these currents are relatively insensitive to 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). Therefore, we assessed whether the swelling-activated anion currents evoked in ClC-2 transfected Sf9 cells could be distinguished from currents endogenous to Sf9 on the basis of a differential sensitivity to these chloride channel inhibitors. In this set of experiments, Sf9 cells were perfused via the whole-cell patch pipette using a more physiological intracellular chloride concentration of 40 mM NMDG-Cl. Superfusion of untransfected Sf9 cells with hypotonic solution (HTS, 50% isotonicity) induced an increase in membrane currents (monitored at −120 mV) within 5 min and maximal currents were achieved at 9–10 minutes (Fig. 3, n = 5). These endogenous hypotonic shock-induced Cl^- currents were reversibly blocked by 100 μM NPPB and 200 μM DIDS (Fig. 3). The chloride currents stimulated by hypotonic shock in untransfected Sf9 cells were outwardly rectifying and DIDS effectively inhibited currents evoked by both hyperpolarizing and depolarizing voltage steps.

In contrast, we found that large swelling-activated currents could be observed in ClC-2 transfected cells in the presence of 200 μM DIDS, the same concentration of inhibitor which almost completely abolished swelling-activated currents endogenous to Sf9 cells (Fig. 3 and 4, n = 15). This DIDS-insensitive Cl^- current activated by HTS in ClC-2 transfected cells was reversibly blocked by the chloride channel antagonist, NPPB (100 μM, n = 5). The difference in the *I-V* relationship for ClC-2 mediated currents shown in Fig. 2 and Fig. 3 is likely due to the difference in chloride ion gradients used in the two experiments. ClC-2 mediated currents in experiments with symmetrical chloride ion gradients show inward rectification (Fig. 2) whereas ClC-2 mediated currents in experiments with asymmetrical chloride ion gradients: Cl (in) = 40 mM and Cl (out) = 70 mM are weakly rectifying in the outward direction. Figure 3 also shows that DIDS is ineffective at blocking swelling-activated

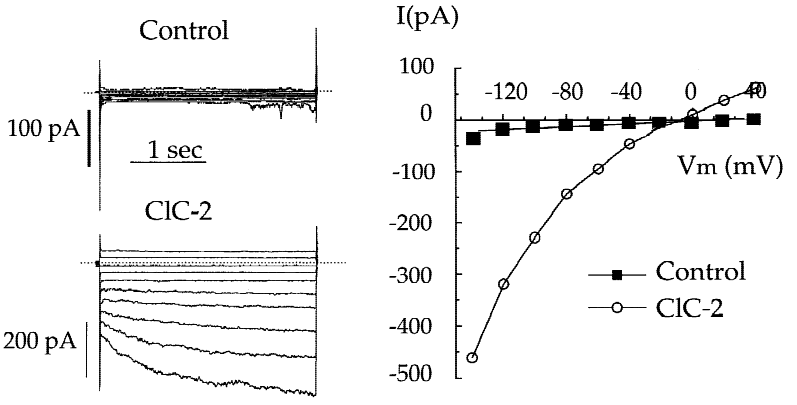

Fig. 2. ClC-2 Transfected Cells Exhibit Hyperpolarization-Activated Currents. *Left Panel:* Untransfected Sf9 cells (Control) or Sf9 cells infected with recombinant baculovirus containing ClC-2 (ClC-2) for 20–22 hr were compared using the whole cell patch clamp configuration. The patch pipette and bath solutions both contained 140 mM NMDG-Cl solutions (more details of buffers in Materials and Methods). The applied voltage clamp protocol steps the membrane potential from V_{hold} = 0 mV through −140 to +40 mV by 20 mV increments. Voltage dependent currents were detected only in the ClC-2 transfected Sf9 cells. *Right Panel:* In contrast to the current-voltage (*I-V*) relationship for the untransfected Sf9 cells, the *I-V* relationship for ClC-2 transfected cell is inwardly rectifying.

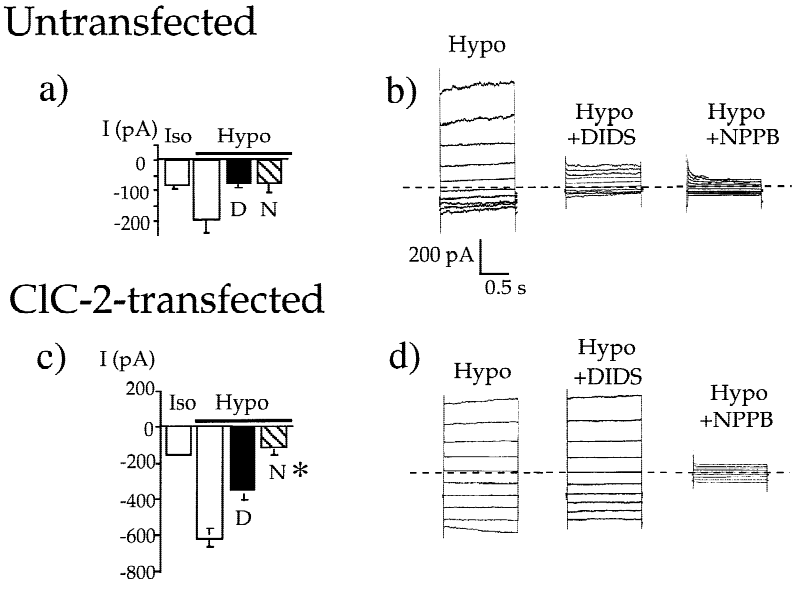

Fig. 3. Extracellular hypotonicity activates a chloride conductance endogenous to Sf9 cells. Panel *a* compares the mean currents (±SE) in isotonic (Iso) or 10 min after exposure to hypotonic (Hypo) solutions at a holding potential of −120 mV in untransfected Sf9 cells (n = 13). DIDS (D, 200 μM) significantly inhibited these currents (n = 7) as did NPPB (N, 100 μM), (n = 6). *Panel b* shows the effect of DIDS and NPPB on currents stimulated by hypotonic solutions in untransfected Sf9 cells. Voltage steps from −120 mV through to +60 mV by 20 mV increments were applied to generate these currents. The holding potential was −60 mV and the cells were exposed to asymmetrical chloride ion gradients: $[Cl^-]$ $(mM)_{in}/[Cl^-]$ $(mM)_{out}$ = 40/70 mM. Panel *c* compares the mean currents (±SE) in isotonic or hypotonic solutions at a holding potential of −120 mV in ClC-2 transfected Sf9 cells (n = 12). DIDS (D, n = 6) partially inhibited and NPPB (N, n = 6) completely inhibited swelling activated currents in ClC-2 transfected cells. Panel *d* shows the effect of DIDS and NPPB on currents stimulated by hypotonic solutions in ClC-2 transfected Sf9 cells. The effect of DIDS and NPPB on swelling-evoked currents in untransfected and ClC-2-transfected Sf9 cells are statistically significant ($P < 0.05$). The difference between the effects of DIDS and NPPB in ClC-2 transfected cells is significant ($P < 0.05$) (*).

chloride currents in ClC-2 transfected cells at all applied potentials, except at potentials more hyperpolarized than −80 mV. The experiments documented in Fig. 4 confirm that the swelling activation of DIDS-insensitive currents in ClC-2 transfected cells occurs at a normal resting potential of −60 mV.

To assess more directly whether the volume-activated chloride currents detected in ClC-2 transfected Sf9 cells were conferred by ClC-2 expression, we determined whether this function could be modified by an anti-ClC-2 antibody. As evident in Fig. 5, intracellular delivery of ClC-2 N-terminal antibody via the patch pipette, specifically inhibited swelling-activated currents associated with ClC-2 expression. Swelling-activated chloride currents in untransfected Sf9 cells were not affected by intracellular application of the ClC-2 antibody. In these experiments we found that currents were similarly activated by hypotonic shock in untransfected Sf9 cells with (440 ± 65 nA, n = 7) or without (385 ± 95 nA, n = 5) intracellular perfusion with the anti-ClC-2 antibody. On the other hand swelling-activated, DIDS-insensitive, chloride currents in ClC-2 transfected cells were significantly inhibited in cells perfused with the anti-ClC-2 antibody. This inhibitory effect of the anti-ClC-2 antibody is specific, as inoculate containing preimmune IgG antibodies (500 ng/μl) were ineffective in suppressing swelling-activated ClC-2 currents (n = 7).

As ClC-2 channel activity is stimulated by hypotonic shock, we designed experiments to assess its contribution to the volume regulatory response of the cell.

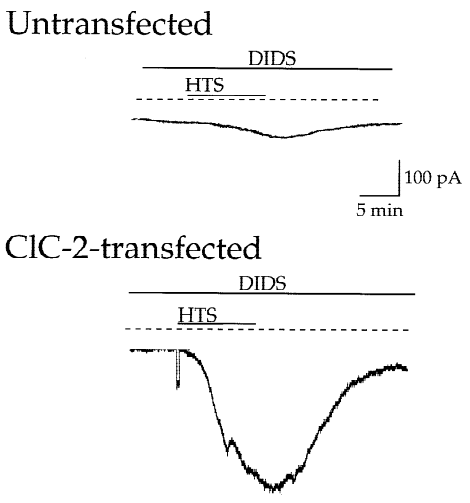

Fig. 4. Extracellular hypotonicity activates a chloride conductance endogenous to Sf9 cells. The time course for activation of currents by hypotonic solutions in untransfected and ClC-2 transfected Sf9 cells at −60 mV. Both untransfected and ClC-2 transfected cells were pretreated with DIDS (200 μM).

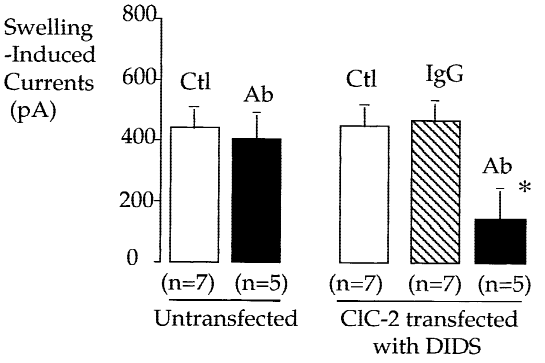

Fig. 5. Swelling-activated Cl^- currents associated with ClC-2 expression are inhibited by an anti-ClC-2 antibody. Bar graphs show mean ±SE currents evoked by hypotonic solutions (difference between currents prior to and following hypotonic shock) at +40 mV in untransfected Sf9 cells and in DIDS (200 μM)-treated ClC-2 transfected Sf9 cells. The cells were exposed to asymmetrical chloride ion gradients: $[Cl^-]$ $(mM)_{in}/[Cl^-]$ $(mM)_{out}$ = 40/70 mM. DIDS was added to ClC-2 transfected Sf9 cells in order to minimize contribution of endogenous currents. Intracellular perfusion with the anti ClC-2 polyclonal antibody (Ab) via the patch clamp pipette (solid bar) did not inhibit swelling-activated currents in untransfected cells but significantly inhibited swelling-activated currents in ClC-2-transfected cells ($P < 0.02$,*). Nonspecific preimmune IgG ($n = 7$) failed to inhibit swelling-activated currents in ClC-2 transfected cells (hatched bar).

Continuous changes in mean cell size of Sf9 cells in suspension (1×10^6 per assay) were monitored using the light scattering assay in a Beckman spectrofluorometer as described in previous experiments [7]. For these experiments, mock-transfected Sf9 cells (cells infected with wild-type baculovirus) or ClC-2 transfected Sf9

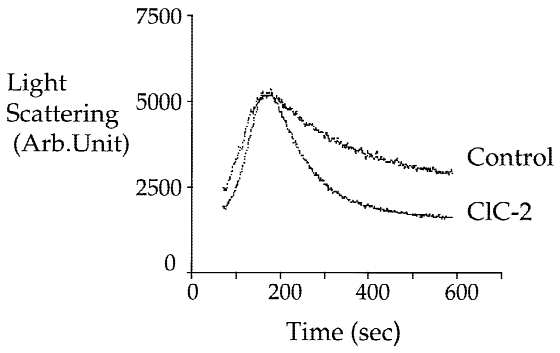

Fig. 6. ClC-2 Expression Modulates Regulatory Volume Decrease. Continuous changes in cell volume of Sf9 cells in suspension (approximately 10^6 cells/ml) were monitored by measuring 90° light scattering using a spectrofluorometer maintained at 27°C. The data traces do not intersect with zero on the time axis as there was a discontinuity while isotonic solutions were exchanged for hypotonic solutions (67% isotonicity). After solution changes were complete, peak light scattering (maximal mean cell size) was attained in both mock-transfected and ClC-2-transfected cells in approximately 50–80 sec. Decreases in light scattering, corresponding to regulatory volume decrease occurred over the subsequent 6–7 min. The light scattering trace obtained using ClC-2 transfected Sf9 cells showed a more rapid volume decrease than the trace obtained using control-transfected Sf9 cells.

cells in suspension were subjected to hypotonic shock (67% of isotonicity) and the resulting cell swelling and regulatory volume decrease (RVD) monitored. We did not use untransfected Sf9 cells as controls in these experiments as they do not swell by the same degree as transfected cells with hypotonic shock, suggesting that the virus alone affects the water permeability of the membrane. Using the Coulter counter to measure cell diameter, we determined that untransfected Sf9 cells swell by 117.5 ± 2.5% of their initial diameter (16 trials) and ClC-2 transfected cells swell by 130 ± 7% (17 trials), one minute after exposure to 67% hypotonic shock. On the other hand, both mock-transfected and ClC-2 transfected Sf9 cells swell similarly. Suspensions of mock and ClC-2 transfected Sf9 cells, i.e., reached comparable light scattering maxima, when suspended in Na^+- and K^+-free medium (NMDG replacement) (Fig. 6). In the absence of the cationophore, gramicidin (10 μg/ml), both control and ClC-2 transfected Sf9 cells shrink slowly (10 ± 1.5 min for 50% recovery, $n = 4$) due to the loss of intracellular cation, Cl^- and osmotically obliged water (*data not shown*). Addition of gramicidin (10 μg/ml), caused the mock-transfected cells to shrink more rapidly (4.9 ± 0.6 min for 50% recovery, $n = 4$) indicating that normally, the cation conductance of the Sf9 cell membrane is low. We predict that following gramicidin treatment, the rate of cell shrinkage will be limited by chloride conductance. As expected, the rate of cell shrinkage in mock-transfected cells is inhibited by both NPPB and by DIDS (Fig. 7) relative to the effect of the vehicle DMSO alone, inhibitors that we previously determined

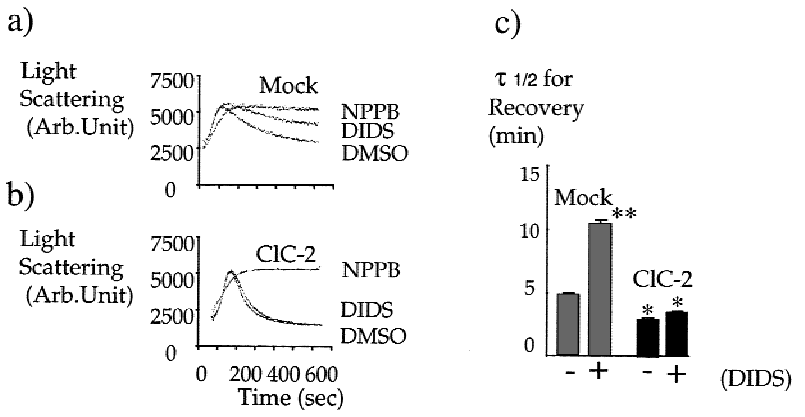

Fig. 7. ClC-2 Expression Modulates Regulatory Volume Decrease by Conferring a Novel Chloride Conductance. Panel *a* shows the light scattering traces obtained after hypotonic shock in suspensions of mock-transfected Sf9 cells and panel *b* shows light scattering traces from ClC-2-transfected Sf9 cells in the presence of chloride channel blockers; NPPB (100 μM), DIDS (200 μM) or drug vehicle (DMSO). In the bar graphs shown in panel *c*, the relative times for 50% volume decrease in mock-transfected (shaded) and ClC-2-transfected Sf9 cells (black), with and without DIDS have been shown. ClC-2 transfected Sf9 cells exhibited shorter τ1/2 times for volume recovery than mock-transfected cells (*, $P < 0.005$). Further, DIDS-treatment delayed recovery in mock-transfected but not in ClC-2-transfected Sf9 cells (**, $P < 0.001$). The mean ± SE for four-five different suspension cultures for each experimental condition has been shown.

to be effective blockers of the swelling-activated chloride conductance path endogenous to the Sf9 cell membrane. Importantly, the rate of cell volume recovery was significantly increased in ClC-2 transfected Sf9 cells to 2.2 ± 0.3 min for 50% recovery ($n = 5$) (Fig. 7). Further, RVD in ClC-2 transfected Sf9 cells was completely inhibited by NPPB whereas the inhibitory effect of DIDS on RVD in Sf9 cells expressing ClC-2 was significant, but relatively minor. These data suggest that ClC-2 expression modulates the volume regulatory response because it confers a novel chloride conductance path.

Discussion

The expression of ClC-2 in various host cell types has been shown to confer a distinct swelling-activated chloride-selective conductance path [6, 8, 10, 11, 18]. The results obtained in the present study suggest that the activation of ClC-2 by cell swelling can mediate regulatory volume decrease, a function which is clearly critical for several essential cellular activities; including intracellular pH regulation, cell proliferation and differentiation [15, 17].

First, we confirmed that ClC-2 expression in Sf9 cells leads to the appearance of swelling-activated chloride currents comparable to those reported for ClC-2 expressed in *Xenopus* oocytes, with regards to its activation by swelling and hyperpolarization, current-voltage relationship and pharmacology [6, 8, 11, 18]. Further, we showed that the swelling-activated conductance conferred with ClC-2 expression in Sf9 cells is distinct from the chloride conductance which is endogenous to the Sf9 cell membrane. Whereas endogenous, swelling-activated chloride currents in Sf9 cells were completely inhibited by pretreatment with DIDS, large chloride currents were activated by swelling in DIDS-pretreated, ClC-2 transfected cells. Furthermore, we found that the cytosolic administration of an antibody generated against the amino terminal sequence of ClC-2 completely inhibited swelling activated currents in DIDS-treated, ClC-2-transfected cells but was ineffective in blocking the DIDS-sensitive, swelling-activated currents in untransfected Sf9 cells. Clearly, ClC-2 expression confers a novel, swelling-activated chloride conductance.

We do not know the molecular basis for the endogenous, swelling-activated chloride current in Sf9 cells. As the anti-ClC-2 antibody is ineffective in blocking this conductance path and does not cross react with any proteins in untransfected Sf9 cells, the endogenous channel is unlikely to share significant sequence similarity in the region of ClC-2 to which the antibody was generated. The sequence in the N′ terminus of ClC-2, against which the antibody was raised, overlaps with a region shown to be critical for volume-sensitive gating of ClC-2 in mutagenesis studies by Gründer et al. [8]. Possibly ClC-3, another volume-activated chloride channel [4], may mediate the endogenous swelling activated chloride conductance in Sf9 cells as ClC-3 shares little sequence similarity with ClC-2 at the N terminus (1–70 aa's), less than 10%. Further, both the swelling-activated conductance endogenous to the Sf9 cells and ClC-3 are outwardly rectifying and inhibited by DIDS. However, we have yet to confirm the molecular basis for the endogenous swelling-activated current in Sf9 cells.

Second, we assessed the role of ClC-2 in regulatory volume decrease (RVD). To date, the role of ClC-2 in mediating RVD has been assessed only in *Xenopus* oocytes [6]. Furukawa et al. showed that ClC-2 expressing *Xenopus* oocytes were less swollen than control oocytes following exposure to hypotonic solutions. Furthermore, oocytes expressing a mutant form of ClC-2 with constitutive activity, i.e., a form in which the N-terminus including the ''essential'' and ''modifier'' regions were truncated, swelled less than ClC-2 expressing oocytes. While the results from this ''swelling assay'' suggest

that ClC-2 expression confers regulatory volume decrease (RVD), it is difficult to separate a potential effect of ClC-2 on cell swelling from regulatory volume decrease. A decrease in the swelling response measured in ClC-2 expressing oocytes could reflect a change in the water permeability of these oocytes. In the present experiments, we found that there was no difference in the extent of swelling induced in mock-transfected and ClC-2-transfected Sf9 cells. Hence, there is no difference in the water permeability of the two groups of virus-infected cells and the degree of tension on the cell membrane or membrane associated proteins caused by 67% hypotonic shock is comparable. Despite similar swelling, the rate of volume recovery was approximately two times greater in the ClC-2 transfected cells than in the mock-transfected cells. As the increase in the rate of volume recovery with ClC-2 transfection was largely DIDS insensitive, our data argues that ClC-2 predominates in these cells to regulate volume decrease.

On the basis of our findings, we speculate that ClC-2 may contribute to regulatory volume decrease in any cell in which it is expressed in the plasma membrane. To date, there are two cell types that possess volume-regulated chloride conductance paths with biophysical features similar to those described for ClC-2 including; human intestinal T_{84} cells [2, 5] and pancreatic acinar cells [3]. The role of ClC-2 in volume regulation in these cells will depend on its expression level relative to other swelling-activated chloride channels. As recently reported by Bond et al., T_{84} cells possesses at least two distinct volume-regulated chloride conductance paths [2] one mediated by ClC-2 and the other by ICl (swell), a swelling-activated outwardly rectifying chloride current. Bond et al. [2] suggests that ICl (swell) rather than ClC-2 mediates regulatory volume decrease in T_{84} cells based on the sensitivity of RVD to specific inhibitors of ICl (swell). Possibly, ClC-2 does not contribute significantly to RVD in T_{84} cells because it is not expressed at the same levels as the channel which mediates ICl (swell). Alternatively, there may be some cell specificity to the regulatory pathways which modulate ICl (swell) and ClC-2 in T_{84} cells. Hence, before we can describe the molecular basis for volume regulation in different cell types, we must identify not only the channels which mediate ion flux but also the signaling pathways which regulate them. This paper describes one experimental system with which we plan to define the molecular basis for ClC-2 channel function and volume regulation.

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