The ClC-3 Cl\(^{-}/H^{+}\) Antiporter Becomes Uncoupled at Low Extracellular pH**

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Adenovirus expressing ClC-3 (Ad-ClC-3) induces Cl\(^{-}/H^{+}\) antiporter current (I_{ClC-3}) in HEK293 cells. The outward rectification and time dependence of I_{ClC-3} closely resemble an endogenous HEK293 cell acid-activated Cl\(^{-}\) current (I_{ClC-acid}) seen at extracellular pH \(\leq 5.5\). I_{ClC-acid} was present in smooth muscle cells from wild-type but not ClC-3 null mice. We therefore sought to determine whether these currents were related. I_{ClC-acid} was larger in cells expressing Ad-ClC-3. Protons shifted the reversal potential \((E_{\text{rev}})\) of I_{ClC-3} between pH 8.2 and 6.2, but not pH 6.2 and 5.2, suggesting that Cl\(^{-}\) and H\(^{+}\) transport become uncoupled at low pH. At pH 4.0 \(E_{\text{rev}}\) was completely Cl\(^{-}\) dependent (55.8 ± 2.3 mV/decade). Several findings linked ClC-3 with native I_{ClC-acid}: 1) RNA interference directed at ClC-3 message reduced native I_{ClC-acid}; 2) removal of the extracellular “fast gate” (E224A) produced large currents that were pH-insensitive; and 3) wild-type I_{ClC-3} and I_{ClC-acid} were both inhibited by (2-sulfonatoethyl)methanethiosulfonate (MTSES; 10–500 \(\mu\)M)-induced alkanethiolation at exposed cysteine residues. However, a ClC-3 mutant lacking four extracellular cysteine residues (C103_P130del) was completely resistant to MTSES. C103_P130del currents were still acid-activated, but could be distinguished from wild-type I_{ClC-3} and I_{ClC-acid} by a much slower response to low pH. Thus, ClC-3 currents are activated by protons and ClC-3 protein may account for native I_{ClC-acid}. Low pH uncouples Cl\(^{-}/H^{+}\) transport so that at pH 4.0 ClC-3 behaves as an anion-selective channel. These findings have important implications for the biology of Cl\(^{-}/H^{+}\) antiporters and perhaps for pH regulation in highly acidic intracellular compartments.

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CIC-3, -4, and -5 are Cl\(^{-}/H^{+}\) antiporters that are expressed primarily in intracellular organelles. They have been proposed to provide shunt conductances for proton current generated by the vacuolar (V-type) H\(^{+}\)-ATPase (V-ATPase)\(^2\) (1). Sufficient ClC-4 and -5 protein localizes to the plasma membrane to readily allow recording of ion currents when these proteins are heterologously expressed in Xenopus oocytes (2). Unfortunately, recombinant ClC-3 currents have been much more difficult to express (2–4) and interpretation of the currents observed (5–8) has been controversial (for review see Ref. 9). ClC-3 localizes to endosomes (10) and lysosomes (11) as well as secretory vesicles of various types (12–14) suggesting that the protein cycles through the plasma membrane. Membrane localization of ClC-3 was quantified in cultured fibroblasts using recombinant protein with both extracellular and intracellular epitope tags. It had a half-life in the membrane of \(~9\) min and about 6% of total ClC-3 protein localized to the membrane at a given time (15). A proportion of ClC-3-eGFP fusion protein clearly appears to localize to the plasma membrane of ClC-3 expressing HEK293 cells (8).

We recently demonstrated that adenoviral-mediated overexpression of ClC-3 produced novel currents in HEK293 cells at neutral pH (8). These currents exhibited very steep outward rectification and time-dependent activation that was reminiscent of ClC-4 and ClC-5 (2, 16) except that the kinetics of activation were slower. Activation was also significantly slower than observed when much smaller currents were elicited by overexpression of ClC-3 plasmids in Chinese hamster ovary-K1 cells (11), although rectification properties and the effect of a specific mutation (E224A) were very similar. Changes in current amplitude and reversal potential observed in response to alterations in extracellular Cl\(^{-}\) and H\(^{+}\) concentration were consistent with ClC-3 acting as a Cl\(^{-}/H^{+}\) antiporter and deviated significantly from the behavior of an anion channel. These results supported predictions, based upon sequence homology, that like ClC-4 and ClC-5, ClC-3 would be a Cl\(^{-}/H^{+}\) antiporter (17, 18).

ClC-3 is required for proper acidification of synaptic vesicles (12), insulin granules (19), lysosomes (11), and endosomes (20) by the V-ATPase. This acidification process leads to intra-or-ganellar pH values ranging from 5.9 to 6.2 for early endosomes to 5.0–6.0 for late endosomes, and 4.6–5.5 for lysosomes (21, 22). A Cl\(^{-}\) channel is well suited to provide charge neutralization for a proton pump. However, the realization that the intracellular ClCs are Cl\(^{-}/H^{+}\) antiporters (8, 17, 18) yielded the surprising requirement that protons move out of the endosome in exchange for Cl\(^{-}\) to provide countercurrent for the V-ATPase. The 2Cl\(^{-}\) to 1H\(^{+}\) stoichiometry of this process (23, 24) makes this mechanism feasible, but has led to speculation that the primary goal of this coupled transport system may be to concentrate Cl\(^{-}\) in the compartment rather than to facilitate acidification (18, 22). This adds an unanticipated layer of complexity to understanding the contribution of CLC proteins to endosomal biology (25).

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\(^\) The abbreviations used are: V-ATPase, vacuolar-type hydrogen-ATPase; eGFP, enhanced green florescent protein; \(E_{\text{rev}}\), reversal potential; I_{ClC-acid}, induced chloride current; I_{ClC-3}, ClC-3 current; VSM, vascular smooth muscle; HEK, human embryonic kidney; RNAi, RNA interference; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; Ad, adenovirus; MTSES, (2-sulfonatoethyl)methanethiosulfonate.

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Following endocytosis, the extracellular surface of plasma membrane proteins faces the vesicular lumen. Lowering extracellular pH to a degree commonly encountered in intracellular vesicles activates an outwardly rectifying chloride current (ICl_{acid}) in HEK293 cells (26, 27), astrocytes (26), Sertoli cells (28), ventricular myocytes (29), and endothelial cells (30). Following endocytosis, this conductance could theoretically provide shunt current for the V-ATPase. The protein responsible for ICl_{acid} has not been identified. It was proposed that it might represent an altered manifestation of the volume-activated Cl current (swelling induced anion current, ICl_{swell}) (27), however, a detailed analysis of ICl_{acid} in HEK293 cells revealed that these two currents are clearly distinct. They differ with respect to anion selectivity, pharmacology, and kinetics (26).

The time dependence and rectification properties of the CIC-3 current are highly reminiscent of native ICl_{acid} in HEK293 cells (26, 27). This raises the possibility that CIC-3 overexpression somehow activates this endogenous current. At first glance it is difficult to imagine that CIC-3 itself produces ICl_{acid} because enhancement of the current at positive holding potentials in response to an increase in the extracellular proton concentration is inconsistent with the behavior of a Cl^−/H^+ antiporter. CIC-3 would have to switch its behavior from that of a Cl^−/H^+ antiporter to an anion channel. Whereas this may seem odd, there is ample evidence that the degree of coupling between Cl^− and H^+ transport by ClC proteins is not fixed, and is less tight in the presence of non-halide anions (23, 31, 32). Furthermore, Cl^− flux through the bacterial protein CLCec-1 varies according to the protonation state of the extracellular glutamate side chain “fast gate” (Glu^{148} of CIC-ec1, Glu^{224} of CIC-3). Neutralization or removal of this negatively charged side chain, which competes with Cl^− for an extracellular anion binding site, uncouples Cl^−/H^+ exchange and yields a protein that only transports Cl^− (33–35). Therefore extracellular H^+ might alter the protonation state and hence the function of this gate. Given the longstanding controversy regarding the nature of CIC-3 currents, we felt it was important to define the relationship between I_{CIC-3} and ICl_{acid}. Our findings demonstrate that CIC-3 expression produces an acid-activated anion current that is indistinguishable from native ICl_{acid}. These results have important implications for the mechanism of ion transport by CIC-3 and potentially for the control of pH in intracellular compartments.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Modification of CIC-3 Expression**—Aortic vascular smooth muscle (VSM) cells were prepared from CIC-3 wild-type and null mice using previously established methods (10, 36). VSM cells were grown in a high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 44 mM NaHCO_{3}, 25 mM HEPES (pH 7.4), 1% minimal essential medium vitamins, and 1% non-essential amino acids (Invitrogen). Aortic VSM cells were allowed to reach no more than 70% confluence prior to use and were passaged less than 10 times. HEK293 cells (HEK293T, adenoviral propagation resistant) were obtained from the American Tissue Culture Collection and maintained in 5% CO_{2} in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Atlanta Biological).

Modification of CIC-3 expression was achieved as previously described (8). Briefly, expression of the short N-terminal isoform of human CIC-3 (Swiss-Prot Q9R279) was driven by the cytomegalovirus promoter in a bicistronic adenoviral construct that also expressed eGFP behind the Rous sarcoma virus promoter. Adenovirus expressing only eGFP was used as a control. The adenovirus shuttle plasmid pacAd5 cytomegalovirus was used for expression of CIC-3 mutants. Site-directed mutations in the pacAd5 clones were created using the QuikChange II kit from Stratagene (La Jolla, CA).

In all experiments in which plasmids were used to express wild-type or mutant CIC-3, RNAi directed against the 5′-untranslated region of endogenous CIC-3 (Dicer duplex system, IDT Inc., Coralville, IA) was used as described previously (8) to suppress endogenous CIC-3 protein levels. Targeted sequences were: 5′-CAUCUGUUUCACAAUAGAGAACCAGCU−3′ or 5′-GAGUAAAGUGGAGGCUUUCACCCAGGA-3′. These sequences do not appear in the CIC-3 clones. This approach allows plasmid expression in the setting of very reduced levels of native CIC-3. Control, scrambled RNAi duplex was provided by the manufacturer. Cells grown in 2% serum were exposed to RNAi at concentrations of 25–50 nM in the presence of Oligo-fectamine (5 μl/ml) and studied 72–96 h later.

**Electrophysiology**—Currents were measured at room temperature (22 °C) using either standard whole cell voltage-clamp methods (37) or perforated patch recording (38) performed with an Axopatch 200B amplifier driven by pClamp 9 software ( Molecular Devices Corporation, Sunnyvale, CA). Pipette resistance was 3–5 mehmohms. Pipette and whole cell capacitance and series resistance compensations were done prior to recording. Currents for 1/V relationships were elicited from a holding potential of −40 mV to test potentials from −100 to +100 mV in 20-mV increments. Test pulses were 1 s in duration delivered at 3-s intervals. Currents were sampled at 5 kHz and filtered at 1 kHz. Ramp protocols for determination of reversal potential (E_{rev}) went from −40 to +40 mV over 900 ms. 10 ramp currents acquired 2 s apart were averaged for each data point.

HEPES-buffered bath solutions contained (mM): NaCl 120, MgCl_{2} 2.5, CaCl_{2} 2.5, HEPES 10, glucose 5.5, pH 4.0–7.35, with NaOH or HCl. 10 mM MES or MOPS were used as alternative buffers in place of HEPES for some experiments. Citrate-buffered solutions contained (mM): NaCl 120, MgCl_{2} 5.0, CaCl_{2} 5.0, sodium citrate 5, glucose 5.5, pH 4.0–7.35, with NaOH or HCl. NaCl was sometimes substituted with 120 mM NaSCN. Osmolality of all solutions was determined using a μ OSMETTE osmometer and all extracellular solutions were titrated to 300 mosmol using 1 mM mannitol. The 435 mM Cl bath solution contained (in mM): NaCl 425, HEPES 10, MgCl_{2} 2.5, CaCl_{2} 2.5, glucose 5.5. The 42 mM Cl− bath solution contained (in mM): NaCl 32, CaCl_{2} 2.5, MgCl_{2} 2.5, HEPES 10, and glucose 240. Liquid junction potentials were minimized by using 3 mM KCl agar bridges and calculated using pClamp 9.0 to be 8.4, 5.0, and 2.4 mV for 435, 130, and 42 mM Cl− buffers, respectively. Pipette solutions for standard whole cell recordings contained (mM): CsCl 120, triethanolamine-Cl 4, EGTA 5, CaCl_{2} 1.187,
MgCl₂ 2, Na-ATP 5, Na-GTP 0.5, HEPES 10, pH 7.2, with CsOH, osmolality at 290 mosmol, and free [Ca²⁺] = 55 nM (calculated using WEBMAXC). The pipette solution for perforated patch recording contained (mM): CsCl 125, MgCl₂ 2.5, HEPES 10, pH 7.2, with CsOH (liquid junction potential 4.4 mV using standard bath solution). Amphotericin was dissolved in dimethyl sulfoxide at a concentration of 60 mg/ml and then 20 μl of this solution was mixed with 5 ml of pipette solution by vortexing. Currents were normalized to cell membrane capacitance and expressed as current density (pA/pF). Identification of GFP positive cells was done immediately prior to cell selection using a fluorescence-equipped inverted microscope (Zeiss Axiovert 25). All chemicals were obtained from Sigma, except for sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES), which was purchased from Anatrace (Maumee, OH). MTSES and dithiothreitol (DTT) were dissolved in dimethyl sulfoxide and diluted at least 1:1000 into experimental solutions.

**Data Analysis and Statistics**—Unless otherwise indicated, steady state currents (measured 5 ms prior to the end of the depolarizing pulse) were used to calculate current-voltage relationships using pClamp 9 software. \( E_{\text{rev}} \) was estimated by either 1) analysis of I/V relationships by fitting a straight line \( (y = mx + b) \) between consecutive steady state current data points that were immediately negative to, and positive to, zero current density, and determining the voltage at which this line crossed 0 pA, or 2) direct measurement of the voltage at which ramp currents crossed 0 pA. Reversal potential comparisons were made after correcting for liquid junction potentials. Activation time courses were fit using one or two exponentials and time constants of activation were determined using the Clampfit module of pClamp 9. Results are expressed as mean ± S.E. Unpaired Student’s \( t \) tests were used to determine statistical significance.

**RESULTS**

**IClacid in Wild-type and CIC-3 Null VSM Cells**—As an initial approach to determining if I_CIC-3 and IClacid are related, we measured currents activated by pH 4.0 in wild-type and CIC-3 null cultured vascular smooth muscle cells (10, 36). IClacid has not previously been characterized in this cell type. Minimal current was observed at pH 7.35 in all cells. Following exposure to pH 4.0, outwardly rectifying currents with time-dependent activation were observed in CIC-3 wild-type but not null VSM cells (Fig. 1). These results encouraged us to pursue a detailed investigation of these two currents in HEK293 cells where others have carefully characterized IClacid (26), and we have studied I_CIC-3 (8).

**Wild-type CIC-3 Currents Are pH-dependent**—HEK293 cells expressing eGFP alone had no significant current at an extracellular pH of 7.35 (Fig. 2A). Reduction of pH to 4.0 elicited outwardly rectifying currents that underwent time-dependent activation (Fig. 2B). The current-voltage relationships for these currents are shown in Fig. 2E. These currents were very similar to the IClacid that was previously characterized under identical conditions (26, 27). Ad-CIC-3-infected HEK293 cells express more CIC-3 protein than either non-infected or Ad-eGFP-infected cells (8). Ad-CIC-3 elicited currents at pH 7.35 as previously described. These currents were smaller than native IClacid at pH 4.0 (Fig. 2, B compared with C and Ref. 8). CIC-3 overexpression greatly enhanced the magnitude of the currents elicited by pH 4.0 (Fig. 2, D and E). The activation time course of IClacid was well fit by 2 exponentials in both control and CIC-3 overexpressing cells. The two time constants at pH 4.0 (Table 1) did not differ significantly with CIC-3 expression and also were not different from the previously published time constants for overexpressed CIC-3 measured at pH 7.35 (8). To ensure that the effect of low pH was not an artifact associated with the internal dialysis process after membrane rupture, several cells were recorded using the amphotericin B-perforated patch technique. The currents elicited by pH 4.0 were indistinguishable from those obtained using dialyzed cells (n = 4, data not shown).

To make direct comparisons to IClacid currents recorded by other groups (26, 27), HEPES was initially used as the bath solution buffer. However, HEPES has virtually no buffering capacity at pH 4.0 (\( pK_b = 7.55 \)). We therefore evaluated the impact of buffer strength on IClacid by comparing currents induced by pH 4.0 in bath solutions containing 10 mM HEPES, 10 mM MOPS
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**TABLE 1**

|          | Slow τ (ms) | Fast τ (ms) | n  |
|----------|-------------|-------------|----|
| Ad-ClC-3 (pH 7.35) | 228.2 ± 53.4 | 16.1 ± 3.3 | 14 |
| Ad-ClC-3 (pH 4.0)  | 193.3 ± 25.5 | 19.3 ± 3.5 | 16 |
| Ad-eGFP (pH 4.0)   | 248.9 ± 47.3 | 18.4 ± 4.4 | 14 |
| E224A (pH 7.35)    | 218.8 ± 49.5 |             | 11 |
| C166_P188del (pH 4.0) | 196.5 ± 20.0 | 17.9 ± 1.6 | 7  |

These experiments are shown in Fig. 3, C and D. These data are consistent with the reported pH sensitivity of ICl_{acid} in HEK293 cells (26, 27). For clarity, examples of the response of individual Ad-ClC-3-expressing cells to both modest acidification and very low pH are provided in supplemental Fig. S2, A and B, and C, which summarize the magnitude of current recorded in Ad-ClC-3 expressing cells exposed to a range of extracellular pH values from 4.0 to 7.35. Activation of current at very low pH cannot be attributed to enhanced Cl^−/H^+ antiport. Intracellular pH was held constant at 7.2 in these experiments, thus increases in extracellular proton concentration should resist proton efflux, which is required for the Cl^−/H^+ exchange current at positive voltages.

**Low pH Uncouples Cl^− and H^+ Transport**—Proton dependence of the CIC-3 current was demonstrated previously by shifts in reversal potential when the proton gradient was altered (8). I_{CIC-3} activation at low pH would seem to require a transition from antiporter to “channel,” or at least to a much less tightly coupled antiporter. Changes in current magnitude are not a useful indicator of where this transition occurs, as protons may affect not only coupling but other parameters such as gating or conductance. We therefore compared the proton dependence of E_{rev} across a wide pH range. We hypothesized that if Cl^− and H^+ transport became uncoupled, further elevations in extracellular proton concentration would no longer affect E_{rev}.

We measured the currents elicited by ramp depolarizations from −40 to +40 mV in cells overexpressing CIC-3 over a pH range from 5.2 to 8.2 (Fig. 4). Only data from cells that were recorded at all pH values were included. As a control for non-specific effects of pH we performed these experiments using either Cl^− or SCN^− as the extracellular anion. CIC antiporters become much less coupled in the presence of non-halide anions (23, 32, 35). Thus the E_{rev} of SCN^− currents would be expected to be much less sensitive to pH. We observed a shift in E_{rev} of I_{CIC-3} in NaCl buffers from −9.3 ± 1.25 to −20.0 ± 3.3 mV as pH increased from 7.2 to 8.2. The magnitude of this change is smaller than that predicted by the 2Cl^−:1H^+ stoichiometry (20 mV), but very similar to the magnitude of the shift previously observed upon alkalization (8). Acidification from pH 7.2 to 6.2 produced a much smaller, but still significant shift in reversal potential to −6.7 ± 0.9 mV. This change is smaller than that previously observed (8) when pH was shifted from 7.35 to 6.35, suggesting that a reduction in coupling is already occurring at pH < 6.35. Interpretation of shifts in E_{rev} over the pH range where the degree of coupling is changing (between pH 6 and 7) is difficult. However, it is very clear that further acidification from pH 6.2 to 5.2 did not cause a significant shift in E_{rev} (−6.7 ± 0.9 to −5.2 ± 0.57 mV). This suggests that at pH 6.2 uncoupling is nearly complete. Of note, no significant differences in estimated reversal potential were observed when comparing ramp protocols with estimates based upon extrapolation of IV plots. For example, the average value obtained at pH 8.2 using ramps (20.0 ± 3.3) agreed well with that obtained previously by extrapolation (−18.9 ± 1.6 mV) (8). In addition, E_{rev} values estimated by I-V analysis of data in this study at pH 4.0 (−6.0 ± 1.0 mV), 5.0 (−6.5 ± 1.0 mV), 5.5 (−5.0 ± 1.5 mV), and
6.0 (−5.8 ± 2.0 mV, n = 5 for all groups) did not differ from that estimated by ramps at pH 5.2 and 6.2.

SCN− not only reduces coupling of Cl− and H+ transport in ClC-4 and ClC-5, but also produces substantially larger currents than does Cl− under otherwise identical conditions. We observed a similar effect on IClC-3 (Fig. 4A). Currents were larger in the presence of extracellular SCN−, however, no significant changes in $E_{rev}$ were observed across the entire pH range tested. Ramp currents obtained from the same cell (Fig. 4B) clearly demonstrates the difference in the effect of pH on IClC-3 when the dominant extracellular anion is changed from Cl− to SCN−.

Native IClacid has previously been shown to be fully Cl−-dependent (26). We also assessed the Cl− dependence of IClC-3. Reversal potentials of currents recorded at pH 4.0 were shifted in response to changes in extracellular Cl− ($−29.7 ± 1.9$ at 435 mM, $−7.2 ± 1.4$ at 130 mM, and $26.1 ± 1.4$ at 42 mM) in a manner consistent with a completely chloride-selective channel. The calculated slope for reversal potential/decade change in [Cl−]o was $55.2 ± 2.3$ mV, very close to the 58.4 mV/decade Nernstian prediction for a chloride-selective channel at 22 °C.

RNAi Directed at ClC-3 Inhibits IClacid—We next attempted to reproduce the impact of knocking out ClC-3 in VSM cells by using RNAi in HEK293 cells. We have previously shown that RNAi targeted to the 5′-untranslated region of Clcn3 mRNA dramatically reduces the abundance of native ClC-3 protein in HEK293 cells (8). Clcn3-targeted RNAi treatment potently suppressed native IClacid, whereas scrambled control RNAi was without effect (Fig. 5). RNAi treatment also provided cells in which it was possible to express ClC-3 plasmids with no background of native ClC-3. These plasmids lacked the 5′-untranslated region sequence targeted by the RNAi, thus allowing reconstitution of ClC-3 protein expression as previously documented (8). The response to pH 4.0 was partially reconstituted by transfection with wild-type ClC-3 plasmid.

Neutralization of Glutamate 224 (E224A) Removes pH Sensitivity—Mutation of the extracellular glutamate fast gate in the exchanger-type ClCs, ClC-4, ClC-5 (17, 18), ClC-ec1 (39), and ClC-3 (8), removes sensitivity to extracellular protons and uncouples anion current from proton transport. In addition, this mutation dramatically alters rectification of ClC-3, -4, and -5 currents (8, 11, 17, 18). ClC-3 plasmid lacking the fast gate (E224A) was expressed in HEK293 cells that had been treated with RNAi as described above to remove native IClacid. E224A currents have previously been shown to be completely Cl−-dependent and uncoupled from proton transport (8). These currents also lack the sharp rectification of wild-type ClC-3. E224A mutant currents were present at pH 7.35 and were not further activated by extracellular acidification (Fig. 6). The I-V plot compares currents elicited at pH 4 and with previously published currents elicited in the same cells at pH 7.35 (see Fig. 8 of 8). Note that unlike Ad-ClC-3, plasmid-mediated overexpression of wild-type ClC-3 yielded virtually no current at pH 7.35 (2.3 ± 0.7 pA/pF at +100 mV, Fig. 5, open triangles). In contrast, the E224A plasmid yielded a significant current at neutral pH (48.3 ± 2.7 pA/pF, Fig. 6, open circles). The current density of the E224A mutant was unchanged at pH 4.0 (47.3 ± 1.1 pA/pF, Fig. 6, closed circles) and was still larger than the current
induced by the wild-type plasmid at pH 4.0 (27.4 ± 7.5 pA/pF, Fig. 5, closed triangles). This suggests that either plasma membrane expression of the E224A mutant is higher than wild-type, or that acidity does not activate the current as potently as does mutation of the fast gate. The activation time constants for the E224A mutant currents are shown in Table 1. Unlike ICl\textsubscript{acid} or wild-type I\textsubscript{ClC-3}, these currents were well fit by a single time constant that was not significantly different from the slow time constant measured for I\textsubscript{Clacid} and I\textsubscript{ClC-3}.

**The Effect of MTSES on I\textsubscript{ClC-3} and I\textsubscript{Clacid}**—The experiments described above provide circumstantial evidence that ClC-3 is an ICl\textsubscript{acid}. We felt that more direct evidence might be obtained by employing cysteine-scanning mutagenesis (40, 41). This method takes advantage of the fact that chemical modification of extracellular cysteine residues can alter protein function, including ion channel activity (42). The presence of a cysteine residue in a critical position that is accessible to alkanethiolation by MTS reagents can create a highly specific inhibitor of an ion current. As a prelude to the introduction of cysteine mutations in the pore region of ClC-3, we first studied the effect of the negatively charged reagent, MTSES, on wild-type Ad-ClC-3 expressing HEK293 cells. Surprisingly, a 5-min incubation in 100 μM MTSES completely inhibited I\textsubscript{ClC-3} (Fig. 7). This suggested that an endogenous cysteine in ClC-3 was positioned such that modification by MTS impaired ion transport. Alkanethiolation of cysteine by MTS reagents is reversed under highly reduced conditions, thus specific effects of these reagents should be reversed by DTT. A 5-min incubation in 500 μM DTT restored I\textsubscript{ClC-3}.

We next determined if MTSES affected native ICl\textsubscript{acid} by pre-incubating control HEK293 cells in MTSES for 5 min prior to exposure to pH 4.0. Fig. 8A demonstrates that MTSES caused a dose-dependent decrease in the amplitude of ICl\textsubscript{acid}. At a test potential of +100 mV, MTSES inhibited ICl\textsubscript{acid} by 95, 94, and 69% at 500, 100, and 10 μM concentrations, respectively. The effect of MTSES was not reversed by 10 min of washing with pH 7.35 buffer. Thus the effect of MTSES was virtually complete even though this reagent was no longer present when pH was lowered to 4.0. The effect of MTSES was reversed by a subsequent 5-min incubation in 500 μM DTT (Fig. 8, B and C). Incubation in DTT was performed at pH 7.35. This observation strongly suggests that the mechanism of ICl\textsubscript{acid} activation is not related to recruitment of new protein to the plasma membrane.

Because MTS inhibited both I\textsubscript{Clacid} and I\textsubscript{ClC-3} rather than inserting cysteine residues into ClC-3 we removed them. A ClC-3 mutant (C103_P130del) was created that lacks part of the
extracellular loop that connects transmembrane domains B and C (43). This region contains 4 cysteine residues. Fig. 9 shows that even a high concentration of MTSES (500 μM) did not block the acid-induced current in cells expressing the mutant plasmid. The activation time constants of this current (Fig. 9B) were not significantly different from those of ICl_{acid} or ICl_{C-3} (Table 1). However, the time course of the appearance of ICl_{acid} after exposure to pH 4.0, and the return of current amplitude to baseline after changing back to pH 7.35 were dramatically altered in the Cys^{103-Pro^{130}} mutant. Fig. 10 compares the time course of ICl_{acid} activation and washout of this effect between control HEK293 cells and cells expressing the C103_P130del mutant upon brief exposure to pH 4.0 bath solutions. The time to maximal ICl_{acid} activation was 39.2 ± 3.3 s for controls and 98 ± 6.0 s for mutant expressing cells (n = 5, p < 0.05). The time to half-maximal current levels after pH 4.0 washout was 34.2 ± 1.5 s for control cells and 304 ± 24.3 s for cells expressing the mutant (n = 6, p < 0.05). The observed changes in MTSES sensitivity and acid activation time course induced by this deletion mutation unequivocally identify these currents as ICl_{acid}. The ability of low pH to activate the mutant demonstrates that CIC-3 is indeed an ICl_{acid}.

**DISCUSSION**

CIC-3 Is an ICl_{acid}—This investigation was undertaken because significant similarity was noted between the ion currents induced by Ad-CIC-3 at neutral pH in HEK293 cells (ICl_{C-3}) and an endogenous acid-induced anion current that was previously characterized in these cells (ICl_{acid}) (26, 27). HEK293 cells express native CIC-3 protein (8). We therefore wished to determine whether CIC-3 expression is required for activation of endogenous ICl_{acid} or alternatively, if CIC-3 protein is responsible for ICl_{acid}. The observation that ICl_{acid} is present in wild-type but not CIC-3 null murine VSM cells provided an initial hint that a relationship existed between the two currents. Additional results supporting this conclusion included: 1) ICl_{acid} is much larger in HEK293 cells overexpressing CIC-3; 2) the two time constants of activation for ICl_{C-3} and ICl_{acid} are indistinguishable; and 3) ICl_{acid} is absent in cells treated with RNAi directed against CIC-3. Although these data seem to link ICl_{C-3} and ICl_{acid}, they do not establish whether this relationship is direct or indirect. The first clue that CIC-3 is itself an ICl_{acid} was provided by the behavior of the E224A mutant. This mutant lacks the extracellular “gating” glutamate. Unlike with wild-type plasmid expression, currents were present at pH 7.35 and were easily identified by their markedly reduced rectification. Reduction of extracellular pH to 4.0 did not enhance the mutant current. E224A currents also lacked the faster of the two time constants of activation that characterize ICl_{C-3} and ICl_{acid}. The strongest evidence that CIC-3 is an ICl_{acid} was provided via the use of the negatively charged methanethiosulfonate reagent MTSES, which reacts with thiol groups and modifies accessible cysteine residues. Both wild-type ICl_{C-3} and native ICl_{acid} were inhibited by MTSES. A CIC-3 mutant lacking four extracel-
lular cysteine residues (C103_P130del) was identified by its resistance to inhibition by MTSES and slower response to low pH. However, C103_P130del ClC-3 was still responsive to protons and retained the same activation time constants as wild-type IClC-3 and native IClacid. Thus, we conclude that ClC-3 currents are activated by low pH.

The historical difficulty of expressing recombinant IClC-3 (2–4) suggests that in Xenopus oocytes and in many other cell types, the percentage of ClC-3 that localizes to the plasma membrane is not sufficient to produce identifiable currents, at least not at neutral pH. This percentage may differ between resting and stimulated cells but this has not been routinely tested. We observed no significant current at pH 7.35 in HEK293 cells expressing wild-type ClC-3 plasmid (Fig. 5). However, robust currents are induced by Ad-ClC-3 at this pH (see Ref. 8 and Fig. 2C). This difference may be related to the increased abundance of recombinant protein, or the virus may affect the percentage of protein that localizes to the plasma membrane. In either case, the rapid provocation of current by low pH at room temperature, and the ability of prior exposure to MTSES to completely inhibit IClacid, makes rapid membrane insertion of ClC-3 in response to extracellular protons very unlikely.

**Activation of ClC Current by Protons**—Activation by extracellular protons is a well documented property of ClC-0, ClC-1, and ClC-2 channel-type ClC currents (44–46). Fast gating of all ClC pores has been proposed to be mediated by the same extracellular glutamate (Glu232 of ClC-1, Glu224 of ClC-3). It is thought that in the closed state the charged COO⁻ side chain of this amino acid competes with Cl⁻ for access to its extracellular binding site. Protonation to COOH causes this side chain to swing out of the way and allows Cl⁻ to interact (43). If this COOH is accessible to the extracellular aqueous environment, extracellular pH might be anticipated to impact its protonation state. In exchanger-type ClC proteins, neutralization or deletion mutations of this glutamate

FIGURE 8. **MTSES inhibits native IClacid.** A, I/V relationships for currents recorded at pH 4.0 following incubation in varying concentrations of MTSES at pH 7.35. MTSES was washed off as the bath was changed to pH 4.0. B, reversal of the effect of MTSES (500 μM) by DTT (500 μM). Representative raw currents for these experiments are shown in C. The sequence of bath solution exchanges used to create the I/V relationships in B was as follows: 1) control, pH 7.35; 2) effect of reducing pH to 4.0; 3) return to pH 7.35 and incubation in MTSES; 4) change to pH 4 with wash-off of MTSES; 5) return to pH 7.35 and incubation in DTT; and 6) change to pH 4 after DTT. Error bars indicate ± S.E.

FIGURE 9. **The effect of MTSES (500 μM) on cells expressing the C161_P188del plasmid.** RNAi was used to suppress endogenous ClC-3. Raw current traces from a representative HEK293 cell expressing the C161_P188del mutant are shown at pH 7.35 during incubation in MTSES at pH 7.35 (A) and after changing to pH 4.0 (B). The I/V relationship for these experiments are shown in C. Error bars indicate ± S.E.
render transport activity pH-insensitive and uncouples Cl\(^-\) from H\(^+\) transport (2, 39, 47).

Mutation of the fast gate also alters rectification of both channel (48) and exchanger-type CLCs (2, 33, 39, 47) including E224A ClC-3 (8, 11). The mechanism of the prominent rectification of the mammalian CLC exchangers is not established. The loss of rectification in response to neutralization of the gating glutamate suggests that rectification may result from the voltage dependence of gating. It has also been proposed that protons may much more readily reach the fast gate glutamate from the intracellular side and must be moved there against an energy barrier by voltage (35).

Comparing wild-type ClC-3 plasmid-induced current density at pH 7.35 (Fig. 5, open triangles) to E224A current density at the same pH (Fig. 6, open circles) suggests that there is an increase in transport in the absence of the fast gate. The mutant current is much larger than wild-type and is very similar in magnitude to the wild-type current at pH 4.0 (Fig. 5, closed triangles). E224A currents are not further activated by low pH. Thus, both extracellular protons and the E224A mutation activate the ClC-3 current. Both I_{ClC-3} at pH 4.0 and E224A currents are completely Cl\(^-\) dependent, as is native I_{Cl\text{acid}} (26). However, two properties clearly distinguish E224A currents from wild-type I_{ClC-3} and I_{Cl\text{acid}}. First, whereas both wild-type I_{ClC-3} and I_{Cl\text{acid}} are best fit by two time constants, E224A currents are well fit by a single constant that is not significantly different from the slower of the two wild-type constants (Table 1). This is consistent with the fast gate accounting for the shorter of the two time constants. Second, rectification is dramatically reduced in the E224A mutant, but is unaltered by extracellular protons. Thus, whereas low extracellular pH could impact the protonation state of Glu\(^{224}\), the effect of low pH on current is not readily explained solely by this mechanism. Extracellular protons may also exert acid-induced changes in the conformation of other regions of the extracellular face of ClC-3.

**MTSES Identifies ClC-3 Current**—Both I_{ClC-3} and native I_{Cl\text{acid}} are inhibited by MTSES. This effect is dose-dependent and reversed by DTT as expected for a specific action of the compound. MTSES resistance directly identifies C103_P130del ClC-3 mutant currents. These currents are still acid-activated, and the altered time course of the response to changes in pH also identifies the C103_P130del mutant. I_{ClC-3} is therefore unequivocally activated by protons. This rules out tightly coupled Cl\(^-\)/H\(^+\) antiport as a mechanism. Combined with the lack of acid-induced changes in reversal potential at pH \(\leq 6.2\) and the virtually complete Cl\(^-\) dependence of I_{ClC-3} at pH 4.0, we conclude that low pH must drastically reduce or eliminate the coupling of Cl\(^-\) to H\(^+\) transport. The Cys\(^{103}\) to Pro\(^{130}\) segment provides an interesting candidate region for modulating responsiveness to protons. Future studies will focus on determining which of the four cysteine residues deleted by our mutation (Cys\(^{103}\), Cys\(^{114}\), Cys\(^{115}\), and Cys\(^{129}\)) are important for MTSES sensitivity.

Although we can clearly conclude that by definition ClC-3 is an "I_{Cl\text{acid}}" our data do not definitively demonstrate that the native I_{Cl\text{acid}} in HEK293 cells is endogenous ClC-3. Several lines of evidence strongly suggest this, including similarities in basic biophysical properties (voltage dependence, time constants of activation, rectification, and MTSES sensitivity) and the absence of native I_{Cl\text{acid}} following anti-ClC-3 RNAi treatment. However, these observations do not completely rule out the possibility that native I_{Cl\text{acid}} is a distinct protein that is in some way dependent upon ClC-3 for activation.

**Coupling of Cl\(^-\)/H\(^+\) Antiport**—The stoichiometry of the best understood of the Cl\(^-\)/H\(^+\) antiporters, ClCec-1 is 2Cl\(^-\)/H\(^+\) (23). Based on data supporting the same ratio for ClC-5, this has been suggested to be a general property of CLC antiporters (24). The coupling ratio of ClC-3 at neutral pH has not been rigorously established. However, whereas 2:1 antiport may be the normal mode of function for CLC antiporters, it is clear that this ratio is not tightly fixed. Coupling is structure-dependent and can be altered by mutations in both the extracellular "gating glutamate" and the intracellular "proton glutamate" (24, 33). Surprisingly, channel type CLC proteins actually share a similar mechanism of ion transport with the antiporters. ClC-0 channel gating also requires the transmembrane movement of protons, suggesting that the channel-type CLCs may be modified antiporters in which one of the conforma- tional states is leaky for chloride ions (49, 50). Coupling of Cl\(^-\)/H\(^+\) antiport is also dependent upon the permeant anion with non-halide anions resulting in reduced H\(^+\) countertransport (23, 35). In the presence of NO\(_3\^-\) the stoichiometry of exchange also becomes voltage-dependent (24). Our data sug- gest that pH affects the Cl\(^-\)/H\(^+\) coupling of the ClC-3 current and markedly enhances Cl\(^-\) transport at high extracellular proton concentrations. Although coupling is clearly reduced by low pH, our methods are not adequate to determine the degree
to which this ratio changes or to say if transport is completely uncoupled.

Functional Significance—ClC-3, ClC-4, and ClC-5 have been hypothesized to provide shunt conductances in the membranes of intracellular organelles, permitting intraluminal acidification by the V-ATPase (51). This idea was supported by the finding of impaired endosomal acidification in ClC-3-deficient mice (20). However, the realization that ClC-3 through 7 are all Cl⁻/H⁺ antiporters rather than channels has raised questions regarding the role of these proteins in vesicular electrophysiology (25, 52, 53). The direction of charge flow required to neutralize V-ATPase activity would necessitate removal of protons from the vesicle that is being acidified. Neutralizing the V-ATPase with a transporter that removes one proton from the vesicular lumen for every 3 units of charge moved yields a system that is only 66% efficient. It has been hypothesized that this method of charge neutralization makes more sense if increasing the luminal Cl⁻ concentration is also critical for vesicular biology (18, 22). However, the sharp outward rectification of these currents does not favor Cl⁻ entry into endosomes.

It remains to be determined if pH-dependent changes in coupling of Cl⁻/H⁺ transport by ClC-3 is physically significant. Our ability to interpret the current studies is limited by an inability to directly quantify IClacid in endosomes. We are left to speculate based upon the assumption that current characteristics observed in the plasma membrane can be extrapolated to endosome electrophysiology. If so, the pH of the vesicular lumen of some compartments clearly becomes sufficiently acidic to alter coupling of ClC-3 (21, 22). It is important to note that reducing pH to 5.5, which clearly activates and uncouples ClC-3, potently inhibits both ClC-4 (55) and ClC-5 (18) currents. Thus the ability of ClC-3 to become uncoupled and activated at low pH may have important physiologic implications for vesicles where it is the primary ClC protein. Transition from exchanger to channel mode might create the pure anion shunt that the CICs were previously hypothesized to be (11, 12). More efficient charge compensation of the V-ATPase could enhance the ability of energy-dependent proton pumping to maintain lower pH in vesicles containing ClC-3 compared with those expressing CIC-4 or CIC-5. Consistent with this, CIC-3 protein is specifically enriched in synaptic-like microvesicles of neuronal endocrine tissues (13). This compartment is surprisingly acidic (pH 5.11 versus 5.14 in lysosomes) in rat pinealocytes (54). If, on the other hand, vesicular Cl⁻ uptake is a primary function of endosomal CICs (18, 22), uncoupling of ClC-3 would terminate proton-driven Cl⁻ accumulation. As we learn more about the biology of CIC Cl⁻/H⁺ antiporters, the ability of ClC-3 to uncouple at physiologic proton concentrations may provide important insight into the functional role of the protein.

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