CIC-3 Chloride Channels Facilitate Endosomal Acidification and Chloride Accumulation*

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We investigated the involvement of CIC-3 chloride channels in endosomal acidification by measurement of endosomal pH and chloride concentration ([Cl−]) in control versus CIC-3-deficient hepatocytes and in control versus CIC-3-transfected Chinese hamster ovary cells. Endosomes were labeled with pH or [Cl−]-sensing fluorescent transferrin (Tf), which targets to early/recycling endosomes, or α2-macroglobulin (α2M), which targets to late endosomes. In pulse label-chase experiments, [Cl−] was 19 mM just after internalization in α2M-labeled endosomes in primary cultures of hepatocytes from wild-type mice, increasing to 58 mM over 45 min, whereas pH decreased from 7.1 to 5.4. Endosomal acidification and [Cl−] accumulation were significantly impaired in hepatocytes from CIC-3 knock-out mice, with [Cl−] increasing from 16 to 43 mM and pH decreasing from 7.1 to 6.0. Acidification and Cl− accumulation were blocked by bafilomycin. In Tf-labeled endosomes, [Cl−] was 46 mM in wild-type versus 35 mM in CIC-3-deficient hepatocytes at 15 min after internalization, with corresponding pH of 6.1 versus 6.5. Approximately 4-fold increased Cl− conductance was found in α2M-labeled endosomes isolated from hepatocytes of wild-type versus CIC-3 null mice. In contrast, Golgi acidification was not impaired in CIC-3-deficient hepatocytes. In transfected Chinese hamster ovary cells expressing CIC-3A, endosomal acidification and [Cl−] accumulation were enhanced. [Cl−] in α2M-labeled endosomes was 42 mM (control) versus 53 mM (CIC-3A) at 45 min, with corresponding pH 5.8 versus 5.2; [Cl−] in Tf-labeled endosomes at 15 min was 37 mM (control) versus 49 mM (CIC-3A) with pH 6.3 versus 5.9. Our results provide direct evidence for involvement of CIC-3 in endosomal acidification by Cl− shunting of the interior-positive membrane potential created by the vacuolar H+ pump.

Endosomal acidification is involved in a variety of cellular processes, including receptor and ligand sorting, vesicular fusion and budding, and protein degradation (1–3). Acidification is an active process in which H+ is pumped into the endosome lumen by a bafilomycin-sensitive vacuolar H+ ATPase. Charge balance requires the accompaniment of inward H+ movement by outward movement of cations such as K+ and/or inward movement of anions such as Cl−. Ion substitution, inhibitor, and endosomal pH/Cl− measurements suggest that Cl− is the principal ion in most cells responsible for shunting the interior-positive endosomal potential produced by active H+ entry (4–10). In addition to ion conductances and pump rates, endosomal acidification depends on the magnitude of H+ leak, the buffer capacity and density of fixed charges in the endosome lumen, the pH and ionic content of cytoplasm, and the rate and nature of endosome fusion/budding events.

It has been proposed without direct evidence that CIC-type Cl− channels are responsible for some or all endosomal Cl− conductance in many cell types, and hence they may be a major determinant of endosomal acidification. There is evidence for expression of at least five members of the CIC Cl− channel family (CICs 3–7) in intracellular vesicles, including endosomes (reviewed in Ref. 11). CIC-3 is localized in synaptic vesicles in neurons, where it is thought to provide an electric shunt for the proton pump (12). Studies in transfected cells suggest that a shorter CIC-3 isoform (CIC-3A) is localized in late endosomes and lysosomes, where it may regulate organellar pH (13, 14). The function of a Golgi-localized longer CIC-3 isoform (CIC-3B) is not known (14, 15). CIC-5 is localized primarily in early endosomes in renal proximal tubules, where it appears to be involved in receptor-mediated endocytosis though its function as an electrical shunt has not yet been shown (16). A recent study reported CIC-4 expression in endosomal membranes and a possible role in endosomal acidification and trafficking in epithelial cells (17). Human mutations and/or knock-out mouse studies have implicated the involvement of CIC-3 in hippocampal degeneration (12, 18), CIC-5 in renal stone formation in Dent’s disease (19, 20), and CIC-7 in osteopetrosis and retinal degeneration (21). A variety of mechanisms have been proposed by which defective endosomal Cl− conductance and acidification could produce these defects.

The purpose of this study was to define the role of CIC-3 Cl− channels in endosomal acidification and Cl− accumulation. The kinetics of endosomal pH and [Cl−] were measured in intact cells using two models: primary cultures of hepatocytes from wild-type and CIC-3 knock-out mice, and Chinese hamster ovary (CHO)1 cells after transfection with control or CIC-3A-
encoding cDNA. In addition, measurements were made in Golgi to examine organelle-specific effects in CIC function. Hepatocytes were chosen as a readily cultured cell type that natively expresses CIC-3 (22, 23). CHO cells were chosen as an easily transfected cell type that expresses little or no CIC-3 and in which endosomal acidification and Cl\(^-\) accumulation have been studied extensively (9, 24–27). The methodology for measurement of endosomal [Cl\(^-\)] by ratio imaging fluorescence microscopy was established and validated recently by the Verkman laboratory. Cells are imaged after pulse label-chase with fluorescent ligands (transferrin, Tf, or \(\alpha\)-macroglobulin, \(\alpha\)M) containing Cl\(^-\)-sensitive and -insensitive chromophores (9). We report here that CIC-3 deletion in mice remarkably impairs endosomal acidification and Cl\(^-\) accumulation in hepatocytes and that transfaction of CIC-3A into CHO cells enhances endosomal acidification and [Cl\(^-\)] accumulation.

**EXPERIMENTAL PROCEDURES**

**Hepatocyte Cell Culture from Wild-type and CIC-3-deficient Mice—**Wild-type and CIC-3-deficient mice were generated as described previously (18). The CIC-3 knock-out mice were smaller than litter mates and had a higher mortality at 3–4 weeks. Hepatocytes were isolated by a modification of the two-step liver perfusion method (28). After anesthesia, livers were perfused with liver perfusion buffer (28) and hepatocytes were freshly isolated as described above and washed three times in HepatoZYME-SFM (Invitrogen). Isolated hepatocytes were filtered, washed, and suspended in Williams’ medium E (Invitrogen) containing 5% fetal bovine serum and 2 mM glutamine. Cells were plated on 18-mm-diameter round coverslips that were coated with rat tail collagen (20 \(\mu\)g/cm\(^2\)) and bovine serum albumin (2 mM) and 2 mM glutamine. Cells were plated on 18-mm-diameter round coverslips that were coated with rat tail collagen (20 \(\mu\)g/cm\(^2\)) and bovine serum albumin (2 mM) and 2 mM glutamine.

**CHO Cell Culture and Transfection—**CHO-K1 cells were transfected with cDNA encoding ClC-3A in pcDNA6/Myc-His (Invitrogen), which introduces a c-Myc tag at the NH\(_2\) terminus. The full-length insert was confirmed by sequence analysis.

**Fluorescent Ligands for Endosomal pH and Cl\(^-\) Sensing—**Fluorescent ligands (transferrin, Tf, or \(\alpha\)-macroglobulin, \(\alpha\)M) containing Cl\(^-\)-sensitive and -insensitive chromophores (9). We used Alexa 555-conjugated IgG (Molecular Probes) or sheep anti-rabbit Cy3-conjugated IgG (Sigma) secondary antibodies. For immunoblot analysis, c-Myc antibody and horseradish peroxidase-conjugated secondary anti mouse- IgG antibody (Amersham Biosciences) were used for detection by enhanced chemiluminescence (Amersham Biosciences).

**RESULTS**

**Impaired Acidification and Cl\(^-\) Sensing in CIC-3-deficient Hepatocytes—**Measurements were made in primary cultures of hepatocytes generated from wild-type and CIC-3 null mice. Fig. 1A shows a similar appearance of control and CIC-3-deficient hepatocytes by light microscopy. Fig. 1B shows colocalization of structures labeled with FITC-conjugated Tf (top) and \(\alpha\)M (bottom) with the early (EAA1) and late (Rab7) endosomes, respectively. Fig. 1C shows fluorescence micrographs of hepatocytes after labeling with BAC-dextran-\(\alpha\)M-TMR and BAC-dextran-Tf-TMR at low temperature, followed by chase for 15 min (Tf) or 45 min (\(\alpha\)M) at 37 °C. A typical endosomal pattern was seen in the control and CIC-3-deficient hepatocytes, as found for internalization of Tf and \(\alpha\)M in many cell types (2, 9, 27, 30, 31). A membrane pattern was observed just after labeling, with progressive maturation over time (not shown). Cellular fluorescence was reduced to near zero when up to 100-fold excess unlabeled Tf or \(\alpha\)M was included in the
labeling solution, confirming a receptor-mediated internalization mechanism.

Fig. 2 summarizes the kinetics of endosomal acidification and \( \text{Cl}^- / \text{H}^+ \) accumulation in control and ClC-3-deficient hepatocytes. Initial pH in Tf-labeled endosomes was 7.2, decreasing over 15 min to 6.1 in control and 6.5 in ClC-3-deficient hepatocytes (Fig. 2A, left). Endosomal \( \text{Cl}^- \) increased in parallel from an initial low value of 16 to 46 mM in control and 35 mM in ClC-3-deficient hepatocytes (Fig. 2A, right). As reported for CHO cells (9), the low \( \text{Cl}^- \) early after endocytosis is probably the consequence of a Donnan potential produced by surface negative charges that excludes \( \text{Cl}^- \) during endosome formation. Endosomal acidification and \( \text{Cl}^- \) accumulation were blocked by inclusion of the \( \text{H}^+ \) pump inhibitor bafilomycin in the perfusate. Fig. 2B shows that pH in \( \alpha_2 \text{M} \)-labeled endosomes at 45 min was reduced to 5.4 in control and 6.0 in ClC-3-deficient hepatocytes. Corresponding \( \text{Cl}^- \) was increased to 58 mM in control and 43 mM in ClC-3-deficient hepatocytes. As found for early/recycling Tf-labeled endosomes, acidification and \( \text{Cl}^- \) accumulation were bafilomycin-sensitive and significantly impaired in ClC-3 deficiency.

**Enhanced Acidification and \( \text{Cl}^- \) Accumulation in CIC-3A-Transfected CHO Cells**—RT-PCR and immunostaining analysis showed the absence of CIC-3 transcript and protein in CHO cells. To determine the influence of expressed CIC-3A on endosomal acidification and \( \text{Cl}^- \) accumulation, CHO cells were transfected with cDNA encoding c-Myc epitope-tagged mouse CIC-3A. Control cells were transfected with cDNA encoding c-Myc alone. Immunofluorescence with c-Myc and CIC-3 antibodies showed CIC-3A expression in the transfected CHO cells in an endosomal pattern (Fig. 4A). A diffuse cytoplasmic/nuclear pattern was seen for c-Myc expression in control cells. Immunoblot analysis showed the expected molecular size of 84 kDa for c-Myc-tagged CIC-3A in the transfected cells (Fig. 4A, bottom). Fig. 4B shows fluorescence micrographs of CIC-3A-expressing CHO cells after labeling with BAC-dextran-Tf-TMR and BAC-dextran-\( \alpha_2 \text{M} \)-TMR. The staining patterns of BAC-
dextran-Tf-TMR and BAC-dextran-\( \alpha_2 \)-M-TMR were characteristic of the ligands in early and late endosomes, respectively, as also seen for FITC and TMR labeled with Tf and \( \alpha_2 \)-M (not shown).

Fig. 5 summarizes the kinetics of endosomal pH and [Cl\(^{-}\)] in Tf and \( \alpha_2 \)-M-labeled endosomes in hepatocytes from wild-type and CIC-3 null mice. Time course of endosomal pH (left) and [Cl\(^{-}\)] (right) after labeling at 4 °C with FITC- or BAC-dextran-Tf-TMR (A) and FITC- or BAC-dextran-\( \alpha_2 \)-M-TMR (B) and chase at 37 °C. Hepatocytes from wild-type mice (closed circles) and CIC-3 null mice (open circles) (S.E., 7–10 sets of experiments). Where indicated, 200 nM bafilomycin was present in the incubation solution and perfusate (S.E., 7–9 sets of experiments).

Functional Analysis of Cl\(^{-}\) Transport in Endosomes—The involvement of CIC-3 Cl\(^{-}\) channels in endosomal acidification and Cl\(^{-}\) accumulation by an electrical shunt mechanism requires functional expression of CIC-3 in endosomes. We developed a strategy to measure Cl\(^{-}\) transport directly in fluorescently labeled endosomes contained in a cellular homogenate. Endosomes were labeled with a BAC-dextran conjugate of \( \alpha_2 \)-M.
as done in intact cell studies. After incubation at 37 °C for 45 min, cells were homogenized, centrifuged, and suspended in a low Cl⁻/H¹¹⁰⁰₂, high K⁺/H¹¹⁰⁰₁ buffer containing the K⁺ ionophore valinomycin to make Cl⁻/H¹¹⁰⁰₂ influx (rather than counterion transport) rate-limiting. Cl⁻/H¹¹⁰⁰₂ influx was measured selectively in fluorescently labeled endosomes in the heterogeneous membrane suspension from the kinetics of decreasing BAC fluorescence in response to mixing of the membrane suspension with an isosmolar high Cl⁻/H¹¹⁰⁰₂ buffer. Fig. 6A shows the kinetics of Cl⁻/H¹¹⁰⁰₂ influx measured in endosomes from wild-type and ClC-3-deficient hepatocytes. There was little decrease in fluorescence corresponding to Cl⁻/H¹¹⁰⁰₂ influx in the absence of valinomycin, indicating a conductive Cl⁻ entry mechanism. ClC-3 deletion remarkably reduced Cl⁻/H¹¹⁰⁰₂ influx as seen from the slower decrease in fluorescence signal. Fig. 6B summarizes average Cl⁻/H¹¹⁰⁰₂ influx rates shown as reciprocal exponential time constants fitted to the kinetics of BAC fluorescence. Cl⁻/H¹¹⁰⁰₂ influx was significantly reduced by ~4-fold in endosomes of ClC-3-deficient hepatocytes, although significant residual influx remained. This result indicates that ClC-3 is the main Cl⁻ conductive mechanism in the labeled endosomes. The non-zero Cl⁻ conductance in endosomes from ClC-3-deficient hepatocytes accounts in part for their ability to acidify, albeit less well than hepatocytes from wild-type mice.

**DISCUSSION**

Our results provide direct evidence supporting the involvement of an intracellular ClC-type Cl⁻ channel in organellar acidification. Endosomal acidification and Cl⁻ accumulation were significantly reduced in hepatocytes from ClC-3-deficient versus wild-type mice and enhanced in ClC-3A-transfected versus control CHO cells. Functional ClC-3 Cl⁻ conductance in endosomes was demonstrated in cell homogenates from wild-type versus ClC-3-deficient hepatocytes containing fluorescently labeled endosomes. The data suggest that ClC-3 Cl⁻ conductance provides a quantitatively significant electrical shunt pathway to permit endosomal acidification by the vacuolar H⁺ pump.

ClC-3 is expressed in brain, liver, and other tissues (22) and has been reported to localize in intracellular vesicles (12) and the plasma membrane (36, 37). The phenotype of ClC-3 knockout mice includes blindness and hippocampal neurodegeneration (12, 18). Indirect measurements of ATP-induced acidification by acridine orange uptake/quenching showed reduced uptake in isolated synaptic vesicles of ClC-3 versus wild-type mice (12), and estimated pH in isolated liver vesicles from ClC-3 null mice in the presence of ATP was greater than that of vesicles from wild-type mice (18). However, other reports of ClC-3A and ClC-3B localization (13, 14) and possible function as a swelling-activated Cl⁻ channel (12, 36–38) draw somewhat contradictory conclusions. Here, we have shown that ClC-3 functions as an intracellular ClC-type Cl⁻ channel that facilitates endosomal acidification and Cl⁻ accumulation in intact cells, providing a possible explanation for the phenotypic abnormalities in ClC-3 null mice. Our conclusion about the role...
of CIC-3 in endosomal acidification in liver does not necessarily apply to other organs/cell types where different endosomal ion channels may be expressed. For example, CIC-5 may be important in endosomal acidification and Cl⁻ conductance in kidney proximal tubule cells where impaired endocytosis has been demonstrated in CIC-5 null mice (19) and may provide a mechanism for Dent’s disease in CIC-5 deficiency in humans.

The partial impairment of endosomal acidification in CIC-3 deficient hepatocytes suggests that some, but not all, ion movement accompanying active H⁺ entry involves CIC-3-mediated Cl⁻ entry. The results in Fig. 6 suggest that CIC-3 provides the principal route for conductance of Cl⁻ entry. It is difficult to quantify the fraction of ionic movement through CIC-3 versus other channel pathways because of the complex set of determinants of endosomal acidification as described in the Introduction. Endosomes from hepatocytes may express other types of Cl⁻ channels, such as ClC-4 and/or K⁺ channels, that may be up-regulated as a compensatory response to CIC-3 gene deletion in mice. Also, endosomes in intact cells may differ from those in suspension in that regulatory factors may be different and little endosome fusion occurs. Notwithstanding the difficulties in ascribing the exact fraction of total ionic movement accompanying H⁺ entry, our results here support the involvement of CIC-3 in endosomal acidification.

The CHO cell transfection studies showed that overexpression of CIC-3A in a cell type that does not normally express CIC-3 produced an enhancement in endosomal acidification and Cl⁻ accumulation. Therefore, endogenous endosomal ion conductance (rather than the H⁺ pump) must be a rate-limiting determinant of endosomal acidification. Thus, regulation of the expression and/or function of CIC-3 or other endosomal ion channels can influence acidification kinetics and steady-state pH. Because Cl⁻ entry produces an osmotic gradient leading to endosome swelling (24), whereas K⁺ exit accompanying H⁺ influx would produce endosome shrinkage, regulation of endosomal Cl⁻ or K⁺ conductances is predicted to alter endosome volume. Based on conclusions from our recent study on the role of endosomal Cl⁻ accumulation and swelling on transgene delivery (25), the ability of overexpression of a CIC-type Cl⁻ channel to enhance acidification and Cl⁻ entry provides a potential new tool to enhance gene delivery by non-viral mechanisms.

In summary, measurements of endosomal pH and [Cl⁻] provide direct evidence for the involvement of a CIC-type Cl⁻ channel in endosomal acidification. The approach used here should be applicable to investigation of the role of other putative intracellular Cl⁻ channels, such as CIC-4, CIC-6, CIC-7, and AQP6, in organelar acidification. When available, specific high affinity inhibitors of CIC-type Cl⁻ channels will be useful to resolve the contributions of individual Cl⁻ channels because
quantitative conclusions cannot be rigorously made using cells from knock-out mice or transfected systems.

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