Cystic fibrosis transmembrane conductance regulator contributes to reacidification of alkalinized lysosomes in RPE cells


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Liu J, Lu W, Guha S, Baltazar GC, Coffey EE, Laties AM, Rubenstein RC, Reenstra WW, Mitchell CH. Cystic fibrosis transmembrane conductance regulator contributes to reacidification of alkalinized lysosomes in RPE cells. Am J Physiol Cell Physiol 303: C160–C169, 2012. First published May 9, 2012; doi:10.1152/ajpcell.00278.2011. —The role of the cystic fibrosis transmembrane conductance regulator (CFTR) in lysosomal acidification has been difficult to determine. We demonstrate here that CFTR contributes more to the reacidification of lysosomes from an elevated pH than to baseline pH maintenance. Lysosomal alkalinization is increasingly recognized as a factor in diseases of accumulation, and we previously showed that cAMP reacidified alkalinized lysosomes in retinal pigmented epithelial (RPE) cells. As the influx of anions to electrically balance proton accumulation may enhance lysosomal acidification, the contribution of the cAMP-activated anion channel CFTR to lysosomal reacidification was probed. The antagonist CFTRinh-172 had little effect on baseline levels of lysosomal pH in cultured human RPE cells but substantially reduced the reacidification of compromised lysosomes by cAMP. Likewise, CFTR activators had a bigger impact on cells whose lysosomes had been alkalinized. Knockdown of CFTR with small interfering RNA had a larger effect on alkalinized lysosomes than on baseline levels. Inhibition of CFTR in isolated lysosomes altered pH. While CFTR and Lamp1 were colocalized, treatment with cAMP did not increase targeting of CFTR to the lysosome. The inhibition of CFTR slowed lysosomal degradation of photoreceptor outer segments while activation of CFTR enhanced their clearance from compromised lysosomes. Activation of CFTR acidified RPE lysosomes from the ABCA4−/− mouse model of recessive Stargardt’s disease, whose lysosomes are considerably alkalinized. In summary, CFTR contributes more to reducing lysosomal pH from alkalinized levels than to maintaining baseline pH. Treatment to activate CFTR may thus be of benefit in disorders of accumulation associated with lysosomal alkalinization.

enzyme activity, as the increased retention of partially degraded material may be pathological. In this regard, lysosomal enzymes function optimally over a narrow range of acidic pH values and the predominant lysosomal enzymes of the RPE reflect this sensitivity (11). This sharp pH dependence implies that alkalinizing lysosomes of RPE cells will lower enzyme efficiency and interfere with the degradation of internalized outer segments.

Disruption of lysosomal pH (pH_L) slows outer segment degradation by RPE cells. Administration of the lysosomotrophic compound chloroquine leads to the incomplete digestion of photoreceptor outer segments within RPE cells, an increase in lysosomal-associated organelles, and the accumulation of extracellular debris around adjacent Bruch’s membrane and choriotapillaris (24, 29). Similarly, tamoxifen raises lysosomal pH and slows outer segment clearance in RPE cells (20). In autosomal recessive Stargardt’s disease, a mutation in the ABCA4 transporter leads to the accumulation of N-retinylidene-N-retinylethanolamine (A2E) in RPE cells (1, 27). Addition of A2E to cultured RPE cells increases the lysosomal pH, slows degradation of proteins, and inhibits the translocation of protons into the lysosomes and the degradation of outer segments (3, 15). Lysosomal pH in the RPE cells of ABCA4−/− mice is elevated compared with age-matched controls (20), consistent with the alkalinizing effect of A2E.

As alkalinization of RPE lysosomes leads to incomplete degradation of photoreceptor outer segments, it follows that restoring an acidic pH to the organelle may improve degradation and preserve cell health. A previous study found that stimulation of A1 adenine receptors or β-adrenergic receptors reacidified compromised RPE cell lysosomes; the coupling of both receptors to the Gs protein implicated the second messenger cAMP (20). Cell-permeant chlorophyllthio-cyclic AMP (cpt-cAMP) reacidified lysosomes, and myristoylated protein kinase inhibitor prevented acidification, strongly implying a role for protein kinase A. Furthermore, activation of these pathways increased the degradation of photoreceptor outer segments, providing functional verification. Of note, cAMP was able to reacidify the lysosome in RPE cells from ABCA4−/− mice, suggesting that the pathway remained intact in diseased cells.

While the ability of cAMP to restore lysosomal pH in RPE cells and improve outer segment degradation is clear, the underlying mechanisms remain to be determined. The basic physiology of lysosomal acidification identifies two likely candidates. First, the delivery of protons to lysosomes and late endosomes is primarily mediated by the vacuolar proton pump
vHATPase (33). Insertion of vHATPase into the plasma membrane of clear cells is enhanced by cAMP acting through soluble adenylate cyclase (32). While the transport of protons by the vHATPases lowers organelle pH, the accumulation of positive charge eventually establishes an unfavorable driving force across the membrane and makes further acidification energetically unfavorable (28, 30). This excess positive charge is often neutralized by the influx of Cl\(^-\) into the vesicle, making anion transport a second key mechanism in lysosomal pH regulation (10). The most widely distributed cAMP-activated Cl\(^-\) channel is the cystic fibrosis transmembrane conductance regulator (CFTR), where phosphorylation of protein kinase A activates CFTR (9). As functional and immunohistochemical evidence indicates that CFTR is present in RPE cells (4, 36, 37), this study asked whether CFTR contributed to the cAMP-mediated acidification of lysosomes in RPE cells.

**EXPERIMENTAL PROCEDURES**

**Cell culture.** The human ARPE-19 cell line was obtained from American Type Culture Collection (Manassas, VA) and processed as described previously (20). In brief, cells were grown to confluence in 25-cm\(^2\) primary culture flasks (Becton Dickinson) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 3 mM l-glutamine, 100 |µ|g/ml streptomycin, and 2.5 mg/ml Fungizone and/or 50 |µ|g/ml gentamicin and 10% fetal bovine serum (all Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in 5% CO\(_2\) and subcultured weekly with 0.05% trypsin and 0.02% EDTA.

**Lysosomal pH measurement from ARPE-19 cells.** Measurement of lysosomal pH from ARPE-19 cells was performed using a ratiometric lysosomal pH indicator dye (LysoSensor Yellow/Blue DND-160; Invitrogen). The procedure has been described in detail previously (20). Given the temperamental nature of the dye, three key parameters were optimized to identify conditions giving the best signal-to-noise level using the least amount of dye. Dye concentration from 1 to 25 |µ|M was tested, with 5 |µ|M the lowest concentration giving a consistently bright signal. The dye was incubated from 1 to 30 min, with 3 min identified as the shortest incubation time to provide a sufficiently bright signal. To evaluate the possible alkalining effects of the dye and the possible light effects on the fluorescence readings, each of the fluorescence measurements was repeatedly conducted every 40 s for 15 min. While an increased ratio was observed at higher dye concentrations, this effect was minimal with 3–5 |µ|M dye. The stability of the dye was examined with a single set of readings at 5, 10, 30, and 60 min after incubation. The signal slowly decreased, presumably owing to diffusion of the dye. Given this information, we consistently incubated cells with 5 |µ|M LysoSensor Yellow/Blue dye for 3 min at room temperature (RT), washed to remove any residual dye, and light emitted >520 nm in response to excitation at 340 nm and 380 nm was measured for 20 ms every 30 s. Fluorescent levels were the mean of four measurements taken after the removal of the dye; while most measurements were made ~16.5–18.5 min after dye removal, occasional measurements were made ~1–2.5–18.5 min after dye removal, delays occasionally occurred. Cells were bathed in 100 |µ|l isosol solution (IS; in mM: 105 NaCl, 5 KCl, 6 HEPES acid, 4 Na HEPES, 5 NaHCO\(_3\), 60 mannitol, 5 glucose, 0.5 MgCl\(_2\), 1.3 CaCl\(_2\)). Drugs tested for their ability to reverse the effects of alkalining compounds were added to cells 5 min before the addition of the alkalining agents. Drugs were tested in the absence of dye and found to have no significant effect on the light excited at 340 or 380 nm.

Additional steps were taken to minimize variation. For example, measurements were performed on parallel wells in a 96-well plate, and the fluorescence was measured with a fluorescence plate reader at room temperature (Fluroskan; Thermo Fisher Scientific, Waltham, MA). Despite these precautions, variation in ratio and pH values did occur sometimes between preparations. While changes in passage number, cell age, measurement time, and investigator may have increased variation, other unknown factors may have led to small differences. However, the relative levels within a plate were consistent.

Initial experiments were undertaken to confirm that pH measurements made with the ratiometric dye LysoSensor Yellow/Blue DND-160 were an accurate reflection of the lysosomal pH (pH\(_L\)). The LysoSensor dye stained small punctuate regions in ARPE-19 cells that showed considerable overlap with the single wavelength acidic stain LysoTracker Red DND-99 (Fig. 1A). This colocalization confirms the ability of dyes to specifically signal from acidic organelles. While readout is attributed to lysosemes, it should be noted that the output likely originates from a mixture of organelles including lysosomes, phagolysosomes, and perhaps late endosomes. The ratiometric signal from LysoSensor Yellow/Blue was converted into absolute pH values following calibration with ionophores monensin and nigericin (Fig. 1B). Independent pH calibrations were performed simultaneously with each experimental plate to minimize potential confounding of these data by LysoSensor dye instability. The ratio of light excited at 340/380 nm was converted to pH by calibration performed at precisely the same time as the other readout in adjacent wells of the same plate; the extracellular solution was buffered to pH 4.0 to 6.0 in the presence of 10 |µ|M H\(^+\)/Na\(^+\) ionophore monensin and 20 |µ|M H\(^+\)/K\(^+\) ionophore nigericin dissolved in 20 mM (N-morpholino)ethanesulfonic acid (MES), 110 mM KCl, and 20 mM NaCl and adjusted to pH 4.0 to 6.0 with HCl/NaOH.

**Isolation and measurement of lysosomal pH from fresh ABCA4\(^{-/-}\) mouse RPE cells.** ABCA4\(^{-/-}\) mice were a kind gift from Dr. Gabriel Travis of the Jules Stein Eye Institute (Univ. of California, Los Angeles). All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were reared at 5–15 lux and euthanized with a CO\(_2\) overdose. Mouse RPE cells were isolated and processed as described (20). In brief, after enucleation, intact eyes were incubated in 2% dispase and 0.4 mg/ml collagenase IV for 45 min, rinsed, and incubated in growth medium for 20 min (containing DMEM with 1 |µ|g/ml gentamicin and 10% fetal bovine serum; all Invitrogen). Sheets of RPE cells were separated from the choroid and triturated to single cell level. Cells from four to six eyes were pooled, loaded with 2–5 |µ|M LysoSensor Yellow/Blue for 5 min at room temperature, rinsed, and distributed into wells of 384-well UV Star plates (Greiner Bio-One, Monroe, NC) and measured as described above. Although eyes from ABCA4\(^{-/-}\) mice were slightly autofluorescent, the signal from the dye was 100-fold greater, validating the measurements (20). Lysosomal pH was measured within 3 h postmortem. CFTR\(_{Act11}\) was added to the bath 20 min before measurements were taken. Because of the reduced number of cells, measurements from fresh RPE cells were not calibrated but were expressed as ratio of fluorescence excited at 340 vs. 380 nm and emitted >540 nm. As the numbers of cells from each eye were too low to give acceptable signal to noise, three pairs of each genotype were pooled in one trial and two pairs from each genotype were pooled in another.

**Outer segment degradation assay.** Bovine photoreceptor outer segments were isolated on the basis of published protocols (1). As described previously (20), outer segments were labeled with the fluorescent dye calcein to overcome the pH-dependent loss of signal that occurs with FITC; the negative log of acidic dissociation constant (pK\(_a\)) of 6.8 means that fluorescence of FITC is lost in acidic organelles, making actual clearance hard to determine. In contrast, calcein is unaffected by pH or calcium levels. Outer segments were loaded with 5 |µ|M calcein-acetoxymethyl (AM; Invitrogen) in PBS for 10 min and spun twice at 14,000 rpm. The AM form of the dye is nonfluorescent until cleaved by intracellular esterases, reducing background fluorescence. The fluorescence signal reached a maximum within 20 min and was evenly distributed, although its localization in disk membranes compared with cyttoplasm could not be distinguished. Outer segments labeled with calcein were added to cells in 96-well
plates for 2 h. Cells were then washed vigorously and incubated with growth medium for a further 2 h. This 2-h “chase” of cells in the absence of extracellular outer segments was designed to allow sufficient time for the transport of outer segments to lysosomes before drugs were added, to ensure that drugs were targeting the degradation and not internalization or binding steps. Microscopic examination confirmed that the majority of outer segments were in lysosomes after this chase period (see Fig. 5A in RESULTS). Baseline fluorescence levels excited at 485 nm and emitted >520 nm were read at the end of the 2-h chase period. Cells were then incubated with alkalinizing drugs, putative treatments, or control solution for 20 h, after which the cells were washed twice and the final fluorescence reading was taken. The time course was chosen on the basis of previous kinetic analyses showing that most outer segment degradation is complete within 18 h (21). The “%POS” (percentage of photoreceptor outer segments) was defined as the change in fluorescence between final \((t_f)\) and initial \((t_0)\) readings: \(\%POS = 100 \times \frac{(t_0) - (t_f)}{(t_0)}\). The time course is summarized in RESULTS (see Fig. 5B).

To localize outer segments (see Fig. 5A), cells were cultured on glass coverslips. Cells were exposed to calcine-labeled outer segments for 2 h, followed by a 2-h wash. Cells were then incubated with 100 mM LysoTracker Red (Invitrogen). The red fluorescent lysosomal stain was imaged at 540-590 nm (excitation/emission), whereas labeled outer segments were imaged at 480-535 nm on a Nikon microscope (Eclipse 600; Nikon USA, Melville, NY) and imaged with a 3-CCD digital camera (Toshiba America, Irvine, CA). Images were processed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

**Molecular manipulation of CFTR.** One day before transfection, \(2 \times 10^5\) cells/ml ARPE-19 cells were cultured in 96-well black plates (Corning, Corning, NY) using 100 \(\mu\)l of growth medium without antibiotics. The plasmid DNA contained the wild-type CFTR construct used previously (34). The plasmid (0.4 \(\mu\)g) was diluted in 25 \(\mu\)l serum-free Opti-MEM medium and incubated for 5 min at RT. Lipofectamine 2000 (1 \(\mu\)l) was subsequently mixed in 25 \(\mu\)l Opti-MEM medium and incubated for 20 min at RT to allow complex formation. This mixture was added to the same volume of growth medium and loaded into ARPE-19 cells. Plasmids were incubated with cells at 37°C for 4–6 h, after which the transfection mix was replaced with growth medium. Cells were grown for a further 48 h before experiments. Western blotting using the anti-CFTR antibody (M3A7; Upstate Biotechnology) was performed using standard protocols (21). In brief, ARPE-19 cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and protease inhibitor cocktail) and centrifuged at 13,000 g for 10 min at 4°C. Protein concentrations were determined using the bicinchoninic acid protein assay kit. Protein lysate were separated on SDS-PAGE (Bio-Rad), and transferred to polyvinylidene difluoride membrane (Millipore).

Approaches to CFTR depletion were analogous; 0.5 \(\mu\)l of small interfering (si)RNA (20 \(\mu\)M, siGenome SMART Pool, catalog no. M-006425-01, Dharmacon/ThermoFisher) or nontargeting siRNA (siGenome SMART Pool, catalog no. M3A7; Upstate Biotechnology) was performed using standard protocols (21). In brief, ARPE-19 cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and protease inhibitor cocktail) and centrifuged at 13,000 g for 10 min at 4°C. Protein concentrations were determined using the bicinchoninic acid protein assay kit. Protein lysate were separated on SDS-PAGE (Bio-Rad), and transferred to polyvinylidene difluoride membrane (Millipore).

**RESULTS**

cAMP acidifies compromised lysosomes. The regulation of lysosomal pH is complex, with particular mechanisms active only over a particular pH range. As a key goal of these studies is to restore an acidic lysosomal pH in disease states where lysosomal pH is more alkaline than normal, we experimentally elevated the pH\(_{50}\) and tested the ability of various candidates to restore acidity. Chloroquine is a weak base which alkalinized the lysosomes of RPE cells (Fig. 1C). This lysosomal alkalization was reversed by elevating intracellular cAMP with a mixture including cell-permeant cPT-cAMP. Tamoxifen was used as a second approach to alkalinize the pH of lysosomes. Tamoxifen is thought to elevate pH\(_{50}\) through its actions as a weak base and as a mediator of coupled transport of proton/
hydroxide and chloride (6); the alkalinizing effects of tamoxifen on lysosomal pH are independent of the estrogen receptor (20). While tamoxifen alkalinized the lysosomal pH of RPE cells, addition of cpt-cAMP mix also acidified cells treated with tamoxifen. Interestingly, the magnitude of lysosomal acidification produced by cpt-cAMP in cells at baseline was substantially smaller (Fig. 1D). Likewise, the cell-permeant 8-Br-cAMP acidified lysosomes, with the magnitude of this acidification greater in alkalinized lysosomes (Fig. 1E).

Pharmacological identification of CFTR in lysosomal acidification. The ability of cAMP to acidify lysosomal pH over the working range of lysosomal enzymes suggested the activation of a cAMP-dependent transport mechanism. Given that anion entry into the lysosomal lumen will balance the positive charge brought by proton entry, this acidification might be due to activation of a cAMP anion channel and/or transporter. The role of CFTR in lysosomal acidification was thus probed. The sulfonfonylurea glibenclamide inhibits conductance through CFTR (2). Glibenclamide produced a small elevation in baseline pHL (Fig. 2A). A newer generation of drugs with enhanced specificity for CFTR includes the inhibitor CFTRinh-172, which produces a voltage-independent block of CFTR that is not influenced by cAMP levels (23). CFTRinh-172 (10 μM) led to a small but significant elevation in pHL from baseline levels of 4.50 ± 0.02 to 4.58 ± 0.02 (Fig. 2B). However, CFTRinh-172 raised the lysosomal pH substantially more when the cAMP mix was used to treat cells challenged with chloroquine, from 5.07 ± 0.16 to 5.80 ± 0.08 (Fig. 2C). The blocker also raised the pH of cells treated with tamoxifen + cAMP mix from 5.31 ± 0.07 to 5.63 ± 0.11 (Fig. 2D). The magnitude of the effect of 10 μM CFTRinh-172 was thus larger in alkalinized cells treated with cAMP mix.

Specific activators of CFTR were examined for their ability to acidify lysosomes (23). CFTRAct16 led to a small but significant acidification of lysosomal pH in ARPE-19 cells from baseline levels; 10 μM CFTRAct16 dropped pHL from 4.49 ± 0.07 to 4.30 ± 0.03 (Fig. 2E). However, the effect of CFTRAct16 was substantially larger in lysosomes alkalinized by tamoxifen; pHL fell from 5.96 ± 0.05 to 5.57 ± 0.07 (Fig. 2F). This increased magnitude of the acidification by CFTRAct16 in tamoxifen-treated cells compared with baseline levels was analogous to the increased alkalinization by CFTRinh-172 on tamoxifen-treated compared with baseline treated cells.

Fig. 1. cAMP reacidifies compromised lysosomes. Lysosomal pH (pHL) was measured: A: labeling of retinal pigmented epithelial (RPE) cell lysosomes. i and ii: lysosomal pH indicator LysoSensor DND-160 Yellow/Blue (i) and the red fluorescent lysosomal stain LysoTracker Red DND-99 (ii) demonstrated considerable colocalization when simultaneously delivered to cultured RPE cells. iii: differential interference contrast image. iv: overlap of i and ii. A slight shift occurred between images. Scale bar, 20 μm. B: typical calibration of lysosomal pH from ratios in the presence of ionophores monensin and nigericin. F340/380, ratio of fluorescence excited at 340 nm/380 nm and emitted at 520 nm. The curve is a mean of 11 separate measurements. The calibration curve was well fit with a linear regression (r² = 0.98). Calibration was performed separately on each plate throughout the study to minimize variation. C: While chloroquine (CHQ, 10 μM) elevated lysosomal pH of retinal pigment epithelial (RPE) cells, a cAMP-stimulating mix (cAMP mix) of the cell-permeant analog chlorophenylthio (cpt)-cAMP (100 μM), forskolin (10 μM), and IBMX (100 μM) acidified cells exposed to chloroquine (n = 10). D: cpt-cAMP (500 μM) acidified cells treated with tamoxifen (TMX, 10 μM) although cpt-cAMP induced a smaller acidification of untreated cells (n = 27). E: a second cell-permeant analog, 8-Br-cAMP (500 μM), also acidified cells treated with tamoxifen (10 μM) more than cells at baseline pH levels (n = 27). Here and elsewhere, bars on data plots represent means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. chloroquine; **P < 0.05 vs. tamoxifen.
As CFTR<sub>Act16</sub> is reported to raise cAMP, at least some of its effects may have resulted from changes to cAMP (23). In contrast, CFTR<sub>Act11</sub> activates CFTR without affecting cAMP. CFTR<sub>Act11</sub> lowered pH in cells treated with tamoxifen, from 6.03 ± 0.04 to 5.88 ± 0.05 (Fig. 2G). The smaller effect is consistent with the original characterization of these two drugs, where CFTR<sub>Act11</sub> was less efficacious than CFTR<sub>Act16</sub> (23). Taken together, the acidifying actions of CFTR activators CFTR<sub>Act16</sub> and CFTR<sub>Act11</sub>, combined with the alkalinizing effects of CFTR inhibitors glibenclamide and CFTR<sub>inh-172</sub>, strongly support the concept that CFTR contributes to lysosomal acidification in ARPE-19 cells, but that the effect is larger when cells are alkalinized.

Molecular manipulation of CFTR. While the pharmacologic data above consistently suggest a role for CFTR in lysosomal acidification, manipulation of CFTR using molecular tools was undertaken to further test the hypothesis. When examined in untreated cells, depletion of CFTR with siRNA had a small but insignificant effect on lysosomal pH (Fig. 3A). However, Fig. 3. Molecular manipulation of CFTR alters lysosomal pH. A: CFTR small interfering (si)RNA leads to a small but insignificant increase in the baseline lysosomal pH in cultured RPE cells (n = 12). B: siRNA against CFTR blocked the acidifying effect of the cAMP cocktail mix [cAMPmix: 10 μM forskolin, 500 μM cpm-cAMP, and 100 μM IBMX (n = 24)] on lysosomal pH in ARPE-19 cells treated with tamoxifen, 30 μM, n = 18). Scr-siRNA transfection did not alter lysosomal pH (n = 12).

* P < 0.05 vs. tamoxifen control. ** P < 0.05 vs. tamoxifen + cAMP mix. C: RPE cells that overexpressed CFTR (CFTR-transf) demonstrated lower lysosomal pH when exposed to tamoxifen (30 μM), compared with either the nontransfected (non-transf) or empty vector-transfected (Lipofect) cells (n = 3). * P < 0.05 vs. non-transf, ††† P < 0.001 vs. Lipofect. D: top: Western blot showing light band of CFTR in control, with increased expression after transfection with plasmid. D: bottom: quantitative PCR indicating decrease in mRNA for CFTR after treatment with siRNA.
depletion of CFTR with siRNA prevented the acidifying effect of cAMP in cells treated with tamoxifen (Fig. 3B). In the corollary experiment, the effect of CFTR over expression was examined. Transfected cells with plasmid containing CFTR lowered pH_L in cells treated with tamoxifen (Fig. 3C). To confirm the effectiveness of these manipulations, siRNA was shown to reduce CFTR mRNA; levels were 1, 0.93, and 0.59 in nontransfected control, scrambled control, and cells exposed to CFTR siRNA, respectively (Fig. 3D). Likewise, transfecting cells increased CFTR protein as detected by immunoblotting.

**Direct effects of CFTR on lysosomal membrane.** Although activation of CFTR clearly had an acidifying effect on lysosomal pH, the above experiments performed in intact cells could not discriminate between CFTR acting directly on the membrane and CFTR on the plasma membrane acting to modify lysosomal function through indirect means. To determine whether CFTR functioned on the lysosomal membrane, vesicles were isolated and loaded with LysoSensor Yellow/Blue dye (Fig. 4A). While the identity of these vesicles may not be exclusively lysosomal, several controls are consistent with them as acidic lysosomal-like organelles. For example, the pH of the isolated lysosomes was lower in the presence of 5 mM ATP than in its absence (Fig. 4B), consistent with the presence of a vHATPase on the isolated vesicles. As such, ATP was included in subsequent experiments. The pH was elevated by 0.5 μM of the proton pump inhibitor bafilomycin (Fig. 4C), confirming that constitutive activity of the vHATPase lowers the pH in the isolated vesicles. To evaluate the contribution of CFTR to this pH, vesicles were bathed in ATP with and without CFTRinh-172. CFTRinh-172 alkalinized the isolated lysosomes (Fig. 4D). This suggested that CFTR was functioning on the isolated membrane.

ARPE-19 cells expressed both CFTR and Lamp1 in organelle-type structures; both antigens were expressed alone and together, suggesting that at least some of the CFTR in RPE cells is associated with acid vesicles (Fig. 4E). However, colocalization was not increased by treating the cells with cAMP (Fig. 4F). It should be stressed that while colocalization of CFTR on the lysosomal membrane is supportive, many proteins eventually end up in the lysosome, and the resolution of light microscopy cannot distinguish between CFTR present on the lysosomal membranes or in the lumen awaiting degradation. However, the functional data on isolated lysosomes implies that CFTR is not only present, but also active in the membrane of isolated lysosomes.
CFTR contributes to lysosomal clearance. The regulation of lysosomal pH is likely of great importance to RPE cells as many degradative lysosomal enzymes function maximally at a pH between 4 and 5. The lysosomal environment is critical for activity of these enzymes, so lysosomal enzyme activity cannot be determined using standard lysed cell assays where cells are ruptured and enzyme activity measured in a tube. As RPE cells are responsible for the degradation of photoreceptor outer segments in vivo, the contribution of CFTR to this degradation was determined. Cells were incubated with calcine-labeled outer segments for 2 h, followed by a 2-h wash to enable internalization. Images taken after this 2-h chase period indicated that the majority of outer segments colocalized with LysoTracker Red at this point (Fig. 5A). Baseline fluorescence was determined at this point, $t = 0$. Drugs shown above to alter lysosomal pH were then added, and the fluorescence remaining 20 h later was determined ($t = 20$, Fig. 5B). The effect of drugs added at this point would primarily reflect their effect on outer segment degradation, as most outer segment binding, internalization, and delivery to the lysosomes has already occurred.

The majority of outer segment fluorescence was eliminated after 20 h in control conditions; remaining fluorescence was <20% of that present at baseline. However, CFTR$\text{inh-172}$ increased the amount of fluorescence remaining in a concentration-dependent fashion, with 3 $\mu$M and 10 $\mu$M CFTR$\text{inh-172}$ leading to a significant increase in fluorescence (Fig. 5C). Tamoxifen reduced the rate of outer segment clearance, but CFTR$\text{Act11}$ enhanced the clearance of fluorescent outer segments, presumably by activation of CFTR (Fig. 5D). This supports the presumption that an elevation in lysosomal pH reduces the efficiency of lysosomal enzymes, and it implies that CFTR promotes outer segment degradation/clearance by lysosomes.

**CFTR activation lowers lysosomal pH in RPE from ABCA4$^{-/-}$ mice.** As the findings above suggest that CFTR may be well suited to lower lysosomal pH in conditions where the pH was

![Image](https://example.com/image.png)
pathologically elevated, the effects of CFTR activation on RPE cells from the ABCA4<sup>−/−</sup> mouse were examined. The ABCA4 gene is mutated in Stargardt’s disease, and RPE cells in the mouse have high levels of A2E (1, 26). A2E raises the lysosomal pH of cultured RPE cells (15), and the lysosomal pH of RPE cells from ABCA4<sup>−/−</sup> mice increases with animal age (20). We thus asked whether stimulation of CFTR could lower lysosomal pH in RPE cells from ABCA4<sup>−/−</sup> mice. Stimulation of CFTR with CFTR<sub>Act</sub>11 lowered the lysosomal pH in RPE cells from ABCA4<sup>−/−</sup> mice (Fig. 6). Of note, these eyes were from elderly mice, with a mean age of 465 days. This suggests that activation of CFTR may be effective even with older individuals where the lysosomes have been subjected to damages for a prolonged time.

DISCUSSION

The identification of CFTR as a component of the acidification mechanism in lysosomal-like organelles of RPE cells is supported by multiple observations. First, CFTR inhibitors glibenclamide and CFTR<sub>inh</sub>-172 led to a small increase in lysosomal pH. When cAMP was used to reduce acidity to lysosomes treated with chloroquine or tamoxifen, this lysosomal reacidification was also inhibited by CFTR<sub>inh</sub>-172. Of note, CFTR<sub>inh</sub>-172 was effective in cells treated with both tamoxifen and chloroquine, suggesting that the CFTR is involved in a general acidification that is independent of the method used to alkalinize the cells. The ability of CFTR activators CFTR<sub>Act</sub>16 or CFTR<sub>Act</sub>11 to acidify the lysosomes of RPE cells strengthens a role for CFTR. CFTR<sub>Act</sub>16 and CFTR<sub>Act</sub>11 are more selective activators that were identified using an elegant high-throughput screening approach (23). For instance, CFTR<sub>Act</sub>16 activated cloned CFTR in Fisher rat thyroid cells, with an approximate EC<sub>50</sub> of 200 nM, compared with 500 nM for CFTR<sub>Act</sub>11, although the former also elevated cAMP. The increased acidification of RPE lysosomes by CFTR<sub>Act</sub>16 compared with CFTR<sub>Act</sub>11 is consistent with their relative effects on the cloned CFTR, while the acidification of these organelles by CFTR<sub>Act</sub>11 implies that the actions may be specific for CFTR. It should be noted that in contrast to the experiments on cloned CFTR, the experiments here were performed in the absence of forskolin, so CFTR activation in RPE cells presumably relies on endogenous levels of cAMP.

The overexpression of CFTR acidified lysosomes, while, lastly, the acidifying effects of cAMP were inhibited by CFTR depletion by siRNA. Together, these results provide strong molecular and pharmacological evidence that CFTR contributes to the acidification of lysosomes of RPE cells.

CFTR and organelle pH. A considerable controversy exists regarding a possible contribution of CFTR to organelle acidification. While defective acidification of intracellular vesicles was originally proposed to contribute to the pathology of cystic fibrosis nearly two decades ago (2), subsequent studies were unable to substantiate a role for CFTR in this process, suggesting that the contribution may vary with cell type and organelle (31, 35). A role for CFTR in vesicle acidification has once again been proposed in recent years, attracting an even more enthusiastic denouncement. Lysosomes were alkalinized in macrophages from CFTR<sup>−/−</sup> mice and by CFTR<sub>inh</sub>-172 (8). Using FITC-conjugated probes and dextran-conjugated pHR60/rodamine green, lysosomal alkalinization was shown in alveolar macrophages from CFTR<sup>−/−</sup> mice and, to a lesser extent, in alveolar macrophages from cystic fibrosis mice harboring the AF508 and G551D mutants (12). The absence of CFTR impeded phagolysosome fusion (24). However, several other groups were unable to identify a role for CFTR in vesicle acidification (13, 18, 20, 22).

The substantial data from groups both supporting and denying a role for CFTR in organelle acidification suggest that the mechanisms which regulate CFTR’s contribution to this process may be quite complex. It is thus likely that the small experimental differences that lead to disparate results actually hold clues to unravel this mystery (21). Cellular differences are clear, with alveolar macrophages displaying a role for CFTR while peritoneal macrophages do not, even in the same experimental system (12). The relative distribution of proton and anion transporters likely varies, with anion influx the rate-limiting step for organelle acidification in some cells, while the non-CFTR counter ion permeability is more substantial (and not limiting) in other cell types (20). Use of CFTR<sub>inh</sub>-172 reconstituted from frozen as a stock solution in DMSO was significantly less effective than freshly dissolved inhibitor (12). It is also clear that single wavelength probes are problematic given a relationship between pH and vesicle size (22). Probes based on FITC are also of questionable value in measuring the pH of lysosomes, given the pK<sub>a</sub> of 6.2. The use of pH-sensitive probes selectively attached to CFTR and delivered to organelles through the endocytosis of CFTR is an ideal technique to assess the role of CFTR in recycling endosomes (20), but it may not provide the best assessment of lysosomal pH if CFTR arrives via an alternate route such as straight from the Golgi.

Likewise, bulky accessory compounds such as Zymosan or dextran may deliver the pH-dependent probes to one subgroup of organelles, while lysosomotropic probes such as LysoSensor Yellow/Blue may preferentially target a different subgroup. As lysosomes may well be derived from multiple pools (5), the apparent contradictions in regard to an intracellular role of CFTR might actually identify functional differences in subgroup composition. In addition, additional pathways activated by cAMP may also contribute to organelle acidification.

In this regard, our demonstration that the CFTR contributes little to the baseline levels of lysosomal acidity but aids in the regulated acidification in response may explain some of the discrepancy. The alkalinizing effect of CFTR<sub>inh</sub>-172 was larger in compromised cells treated with cAMP than under baseline
conditions, and the effect of activating CFTR was greater in
aliquotized cells (Fig. 2). The most likely explanation for this
is the reduced open probability of the CFTR channel in acidic
solutions. Single channel recording indicated that the open
probability of CFTR dropped by 47% when the extracellular
pH was decreased from 7.4 to 4.5 (32). While this decreased
open probability is consistent with the enhanced contribution
of CFTR in more alkaline conditions, other factors may con-
tribute. For example, CFTR may make a larger contribution
in cells treated with cAMP, or Cl\textsuperscript{−} influx could be more rate-
limiting after treatment with weak bases.

The ability of antagonist CFTR\textsubscript{inh}-172 to directly raise the
pH in isolated organelles (Fig. 4) provides important mecha-
nistic support for the model by which CFTR residing on the
lysosomal membrane enables Cl\textsuperscript{−} influx to counterbalance the
positive charge accompanying proton import (2). While acti-
vation of CFTR on the plasma membrane could increase
cytoplasmic Cl\textsuperscript{−} sufficiently to increase the driving force for
Cl\textsuperscript{−} into the organelle, localization of CFTR directly on the
lysosomal membrane is predicted to be much more effective
given the voltage difference across organelle membrane. When
trying to understand the action of isolated lysosomes it may be
relevant that adenylate cyclase III is localized to endosomal-
like organelles (15), and membrane-bound adenylate cyclase is
coupled to A-kinase anchoring proteins (AKAPs) (14). As the
magnitude of baseline pH was not determined in isolated
lysosomes, it is not clear whether the effect would have been
larger on alkalized lysosomes. Regardless, these experiments
do identify a direct contribution of CFTR to the regulation of
lysosomal pH. However, the inability of cAMP to alter colo-
calization of CFTR and Lamp implies that the predominant
contribution of cAMP is to the gating of CFTR and not in
trafficking of CFTR to the lysosome. However, it is likely that
cAMP acts to enhance other parallel pathways of organelle pH
regulation.

**Physiological implications.** We propose that CFTR does not
make a major contribution to the constitutive acidification of
lysosomes but plays a more subtle role in rapidly regulating the
pH. While the rate-limiting effect of anion counter transport
may mask other contributions, it is likely that cAMP also
activates alternative mechanisms in parallel to reacidify lys-
osomes. Members of the CIC family of chloride transporters
such as CIC-7 are clearly implicated in lysosomal acidification,
while CIC-3, CLC-4, and CLC-5 are involved in endosomal
acidification (16, 17). The critical role of organelle acidifica-
tion in numerous distinct processes may explain the existence
of several backup systems. In this case, CFTR may contribute
more to receptor-driven acidification in response to certain
stimuli than to the basic maintenance to pH. Regardless, this
still implies that pharmacological stimulation of CFTR may
help boost lysosomal activity under conditions or in disease
states where lysosomal pH is compromised. As a primary role
of acidic lysosomal enzymes in RPE cells is to degrade
photoreceptor outer segments, analysis of outer segment deg-
radation provided quantitation of the functional effects of
CFTR manipulation (Fig. 5). The concentration-dependent
decrease in outer segment clearance by antagonist CFTR\textsubscript{inh}-
172 strongly implicates a role for CFTR in the normal degra-
dation of outer segments, and the ability of activators CFTR\textsubscript{Ac}-16 and CFTR\textsubscript{Ac}-11 to enhance turnover of outer seg-
ments identifies CFTR as a potential regulatory target.

The ocular response is defective in CFTR\textsuperscript{−/−} mice, with the
C-wave, fast oscillations, and light peak reduced (4, 37). While
these changes in short-term responses are consistent with
presence of a defective Cl\textsuperscript{−} channel in the plasma membrane,
examination of RPE cells from older CFTR\textsuperscript{−/−} mice is needed to
determine whether they also display effects of lysosomal
dysfunction or whether a compensatory mechanism is upregu-
lated. Regardless, the ability of CFTR\textsubscript{Ac}-11 to restore lysoso-
mal pH to RPE cells in ABCA4\textsuperscript{−/−} mice suggests that this
CFTR anion permeability is still functioning in diseased RPE
cells. The mice used in this study were over 15 mo old, and the
accumulation of A2E in ABCA4\textsuperscript{−/−} mice will be substantial at
this point (20). The actions of CFTR\textsubscript{Ac}-11 imply that, despite
the sustained insults, the lysosomes retain sufficient CFTR and
other transport mechanisms to respond. As such, this identified
CFTR activation as a potentially useful target to improve
ylosomal clearance in a variety of cells.

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**AUTHOR CONTRIBUTIONS**

research; J.L., W.L., S.G., G.C.B., and E.E.C. performed the experiments; J.L.
and C.H.M. analyzed the data; J.L., A.M.L., W.W.R., and C.H.M. edited and
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approved the final version of the manuscript; W.L., R.C.R., and C.H.M.
interpreted the results of the experiments; C.H.M. drafted the figures; C.H.M.
drafted the manuscript.

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