Chloride Concentration in Endosomes Measured Using a Ratioable Fluorescent Cl⁻ Indicator

EVIDENCE FOR CHLORIDE ACCUMULATION DURING ACIDIFICATION*

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A novel long wavelength fluorescent Cl⁻ indicator was used to test whether endosomal Cl⁻ conductance provides the principal electrical shunt to permit endosomal acidification. The green fluorescent Cl⁻-sensitive chromophore 10,10'-bis(3-carboxypropyl)-9,9'-bicarbocyanine dinitrate (BAC) was conjugated to aminodextran together with the red fluorescent Cl⁻-insensitive chromophore tetramethylrhodamine (TMR). BAC fluorescence is pH-insensitive and quenched by Cl⁻ with a Stern-Volmer constant of 36 M⁻¹. Endosomes in J774 and Chinese hamster ovary (CHO) cells were pulse-labeled with BAC-TMR-dextran by fluid-phase endocytosis. Endosomal [Cl⁻] increased over 45 min from 17 to 53 mM in J774 cells and from 28 to 73 mM in CHO cells, during which time endosomal pH decreased from 6.95 to 5.30 (J774) and 6.92 to 5.60 (CHO). The acidification and increased [Cl⁻] were blocked by bafilomycin. Together with ion substitution and buffer capacity measurements, we conclude that Cl⁻ transport accounts quantitatively for the electrical shunt during vacuolar acidification. Measurements of relative endosomal volume by a novel ratio imaging method involving fluorescence self-quenching indicated a 2.5-fold increase in volume during early acidification and Cl⁻ accumulation, which was blocked by bafilomycin. These experiments provide the first direct measurement of endosomal [Cl⁻] and indicate that endosomal acidification is accompanied by significant Cl⁻ entry and volume increase.

Progressive acidification of vesicles in the endosomal pathway is important for receptor and ligand sorting and vesicular fusion (1, 2). Endosomal acidification is driven by a vacuolar-type H⁺ pump that is present in the endosomal-limiting membrane. To maintain electroneutrality, H⁺ entry into the endosomal aqueous lumen must be accompanied by anion entry and/or cation exit. The principal transportable intracellular anion is Cl⁻ and cation is K⁺. Studies of organelar pH in living cells and isolated vesicles have provided evidence that Cl⁻ entry may be the rate-limiting passive conductance in permitting active H⁺ entry in endosomes (3–9). In isolated endocytic vesicles from kidney proximal tubule (10) and liposomes constituted with proteins from clathrin-coated vesicles (11), a protein kinase A-activated Cl⁻ conductance was characterized, and it was proposed that activation of Cl⁻ channels might regulate endosomal acidification by providing a shunt to dissipate the interior-positive potential produced by the H⁺ pump. There is also evidence that Na⁺/K⁺ pump activity in early endosomes may alter the driving force for H⁺ entry and thus regulate acidification (12–14). In Golgi, there is evidence that both Cl⁻ and K⁺ conductances may contribute to acidification (15–17), whereas acidification of secretory granules in synaptic vesicles appears to require the expression of a specific Cl⁻ channel (18). Although measurements of endosomal pH have been reported utilizing ratioable pH-sensitive fluorescent indicators (19–22), there have been no measurements of ion concentrations in the endosomal lumen.

Physico-chemical considerations indicate that endosomal [Cl⁻] and pH should depend on the activity of the vacuolar H⁺ pump, the magnitude of endosomal cation (K⁺, Na⁺, and H⁺) and anion (Cl⁻ and HCO₃⁻) conductances, endosomal membrane potential, buffer capacity, Donnan potential, and cytoplasmic pH and ion concentrations. Although attempts have been made to model endosomal/organelle acidification mathematically (23, 24), the paucity of information about key endosomal parameters precludes meaningful predictions about endosomal regulatory processes. Taken in reference to endosomal pH and cytoplasmic pH/[Cl⁻], endosomal [Cl⁻] is a particularly important parameter because of its implications for relative endosomal ion conductances and membrane potential. If Cl⁻ conductance is the rate-limiting ion conductance in endosomal acidification, then the interior-positive endosomal electrical potential should produce marked Cl⁻ accumulation in the endosomal aqueous lumen during acidification.

The purpose of this study is to develop and apply methodology to measure endosomal [Cl⁻] quantitatively in living cells. For these measurements, we synthesized a ratioable long wavelength fluorescent Cl⁻ indicator that is brightly fluorescent, pH-insensitive, sensitive to [Cl⁻] from 0 to >100 mM, biochemically stable, and membrane-impermeant. The endosomal aqueous lumen in cultured cells was stained with Cl⁻ and pH indicators by fluid-phase endocytosis, and the kinetics of endosomal [Cl⁻] and pH were measured by ratio image analysis. Pulse labeling, inhibitor addition, and ion substitution maneuvers established quantitatively the role of Cl⁻ conductance in endosomal acidification. An unexpectedly low [Cl⁻] early after endocytosis led us to postulate that endosomal volume increases substantially during acidification, which was supported experimentally using a novel ratio imaging strategy to measure relative endosomal volume.

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MATERIALS AND METHODS

Reagents

All chemicals for synthesis were purchased from Aldrich. Aminodoxtran (Mw 40,000), 5- and 6-carboxyfluorescein (CF)1 succinimidyester, 5- and 6-carboxytetramethylrhodamine succinimidyester (TMR-SE), CF-carboxytetramethyl-rhodamine (TMR)-dextran (CF-TMR-dextran), calcein, sulforhodamine B, 6-methoxy-N-[3-sulfopropyl] quinolinol (SPQ), and 2′,7′-bis[2-carboxethyl]-5- and 6-carboxyfluorescein ace-toxyl ester (BCECF-AM) were obtained from Molecular Probes (Eugene, OR). Nigericin, bafilomycin, valinomycin, monensin, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were obtained from Sigma.

Cell Culture

J774.1 macrophages (ATCC no. TIB-67) were obtained from American Type Cell Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagles medium DME-H21 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. CHO-K1 cells (ATCC no. CCL-61) were also obtained from the ATCC and grown in Ham’s F12K medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured on 18-mm diameter round glass coverslips at 37 °C in a 95% air, 5% CO2 incubator and used just prior to confluence.

Synthesis of Ratiometric Fluorescent CI− Indicator

Synthesis of 10,10′-Bis[3-carboxypropyl]-9,9′-bicipridinium Dinitrate (BAC) (Compound 2)—To a stirred suspension of 10-[3-carboxypropyl]-9(10H)-acridone (Fig. 1A, compound 1) prepared according to Ref. 25 (2.8 g, 10 mM) in acetone (80 ml) was added zinc dust (13.3 g, 201 gram atom). The mixture was stirred for 20 min at 30-40 °C. The flask was cooled in ice-cold water, and 37% HCl (121 g, 1226 mM) was added dropwise over 4 h under inert atmosphere at 10 °C. The reaction mixture was stirred overnight at room temperature, and 50 ml of degassed water was added. A bright yellow precipitate was collected by suction filtration, rinsed with water, and dissolved in 50 ml of 5% aqueous NaOH. The mixture was filtered, and the filtrate was neutralized with acetic acid to give a bright yellow precipitate. The precipitate was filtered, washed with water, dried, and recrystallized from hot ethanol to yield 4.39 g (85%) of 10,10′-bis[3-carboxypropyl]-9,9′-bicipridinium dinitrate (compound 2) as a yellow crystalline solid.

A suspension of compound 2 (5.3 g, 10 mM) in 2 N nitric acid (250 ml) was heated for 1 h at 120 °C until most of the brownish mass was dissolved. After cooling and filtration, the precipitate was washed with dilute nitric acid, dried, and recrystallized from dilute nitric acid to yield 4.81 g (74%) of compound 3 as a yellow crystalline solid (absorption maximum, 435 nm; molar extinction coefficient (μM−1 cm−1) was 216). For synthesis of BAC-TMR-dextran, 10-methylrhodamine; SPQ, 6-methoxy-N-[3-sulfopropyl] quinolinol, CHO, Chinese hamster ovary; BCECF, 2′,7′-bis[2-carboxethyl]-CF. mmol) was stirred with amino dextran (1.0 g, 0.025 mmol, Mw 40,000) in aqueous NaHCO3 (25 ml, 0.1 M, pH 8.3–8.5) at room temperature for 2 h. The dextran conjugate was purified by dialysis (25,000-Da cut-off) for 24 h against 0.1 M NaHCO3 and then against water for 36 h at 4 °C. The TMR-dextran was lyophilized and monitored using TMR/dextran, 0.18 CaCl2, 0.054 mg/ml for synthesis of BAC-TMR-dextran, TMR-dextran (0.5 g, 0.0125 mmol) was reacted with compound 4 (0.25 g, 0.295 mmol) in aqueous NaHCO3 (50 ml, 0.1 M, pH 8.3–8.5) at room temperature for 3 h. The reaction mixture was worked up as above to yield 0.46 g of BAC-TMR-dextran (92% with respect to dextran); molar labeling ratio bicriudinium/TMR/dextran, 6:68:108:1.

Characterization of the Ratiometric Fluorescent CI− Indicator

Fluorescence spectra, molar extinction coefficient, and quantum yield were measured by standard procedures using a Fluoromax-3 fluorimeter. Fluorescence quenching measurements were carried out at peak excitation and emission wavelengths. Microliter aliquots of NaCl (1 m stock) were added to 3 ml of compound (10 μM in 5 mM NaH2PO4–Na2HPO4) at pH 7.4. Stern-Volmer quenching concentration (Ksv) were calculated from the slope of F/F0 = 1 versus [CI−] (F 1 plts, F/F0 = 1 = Ksv [CI−] where F is fluorescence in the absence of CI− and F in the presence of CI−).

Endosome Labeling and Cell Perfusion

Endosomes were labeled by incubation of cells on coverslips with BAC-TMR-dextran (18 mg/ml) or CF-TMR-dextran (3.5 mg/ml) for 2 min in phosphate-buffered saline (PBS; 135 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 10 mM Na2HPO4–NaH2PO4) at 37 °C. Coverslips were then washed five times in ice-cold PBS containing 2% bovine serum albumin and mounted in a custom-built perfusion chamber maintained at 37 °C in a PDMM-2 microincubator (Harvard Apparatus, Holliston, MA). Cells were generally perfused with PBS. In some experiments the perfusate contained bafilomycin A1 (200 nm) or NH4Cl (5 or 10 mM). For CI− free experiments buffer CI− was replaced by Na2SO4.

Calibration Protocols

For in vivo CI− calibrations (BAC/TMR fluorescence ratio versus [CI−]), perfusate and endosomal [CI−] were equilibrated by incubation of cells for up to 1 h at 37 °C in 120 mM KCl/KNO3, 20 mM NaCl/NaNO3, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES pH 7.4, with [CI−] from 0–100 mM (NO3− replacing CI−). Solutions contained the ionophores nigericin (10 μM), valinomycin (10 μM), CCCP (5 μM), monensin (10 μM), and the H+ pump inhibitor bafilomycin (200 μM). For in vivo pH calibrations (TMR/CF fluorescence ratio versus pH) cells were incubated with 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM MES, 120 mM KCl, 20 mM NaCl, and the ionophore/bafilomycin mixture with pH adjusted to 4.5–8.0 (in 0.5-pH unit intervals) (21).

Cytoplasmic pH was measured using BCECF by excitation ratio imaging (440 and 490 nm). Cytochrome (CI−) was measured using SPQ as described previously (27).

Kinetics of Endosomal [CI−] and pH

After fluid-phase pulse labeling (2 min) with BAC-TMR-dextran or CF-TMR-dextran and washing, the cells were perfused briefly with ice-cold PBS in the perfusion chamber and then with PBS at 37 °C. Sets of BAC and TMR images (for CI−) or CF and TMR images (for pH) were acquired at specified times (generally 0, 5, 15, 30, and 45 min). In some experiments bafilomycin (200 nm) was added to the perfusate from the beginning of the experiment or 45 min after pulse labeling.

Endosomal Buffer Capacity

Buffer capacity (β) was determined from the rapid increase in endosomal pH in response to addition of 5 mM NH4Cl to the perfusate (5 mM NH4Cl, 120 mM KCl/NaCl, 2.7 mM KNO3, 130 mM Na2HPO4–NaH2PO4) for 2 min. In some experiments the perfusate contained 200 mM HEPES pH 7.4. β was computed from the equation: β = ([NH4Cl]/ΔpH)10(β/HpH0) – pHTotal), where ΔpH is the pH increase just after NH4Cl addition, pHpH0 is perfusate pH, and pHTotal is endosomal pH after NH4Cl addition (28, 29).

Endosome Volume Changes

Endosomes were pulse-labeled with the mixture of calcine (at self-quenching concentration, 30 mM) and sulforhodamine 101 (at low concentration, 2 mM) in PBS (adjusted to 290 mosM) for 1 min followed by washing with ice-cold PBS. The coverslip was transferred to the perfusion chamber as described above. A series of calcine (green) and sulforhodamine 101 (red) images were obtained at specified times. Endosome expansion produced increased calcine fluorescence because of volume dilution without change in sulforhodamine 101 fluorescence. In

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1 The abbreviations used are: CF, 5- and 6-carboxyfluorescein; BAC, 10,10′-bis[3-carboxypropyl]-9,9′-bicipridinium dinitrate; TMR, tetramethylrhodamine; SPQ, 6-methoxy-N-[3-sulfopropyl] quinolinol; CHO, Chinese hamster ovary; BCECF, 2′,7′-bis[2-carboxethyl]-CF.
some experiments bafilomycin (200 nm) was added to the perfusate. An in vitro calibration of green-to-red fluorescence versus dilution factor curve was obtained by collecting sets of images of thin films (sandwiched between coverslips and glass slides) of PBS containing calcein (30 mM) and sulforhodamine 101 (2 mM) after specified dilutions with PBS.

Fluorescence Microscopy

Experiments were carried out using a Leitz upright fluorescence microscope equipped with a coaxial-confocal attachment (Technical Instruments, San Francisco, CA) and a 14-bit cooled (−30 °C) CCD camera (Photometrics) with a Tektronix back-thinned detector as described previously (19). Cell fluorescence was viewed with a ×100 oil immersion objective with a numerical aperture of 1.4 (Plan-apo, working distance 0.17 mm, Nikon, Garden City, NY). The light source was a stabilized Hg-Xe arc lamp (100 watt) lamp attenuated 5–50-fold using neutral density filters. Cells were identified by transillumination using dim red light (to avoid photobleaching), and focus was adjusted. Images (500-ms acquisition time) were obtained using appropriate filter sets for the BAC, TMR, CF, calcein, sulforhodamine 101, and BCECF chromophores. Electronic shutters (Uniblitz, model D122, Vincent Associates, Rochester, NJ) in the illumination and detection paths minimized sample illumination. Custom filter sets (Chroma, Brattleboro, VT) were used for detection of BAC fluorescence excitation, 470 ± 5 nm; dichroic, 505 nm; emission, 535 ± 20 nm) and TMR fluorescence (excitation, 546 ± 5 nm; dichroic, 565 nm; emission, 590-nm cut-on). Serial image acquisitions indicated 1–3% BAC photobleaching per image acquisition and <1% photobleaching for other chromophores. In time course studies endosomes in different cells were imaged for different time points.

Ratio Image Analysis

Custom software was written in Labview to compute area-integrated background-subtracted pixel intensities. In each TMR (red) image, four regions of cells containing well demarcated endosomes were identified by rectangular boxes, and for each region four nearby regions outside of the endosome/cell were identified for background computation. The same regions were automatically identified in the BAC (green) image and displaced slightly if necessary because of chromatic effects or slight sample movement. The average background was computed for each selected endosome region from the per-pixel intensities of the three lowest of the four selected background regions. After background subtraction, red-to-green (R/G) intensity ratios were computed from area-integrated intensities in each image. Three pairs of images were analyzed for each time point. The same analysis routine was used with CF-TMR images for pH measurements. Calcein/sulforhodamine images for relative volume measurements were analyzed by measuring area-integrated pixel intensities over individual endosomes in green (calcein) and red (sulforhodamine) images. Background values were taken from three areas without endosomes within the analyzed cell. Ratios were calculated for four endosomes in each image pair, and at least two image pairs were analyzed per time point.

RESULTS

Characterization of a Ratioable Cl− Indicator for Measurement of Endosomal [Cl−]−

The ratioable fluorescent Cl− indicator BAC-TMR-dextran (Fig. 1A, compound 5) was synthesized by covalently linking the chloride-sensitive chromophore BAC (synthesized in five steps from 9(10H) acridon and γ-butyrolactone) and the Cl−-insensitive chromophore TMR to amino dextran (reaction scheme shown in Fig. 1A). BAC fluorescence was sensitive to Cl− in the range 0 to >120 mM with a Stern-Volmer quenching constant of 36 M−1 (Fig. 1B, left). BAC fluorescence was insensitive to pH in the range appropriate for cellular measurements (Fig. 1B, top right) and insensitive to cations, non-halide anions (nitrate, phosphate, bicarbonate, and sulfate), and albumin. The green-fluorescing BAC chromophore has fluorescence excitation maxima at 365 and 434 nm and a broad emission spectrum with peak at 505 nm (Fig. 1B, bottom right). The red-fluorescing TMR chromophore is not sensitive to pH or ion concentrations so that the ratio of red TMR fluorescence to green BAC fluorescence gives [Cl−].

Cl− Accumulation in Endosomes—Fig. 2A shows fluorescence emission spectra of BAC-TMR-dextran, indicating the Cl−-sensitive green BAC fluorescence and the Cl−-insensitive red TMR fluorescence. For cell studies, two cell lines with different properties were used (J774 macrophages and CHO cells). Fig. 2B (left) shows a calibration of BAC-TMR-dextran red-to-green fluorescence ratio (R/G) versus [Cl−]− in aqueous solution (filled circles) and 774 cells (open circles). CI− was replaced by nitrate in the solution measurement. For cell measurements, endosomes were labeled by fluid-phase endocytosis with a 2-hr incubation with BAC-TMR-dextran in saline. Representative cell images are shown in Fig. 2B (right). As found in previous studies (20–32), endosomes were seen as distinct bright spots on a dark background. The fluorescent spots became larger with increasing chase time, corresponding to progression from early endosomes to multivesicular bodies to...
lyosomes. The red TMR fluorescence seen in Fig. 2B (right) colocalized well with the green BAC fluorescence. Endosome labeling was not seen when cells were incubated for 2 min with BAC-TMR-dextran at 4 °C instead of 37 °C (images not shown).

Calibration of R/G versus [Cl] - in endosomes was done by incubating BAC-TMR-dextran-loaded cells with a high K+ buffer containing an ionophore/bafilomycin mixture to equalize solution and endosomal [Cl] - as described in “Materials and Methods.” R/G was measured in endosomes from 8–10 different cells in three separate cell cultures. Fig. 2B (left) shows that R/G versus [Cl] - in endosomes is not significantly different from that in solution, indicating that BAC-TMR-dextran fluorescence is Cl - selective and thus insensitive to other components present in the endosome lumen.

The R/G versus [Cl] - calibration was used to determine endosomal [Cl] - under physiological conditions. Fig. 3A shows TMR and BAC images of J774 cells at 0, 15, and 45 min after pulse labeling together with a pseudocolored ratio image with a [Cl] - scale. Endosomal [Cl] - was fairly uniform in each image and increased with chase time. Fig. 3B summarizes the kinetics of endosomal [Cl] - in J774 and CHO cells as a function of chase time at 37 °C. Endosomal [Cl] - progressively increased and attained steady-state values after an ~45-min chase time. Interpretation of these data in terms of driving forces requires knowledge of endosomal pH as well as cytoplasmic pH and [Cl] - (see below). Fig. 3C shows that Cl - accumulation in endosomes was reversed by the addition of the vacuolar H+ pump inhibitor bafilomycin A1. Here the bafilomycin was added after the 45-min chase period. These data suggest that H+ pump activity drives Cl - accumulation in endosomes.

Relationship between Endosomal Acidification and Cl - Accumulation—Endosomal pH was measured under the same conditions used in endosomal [Cl] - measurements. The ratioizable pH indicator CF-TMR-dextran was used in which CF green fluorescence is pH-sensitive and TMR red fluorescence is pH-insensitive (21). Fig. 4A shows a calibration plot of TMR-to-CF red-to-green fluorescence ratio (R/G) versus pH in endosomes. The perfusate contained high [K+] - and ionophores/bafilomycin to equalize external and endosomal pH. R/G was sensitive to pH in the range appropriate for endosomal measurements. Fig. 4B summarizes the time course of endosomal pH in J774 and CHO cells as a function of chase time. Endosomes were labeled with CF-TMR-dextran for 2 min as done for the [Cl] - measurements using BAC-TMR-dextran. As reported in other cell types (19–21) endosomes progressively acidified. Bafilomy-
cin addition to the perfusate alkalinized the endosomal lumen as expected (33).

To relate changes in [Cl\textsuperscript{\textnormal{-}}] and pH quantitatively, endosome buffer capacity was measured. Buffer capacity was determined from the prompt pH increase following addition of NH\textsubscript{4}Cl (5 or 10 mM) to the perfusate (Fig. 4C) (34). Rapid entry of NH\textsubscript{3} produces luminal alkalinization, which depends on the amount of NH\textsubscript{4} entry and endosomal buffer capacity. The endosomal buffer capacities of 36 ± 1 mM/pH unit for J774 cells and 50 ± 3 mM/pH unit for CHO cells (Fig. 4C, inset) were similar to that of 43 mM/pH unit measured in opossum kidney cells (29).

Unlike the bafilomycin-induced alkalinization, NH\textsubscript{4}Cl-induced alkalinization occurs without net movement of charge across the endosomal-limiting membrane. If Cl\textsuperscript{-} exit from endosomes accompanying bafilomycin-induced alkalinization is the result of charge coupling to maintain electroneutrality, then it is predicted that NH\textsubscript{4}Cl-induced alkalinization should not be accompanied by Cl\textsuperscript{-} exit. Fig. 5A shows that after a 45-min chase following BAC-TMR-dextran pulse labeling, bafilomycin addition resulted in Cl\textsuperscript{-} exit, whereas comparable alkalinization produced by NH\textsubscript{4}Cl addition did not affect endosomal [Cl\textsuperscript{-}]. This finding in cells also supports the conclusion in solution studies (Fig. 1B, top right) that BAC-TMR-dextran fluorescence is pH-insensitive.

The time courses of endosomal acidification (Fig. 4B) and [Cl\textsuperscript{-}] increase (Fig. 3B) suggest that the entry of positive charge due to H\textsuperscript{+} influx during endosomal acidification can be accounted for by the entry negative charge caused by Cl\textsuperscript{-}. For example, a decrease in pH from 6.5 to 6.0 (where buffer capacity measurements are accurate) in J774 cells produces an H\textsuperscript{+} influx of 18 mM (\Delta pH = 36 mM/pH unit × 0.5 pH unit), similar to the measured increase in [Cl\textsuperscript{-}] of 15 mM. As a further test of the conclusion that Cl\textsuperscript{-} conductance is the major endosomal conductance, the time course of endosomal [Cl\textsuperscript{-}] was measured in the continuous presence of bafilomycin in the perfusate after pulse labeling (Fig. 5B, closed circles). Endosomal [Cl\textsuperscript{-}] was < 10 mM initially, so that there was a substantial [Cl\textsuperscript{-}] gradient driving Cl\textsuperscript{-} entry (cytoplasmic [Cl\textsuperscript{-}] ~ 45 mM, see below). A significant counterion conductance to K\textsuperscript{+} or other ions would have produced an increase in [Cl\textsuperscript{-}]. Fig. 5B also shows the time course of endosomal [Cl\textsuperscript{-}] after 2 min of pulse labeling with BAC-TMR-dextran in a zero Cl\textsuperscript{-} solution (inset, replacing Cl\textsuperscript{-} with nitrate, open circles). Endosomal [Cl\textsuperscript{-}] increases as in Fig. 3B, except that [Cl\textsuperscript{-}] are lower than those obtained when BAC-TMR-dextran is internalized in the Cl\textsuperscript{-}-containing PBS.

Cytoplasmic pH and [Cl\textsuperscript{-}]—Cytoplasmic pH was measured by excitation ratio imaging (440 nm/490 nm) using the fluorescent pH indicator BCECF. Fig. 5C shows that cytoplasmic pH was 7.31 ± 0.04 in J774 cells and 7.35 ± 0.04 in CHO cells. Cytoplasmic [Cl\textsuperscript{-}] was measured using the quinolinium-type Cl\textsuperscript{-} indicator SPQ. Cells were labeled with SPQ by overnight incubation, and SPQ fluorescence was measured continuously. Cells were initially perfused with a physiological buffer used for the endosomal [Cl\textsuperscript{-}] and pH measurements and then with a series of calibration solutions containing high K\textsuperscript{+}, ionophores, and specified [Cl\textsuperscript{-}] (Fig. 5D). Cytoplasmic [Cl\textsuperscript{-}] was 44 ± 2 mM in J774 cells and 47 ± 3 mM in CHO cells.

Endosomes Swell during Acidification and Cl\textsuperscript{-} Accumulation—The low [Cl\textsuperscript{-}] just after BAC-TMR-dextran internalization was an unanticipated observation because endosomes presumably sample the perfusate [Cl\textsuperscript{-}] of >130 mM. Because it seems unlikely that a mechanism could or should exist for the rapid pumping of Cl\textsuperscript{-} out of endosomes just after internalization, we postulated that quasi-static physico-chemical mechanisms are responsible for the low [Cl\textsuperscript{-}]. This may occur, for example, if endosomes are in a relatively low volume, collapsed state as they form, so that the Donnan effects of membrane proteins might create an interior-negative potential that excludes Cl\textsuperscript{-} during the budding process. Subsequent H\textsuperscript{+} and Cl\textsuperscript{-} entry would result in endosome swelling and decreased density of fixed negative charges. Indeed, there is morphometric evidence that endosome volume increases soon after internalization (35, 36).

To test the hypothesis that endosomes swell after internalization, we adapted a ratio imaging approach developed recently to measure osmolality in microcompartments (37). Endosomes were pulse-labeled with a mixture of calcine at self-quenching concentration (30 mM) and sulforhodamine 101 at low (non-self-quenching) concentration (2 mM). The ratio of calcine green fluorescence to sulforhodamine 101 red fluorescence (G/R) provides a semi-quantitative measure of relative endosome volume. Fig. 6A shows the dependence of G/R on dilution of the internalization solution. Dilution produces an increase in G/R because of decreased calcine self-quenching.

Fig. 6B shows fluorescence micrographs at 0 and 15 min after endosome pulse labeling with the calcine-sulfurohodamine 101 mixture. The green calcine fluorescence (relative to red sulfurohodamine 101 fluorescence) was greater at 15 min, indicating less self-quenching and hence increased endosome volume. Quantitative analysis of G/R ratios in Fig. 6C showed an ~2.5-fold increase in relative endosomal volume (V/V\textsubscript{0}) over 15 min, after pulse labeling. The increase in endosome volume was largely blocked by inhibition of acidification by bafilomycin.

**DISCUSSION**

The purpose of this study was to measure endosomal Cl\textsuperscript{-} concentration to determine whether endosomal acidification is
accompanied by Cl⁻ accumulation. As described in the Introduction, Cl⁻ is the major intracellular anion, and its transport across organellar membranes has been proposed to regulate endosomal acidification. It is not possible to predict endosomal [Cl⁻] a priori because of its many determinants including endosomal H⁺ pump activity, ion permeabilities, buffer capacity, membrane and Donnan potentials, and fusion/budding dynamics. For example, endosomal [Cl⁻] can be driven above its electrochemical equilibrium concentration if Cl⁻ is the principal transported ion that accompanies active H⁺ influx, provided that initial endosomal [Cl⁻] is fairly high and/or buffer capacity is low. To measure endosomal [Cl⁻] we synthesized and validated a fluorescent fluid-phase marker of endocytosis suitable for quantitative ratio image analysis. The new indicator was applied to measure the kinetics of endosomal [Cl⁻] in two cell lines, and the results were evaluated mechanistically by analysis of bafilomycin effects, ion substitution, endosomal volume changes, and cytoplasmic [Cl⁻] and pH.

**BAC-TMR-Dextran as a Ratioable Indicator for Measurement of Endosomal [Cl⁻]—**There were a number of requirements for the ratioable Cl⁻ indicator. Bright (high molar extinction and quantum yield) long wavelength fluorescence was needed to obtain adequate fluorescence signals from small endosomes on an autofluorescent cellular background. Quinolinium-type Cl⁻ indicators, which have been used extensively to measure cytoplasmic [Cl⁻] (38), were not suitable for measurement of endosomal [Cl⁻] because of the imperfect Cl⁻ specificity. Strict insensitivity of the indicator to pH and good sensitivity to [Cl⁻] in the range 0–100 mM was needed for measurement of endosomal [Cl⁻] as well as chemical stability in the endosomal environment. Yellow fluorescent protein-

**FIG. 5. Mechanistic analysis of Cl⁻ accumulation in endosomes.** A, J774 cells were pulse-labeled with BAC-TMR-dextran, chased at 37 °C for 45 min, and perfused with PBS containing 200 nM bafilomycin (filled circles) or 5 mM NH₄Cl (open circles) (S.E., n = three sets of experiments). [Cl⁻] was determined from R/G (right hand axis). B, J774 cells were pulse-labeled as in A except that bafilomycin (200 nM) was present in the perfusate (filled circles), or PBS was replaced by Cl⁻-free PBS (nitrate-replacing Cl⁻) during the 2-min pulse labeling. C, cytoplasmic pH was measured using 440 nm/490 nm excitation ratio imaging of the pH indicator BCECF. Calibration of excitation ratio versus pH shown together with measurements on J774 and CHO cells (S.E., n = 3) (errors smaller than circle size). Intracellular pH was manipulated using ionophores in high [K⁺] solutions as described under “Materials and Methods.” D, cytoplasmic [Cl⁻] measured using the Cl⁻-sensitive fluorescent indicator SPQ. Top, representative experiments showing the time course of cytoplasmic SPQ fluorescence in response to perfusion of J774 cells with PBS followed by calibration solutions containing high [K⁺] and ionophores with indicated [Cl⁻]. Bottom, average cytoplasmic [Cl⁻] for J774 and CHO cells (S.E., n = 3).

**FIG. 6. Time course of relative endosomal volume.** A, in vitro calibration of green-to-red fluorescence ratios (G/R) of PBS containing calcine (30 mM) and sulforhodamine 101 (2 mM) diluted by indicated factors with PBS (S.E., n = 3). B, fluorescence micrographs showing green calcine and red sulforhodamine 101 fluorescence at 0 and 15 min after 2-min pulse labeling with 30 mM calcine and 2 mM sulforhodamine 101. C, time course of relative endosomal volume (V/V₀) deduced from G/R (right hand axis) in J774 macrophages pulse-labeled as in B (S.E., n = 3). Where indicated, bafilomycin (200 nM) was present in the perfusate.
based Cl\(^{-}\) indicators were not suitable because of the strong pH sensitivity and relatively low Cl\(^{-}\) sensitivity (39). The biacridinium-dextran (BAC-TMR-dextran) conjugate synthesized here had the required bright long wavelength fluorescence, pH insensitivity, Cl\(^{-}\) sensitivity, and chemical stability. The main limitation was the susceptibility of the biacridinium chromophore to photobleaching, which was several times greater than that for fluorescein. However, adequate biacridinium images could be obtained with <3% photobleaching using relatively high concentrations during pulse labeling and using brief low intensity illumination.

Our approach for measuring endosomal [Cl\(^{-}\)] involved quantitative ratio imaging of fluorescently labeled vesicles in adherent cells. An important component of the measurement process was the sensitive imaging hardware, consisting of an objective with efficient light collection (numerical aperture, 1.4) and low autofluorescence, electronic shutters to minimize light exposure, and a high quantum efficiency-cooled CCD camera detector. Further, custom-written software for determination of background-subtracted endosome fluorescence made possible the quantitative determination of endosomal [Cl\(^{-}\)], pH, and relative volume by ratio imaging.

**Endosomal Cl\(^{-}\) Accumulation Parallels Acidification**—Endosomal acidification in J774 and CHO cells was accompanied by accumulation of Cl\(^{-}\). Cl\(^{-}\} entry was blocked by bafilomycin, even though a substantial cytoplasmic-to-endosomal Cl\(^{-}\} concentration gradient was present. The presence of a significant parallel conductance (e.g. K\(^{+}\}, H\(^{+}\}) would have permitted Cl\(^{-}\} entry. After endosomal acidification and Cl\(^{-}\} accumulation, alkalinization by bafilomycin but not NH\(_{4}\)Cl resulted in Cl\(^{-}\} exit, supporting the conclusion that charge coupling is responsible for parallel Cl\(^{-}\} and H\(^{+}\} transport. The molar quantity of Cl\(^{-}\} entry was comparable with that of H\(^{+}\} entry as determined from measurements of endosomal acidification and buffer capacity. Similar results were obtained when internalization was done with Cl\(^{-}\}-free extracellular solution; although absolute endosomal [Cl\(^{-}\}] was lower than that when internalization was done in Cl\(^{-}\}-containing solutions, the increases in [Cl\(^{-}\}] were comparable. Together, these findings indicate that Cl\(^{-}\} is the major conductance in endosomes of J774 and CHO cells and that Cl\(^{-}\} transport closely parallels H\(^{+}\} transport. However, our data do not exclude a small contribution from K\(^{+}\} conductance or H\(^{+}\} leak, the latter occurring during endosomal alkalinization following bafilomycin addition (Fig. 4B). In addition, these results provide the first data on endosomal [Cl\(^{-}\)]. If Cl\(^{-}\} is the principal endosomal conductance, the slightly greater endosomal versus cytoplasmic [Cl\(^{-}\}] at 45 min after endocytosis suggests a mildly interior-positive endosomal membrane potential (10–20 millivolts) in the steady-state. Measurements of endosomal membrane potential and luminal [K\(^{+}\}] are needed to define experimentally the remaining driving forces to complete a first-order biophysical description of endosomal H\(^{+}\}/ion transport mechanisms.

**Endosomal Acidification Is Accompanied by Increased Luminal Volume**—The low [Cl\(^{-}\}] of 17 mM (J774) and 28 mM (CHO) just after endocytosis was an unexpected observation, because extracellular fluid contains >130 mM Cl\(^{-}\). As shown schematically in Fig. 7, we reasoned that endosomal [Cl\(^{-}\}] should be low at the time of endosome budding from the plasma membrane, probably because of the high interior-negative Donnan potential of a partially collapsed nascent endosome. Subsequent acidification accompanied by Cl\(^{-}\} entry is predicted to be accompanied by a volume increase. It is not possible to predict a priori the magnitude of the volume increase because of the many factors involved (including changing activity/osmotic coefficients, the quantity of impermeant osmolytes, membrane fusion events, etc.). Morphometric studies of labeled endosomes by electron microscopy support the possibility that endosomal volume increases early after internalization (35, 36), although from static morphometry measurements it is difficult to deduce quantitative time course information. We developed an experimental approach to estimate changes in relative endosomal volume based on the self-quenching of the fluid-phase fluorescent indicator calcein (40). It was found that endosome volume increased 2.5-fold over 15 min after internalization and that the volume increase was blocked by inhibition of endosomal acidification by bafilomycin. Endosomal acidification is thus accompanied by active Cl\(^{-}\} accumulation and endosome swelling.

In summary, our results establish for the first time a quantitative ratio imaging method to measure endosomal chloride concentration. The measurement of endosomal [Cl\(^{-}\}] should have numerous biological applications in the functional analysis of putative intracellular Cl\(^{-}\} channels such as CIC3 (18, 41), CIC5 (42, 43), and CFTR (causing cystic fibrosis, Refs. 8 and 44). The “proton sponge” hypothesis (45) for efficient gene delivery by non-viral vectors (proposed to increase endosome buffer capacity and hence Cl\(^{-}\} accumulation and swelling) should be amenable to direct experimental validation. With suitable chemical modification, it should also be possible to measure [Cl\(^{-}\}] in selected endosomal subcompartments (e.g. internalization of labeled transferrin in recycling endosomes) and in organelles of the secretory pathway. Fluorophore labeling approaches have been developed to target fluorescent probes to organellar sites, including retrograde transport of labeled toxins (46), expressed single-chain antibody trapping of labeled hapten complexes (47), TGN38-based retrieval of labeled antibodies (48), expressed avidin trapping of labeled biotin (17), and trapping engineered probes by expressed cysteine-containing helices (49).

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**FIG. 7. Schematic showing increasing endosomal [Cl\(^{-}\}] and volume during progressive acidification.** See “Discussion” for explanations.
Endosome Chloride Concentration

5513

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