Organelle pH studies using targeted avidin and fluorescein–biotin

Minnie M Wu[1]*, Juan Llopis[2]*, Stephen Adams[2]*, J Michael McCaffery[3,4], Markku S Kulomaa[5], Terry E Machen[1], Hsiao-Ping H Moore[1] and Roger Y Tsien[2]

Background: Mammalian organelles of the secretory pathway are of differing pH. The pH values form a decreasing gradient: the endoplasmic reticulum (ER) is nearly neutral, the Golgi is mildly acidic and the secretory granules are more acidic still (~pH 5). The mechanisms that regulate pH in these organelles are still unknown.

Results: Using a novel method, we tested whether differences in H^+ 'leak' and/or counterion conductances contributed to the pH difference between two secretory pathway organelles. A pH-sensitive, membrane-permeable fluorescein–biotin was targeted to endoplasmic-reticulum- and Golgi-localized avidin-chimera proteins in HeLa cells. In live, intact cells, ER pH (pH_{ER}) was 7.2 ± 0.2 and Golgi pH (pH_G) was 6.4 ± 0.3 and was dissipated by bafilomycin. Buffer capacities of the cytosol, ER and Golgi were all similar (6–10 mM/pH). ER membranes had an apparent H^+ permeability three times greater than that of Golgi membranes. Removal of either K^+ or Cl^- did not affect ER and Golgi H^+ leak rates, or steady-state pH_G and pH_{ER}.

Conclusions: The Golgi is more acidic than the ER because it has an active H^+ pump and fewer or smaller H^+ leaks. Neither buffer capacity nor counterion permeabilities were key determinants of pH_G, pH_{ER} or ER/Golgi H^+ leak rates.

Addresses: [1]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA. [2]Department of Pharmacology and Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093, USA. [3]Integrated Imaging Center, Department of Biology, Johns Hopkins University, Baltimore, MD 21218-2685, USA[4] Division of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA 92093, USA. [5]Department of Biology, University of Jyvaskyla, FIN-40351, Jyvaskyla, Finland.

Correspondence: Terry E Machen
E-mail: machen@socrates.berkeley.edu

*These authors contributed equally to this work.

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Introduction

Organelles of the secretory pathway are thought to exhibit a gradient of decreasing pH. This pH gradient begins at the near neutrality of the endoplasmic reticulum (ER; pH 7.1 [1]), becoming mildly acidic in the Golgi (pH 6.2–7.0 [2–4]) and quite acidic within the secretory granules (~pH 5.0 [5]). Many processes in these organelles are dependent upon proper lumenal pH: activation of enzymes and sorting and processing of secretory proteins all require precisely maintained lumenal pH [6–8]. Even pH differences of less than 0.5 between organelles can be essential for compartmentalizing cellular events. For example, <15% of the secretory prohormone pro-opiomelanocortin is cleaved into its mature form (adrenocorticotropic hormone) at pH values greater than 6.0, which is the approximate pH of the *trans*-Golgi network (TGN), whereas at pH 5.5 (which is the expected lumenal pH of secretory granules) more than 85% of the prohormone undergoes processing [8]. The general goal of our studies is to determine the mechanisms that each organelle along the secretory pathway uses to maintain different lumenal pHs.

It has been widely assumed that lumenal pH is controlled by the concentration of H^+ pumps, activity of which is determined by the magnitude of the membrane potential across the organelle membrane. This mechanism (e.g., see [9]) was proposed on the basis of experiments in which endosomal acidification was inhibited by removing Cl^- counterions (the presence of which would decrease membrane potential and facilitate organelle acidification) and inactivating the Na^+/K^+ ATPase (the electrogenic activity of which should impede maximal acidification by the H^+ v-ATPase [10]). From *in vitro* experiments, Al-Awqati and colleagues [11,12] also concluded that the acidic pH of isolated, purified Golgi and secretory granules was maintained by H^+ pumps coupled to parallel Cl^- conductances. More recently, Seksek *et al.* [2] and Llopis *et al.* [4] each showed that removing Cl^- from the bathing solutions caused Golgi pH (pH_G) to become more alkaline. Although these results are consistent with Cl^- playing a significant role in shunting lumen-positive voltages associated with H^+ v-ATPase pumping, there is also contradictory evidence indicating that the pH of some organelles (i.e. TGN [13]) is not limited by Cl^- conductances.

An alternative mechanism was recently proposed by Kim *et al.* [1], who showed that, when the cytosol was acidified, pH_G acidified more slowly than ER pH (pH_{ER}). They

suggested that the Golgi had a much smaller H^+ permeability than the ER. This result, however, was difficult to interpret, because the experiment was done under conditions in which H^+ permeabilities could not be accurately measured, as the Golgi H^+ v-ATPase was active. Furthermore, the slow Golgi H^+ leak they observed was inconsistent with their finding that pH_G alkalinized rapidly when they treated the cells with the H^+ v-ATPase inhibitor bafilomycin (see also [2]). There has therefore been no direct comparison of pH regulation by H^+ pumps to that by leaks among organelles of the secretory pathway and the mechanisms by which organelles regulate their pHs have yet to be defined.

The most systematic way to determine how the different pHs in organelles of the secretory pathway are established is to compare the pH regulatory mechanisms of adjacent organelles along the pathway, using consistent experimental techniques and protocols. We have devised a novel method in which fluorescein–biotin-based pH dyes are targeted to any organelle along the secretory pathway using avidin-chimera proteins. The recently reported targeted-antibody, fluorescent-hapten method of Farinas and Verkman [14] has many of the same advantages as our method in that all secretory pathway organelles can be targeted and the pH dyes are ratiometric. The pH indicators we have developed are, however, not only ratiometric but also have a broad range of pKa values (4–7). No previous approach has satisfied all these criteria [1–5,14,15]. As a proof of principle, we tested whether the ER and Golgi, the first two compartments of the secretory pathway, maintained their different pHs by regulating H^+ pump and leak activities and/or by regulating counterion conductances. A key aspect of our approach was to monitor H^+ leak rates by measuring rates of change of pH_{ER} and pH_G under conditions in which active H^+ pumping was inhibited (by bafilomycin); and the same transmembrane pH gradients (pH 7.4 versus pH 6.0) were applied in both intact and permeabilized cells. These important criteria have not been fulfilled in previous investigations [1–5,14,15]. The role of counterion conductances in regulating rates of H^+ leak across the membranes was determined by measuring rates of H^+ leak into organelle lumens in Cl-containing and Cl-free solutions and rates of H^+ leak out of organelle lumens in K-containing and K-free solutions. Our data indicate that the ER and Golgi maintain different pHs because of their different H^+ v-ATPase and H^+ leak activities.

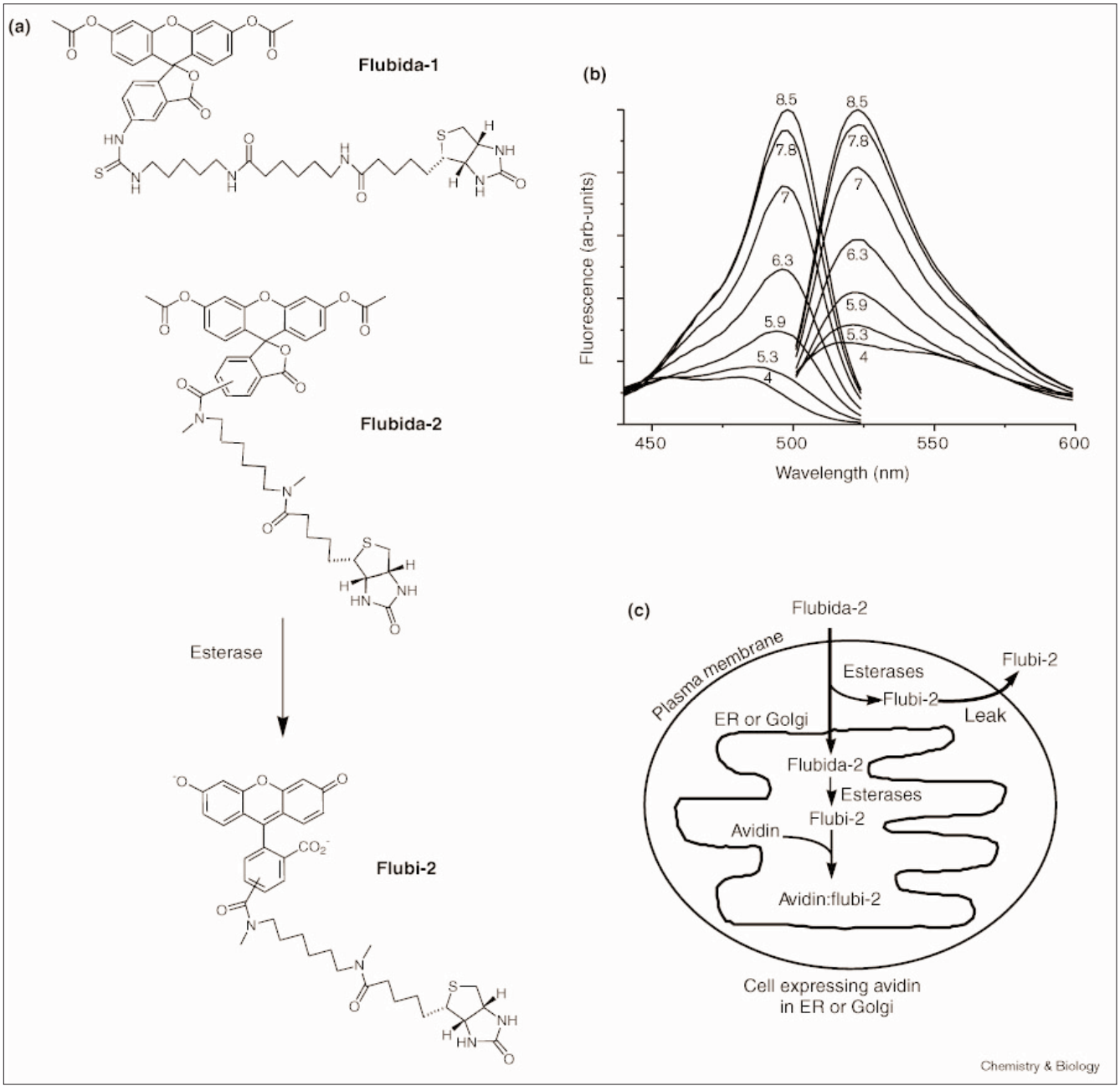
Results

Experimental strategy, pH dependency of fluorescence of Flubi dyes, and targeting of avidin to the ER and Golgi

We have developed the following strategy as a general way to target small fluorescent probes to the ER, Golgi or other organelles (Figure 1c). Chimeras of avidin that have targeting sequences to direct their expression to the organelle of interest are constructed and expressed in mammalian cells by transfection. A fluorescent indicator covalently conjugated to biotin is added to the extracellular medium; any charged groups may require masking (e.g. acetyl esters) to make the molecule less polar and therefore more membrane permeable. The membrane-permeable dye–biotin conjugate then binds to the avidin-chimera expressed in the organelle. The removal of masking groups releases the fluorescent indicator, which is now sensitive to ion concentration but is retained in the organelle. Excess dye–biotin is allowed to exit the cytosol and irrelevant cellular compartments.

We chose fluorescein as a pH indicator because of the commercial availability of a test compound, fluorescein–biotin (termed here Flubi-1). The membrane-permeable diacetate of Flubi-1 (Flubida-1) was synthesized (Figure 1a). Next, we constructed organelle-specific avidin-chimera proteins. Soluble avidin (AV) was appended with the carboxy-terminal tetrapeptide KDEL, an ER retrieval motif, to give AV–KDEL, which was used to target indicators to the ER lumen. The targeting sequence for sialyltransferase (ST), a *trans*-Golgi resident enzyme, was fused to avidin to make a ST–avidin chimera (ST–AV), which we used to label the Golgi lumen. Cells expressing these avidin-chimera proteins were loaded with membrane-permeable (non fluorescent) Flubida and then chased in dye-free medium for a minimum of 2 hours. Cleavage of the acetyl groups by intracellular esterases released the membrane-impermeable (fluorescent) Flubi (Figure 1c). The strong interaction between avidin and biotin ($K_d = 10^{-15}$ M) ensured specific labeling of the avidin-containing compartment by Flubi dyes, with minimal background fluorescence. Any fluorescein–biotin not bound to avidin leaked out of the cells during the subsequent chase. Initial fluorescein–biotin conjugates (e.g. Flubi-1) labeled the organelles that expressed avidin, but dye also accumulated in the cytoplasm. Overnight incubation of the cells in dye-free solution was therefore required to reveal the specific staining. Use of a less polar linker between the fluorescein and biotin moieties such as in Flubi-2 (Figure 1a) allowed brighter specific labeling (Flubi-2 when bound to avidin has a fluorescent quantum yield of ~0.3, compared with 0.17 for avidin-bound Flubi-1) and a more rapid leakage of unbound dye from the cytoplasm (within 2 hours). The pH dependency of the excitation and emission spectra of avidin bound to Flubi-2 *in vitro* is shown in Figure 1b. This probe was advantageous for the present experiments because it had a pK_a of about ~6.7 ($pK_a = 6.8$ for ER *in situ*, $pK_a = 6.6$ for Golgi *in situ*), which allowed it to be used for measuring both pH_G (~pH 6.4) and pH_{ER} (~pH 7.2). Flubi dyes with lower pK values (4.0, 5.0 and 6.2; M.W., J.L., H-P.M., T.M., *et al.*, unpublished observations) can be used to make measurements in more acidic organelles and in any cell that will

Figure 1



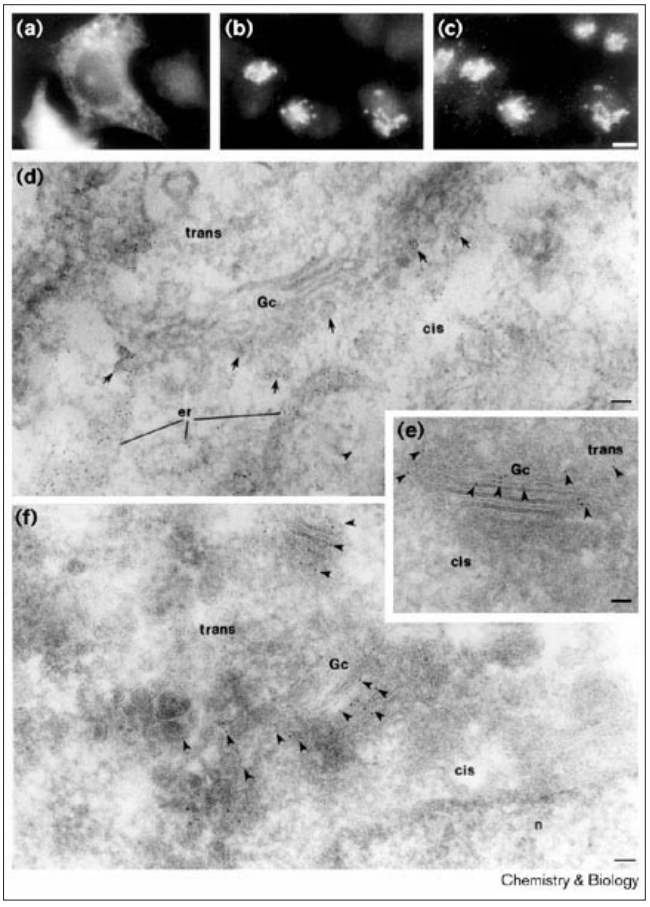
The pH-dependent fluorescence of flubi-2 and a strategy for targeting pH-sensitive dyes to organelles. **(a)** Chemical structures of Flubida-1, Flubida-2 and Flubi-2. **(b)** The pH dependence of Flubi-2's excitation (emission = 530 nm) and emission spectra (excitation = 480 nm). **(c)** A strategy for targeting Flubi-2 to the ER and Golgi compartments.

express the chimera proteins. These Flubi indicators can be used in the ratiometric mode by using dual excitation at 440 and 490 nm (Figure 1b).

Intracellular localization of AV–KDEL, ST–AV and bound Flubi dyes

The subcellular localization of AV–KDEL and ST–AV was determined in HeLa cells using both immunofluorescence and immunogold electron microscopy. Cells were transiently transfected with either AV–KDEL or ST–AV DNA and processed for immunofluorescence. Figure 2a shows AV–KDEL localized to a diffuse, reticular network characteristic of the ER. ST–AV (Figure 2b) was localized to a juxtanuclear structure that co-localized with Golgi-associated, but not cytosolic, coatomer protein β subunit (β-COP; Figure 2c). Treatment with the fungal metabolite brefeldin

Figure 2



The intracellular localization of AV–KDEL and ST–AV. HeLa cells were transiently transfected with either AV–KDEL or ST–AV DNA. Immunofluorescence staining and immunogold labeling were performed on fixed cells. **(a)** AV–KDEL was localized to a diffuse, reticular network characteristic of the ER. **(b)** ST–AV was localized to a juxtanuclear structure that co-localized with **(c)** the Golgi marker β-COP. **(d)** Immunogold labeling of ultrathin cryosections showed abundant AV–KDEL label in the ER, with sparse label in transitional elements (arrowheads). Note the lack of gold particles in the adjacent Golgi. er, endoplasmic reticulum; Gc, Golgi complex; arrows indicate label at/near the *cis* face of the Golgi. **(e,f)** Immunogold labeling revealed that ST–AV was primarily distributed across the *medial-* and *trans-* Golgi cisternae/TGN. n, nucleus; arrowheads indicate gold labeling found *medial* to *trans* in the Golgi complex. Bars = 5 μm (a–c); 0.1 μm (d–f).

A caused ST–AV to redistribute from the Golgi to the ER (data not shown). This behavior is consistent with that of other Golgi resident proteins treated with brefeldin A [16].

Immunogold labeling of ultrathin cryosections showed the localization of abundant label (AV–KDEL) to the ER (Figure 2d) and more sparse localization to the *cis*-Golgi network (CGN) including transitional elements. These observations are consistent with previously reported KDEL localizations. Although immunogold was found consistently on the CGN, label was never observed on the *cis*-most Golgi cisternae (G_C) or beyond this point (i.e., *medial/trans*-Golgi, TGN or plasmalemma). Label of ST–AV (Figure 2e,f) appeared to be distributed primarily across the *medial*-Golgi cisternae and the *trans*-cisternae/TGN, which is consistent with previously observed localizations for sialyltransferase [17].

We next determined whether the Flubi-2 dye localized properly to avidin-chimera-containing ER and Golgi compartments of HeLa cells. Cells were transiently transfected with either AV–KDEL or ST–AV DNA. They were then loaded with Flubida-2, chased and processed for immunofluorescence staining with endogenous ER or Golgi markers. Figure 3a shows Flubi-2 localized to a diffuse, reticular network characteristic of the ER in AV–KDEL expressing cells. This Flubi-2 staining co-localizes very well with calreticulin, an endogenous ER protein (Figure 3b). In ST–AV expressing cells, Flubi-2 was localized to a juxtanuclear structure (Figure 3c) that co-localized with Golgi-associated β-COP (Figure 3d).

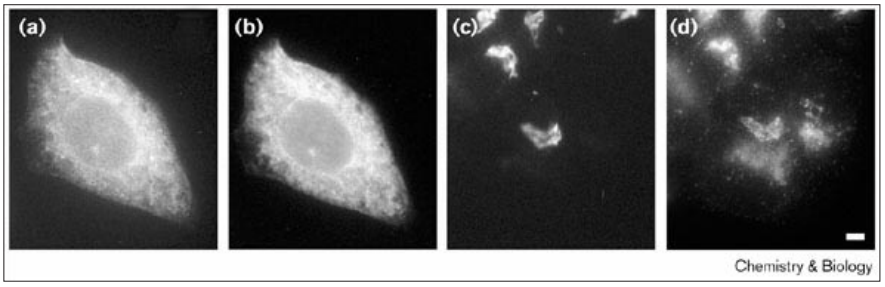
pH titration of Flubi-2 in the ER and Golgi of intact cells

The pH dependence of the 490 nm/440 nm excitation ratio of Flubi-2 *in situ* within living cells is shown in Figure 4. The ER (AV–KDEL) and Golgi (ST–AV) were labeled with Flubi-2, and the pH in these compartments was clamped to the different values shown using solutions containing the ionophores nigericin and monensin. The 490 nm/440 nm fluorescence ratio was plotted against the pH of the solution to generate calibration curves for both the ER and Golgi. Figure 4 shows the average calibration curves for each organelle. Flubi-2 has a very similar pH dependence whether it is expressed in the lumen of the ER bound to AV–KDEL (Figure 4a; pK_a 6.8 ± 0.03, n = 13), in the Golgi lumen bound to ST–AV (Figure 4b, pK_a 6.6 ± 0.04, n = 43), or *in vitro* bound to avidin (Figure 1b, pK_a 6.5). Average pH_{ER} and pH_G values were determined by averaging the steady-state pH of ER and Golgi compartments of cells bathed in pH 7.4 Ringer's solution. The average pH_{ER} was 7.20 ± 0.23 (n = 26 cells), close to the average cytosolic pH (pH_C) measured with 7′-bis-(2-carboxyethyl)-5-(and 6)-carboxy fluorescein acetoxymethylester (BCECF-AM) and fluorescein isothiocyanate (FITC)–dextran (7.4 ± 0.2, n = 119), whereas steady-state pH_G averaged 6.36 ± 0.29 (n = 46; Figure 4).

ER and Golgi have similar buffer capacities but different recoveries from NH_3/NH_4^+ treatments

At least four mechanisms affect organelle pH: buffer capacity, counterion conductances, and activities of H^+ pumps and H^+ leaks. We began by comparing the buffer capacities of the cytosol, ER and Golgi. pH_C of intact HeLa cells was measured using BCECF-AM; pH_{ER} and pH_G were measured using Flubi-2 in intact HeLa cells (Figure 5). NH_4Cl treatment caused all three

Figure 3



Intracellular localization of Flubi-2 in AV–KDEL and ST–AV expressing HeLa cells. HeLa cells transiently transfected with either AV–KDEL or ST–AV DNA were loaded with 3 μM Flubida-2 overnight, chased for 2 h in dye-free media, fixed, permeabilized, and then stained for endogenous ER and Golgi markers. **(a)** Flubi-2 co-localized with **(b)** an ER resident protein, calreticulin, in AV–KDELexpressing cells. In ST–AVexpressing cells, **(c)** Flubi-2 co-localized with **(d)** Golgi-associated β-COP. Bar = 5 μm.

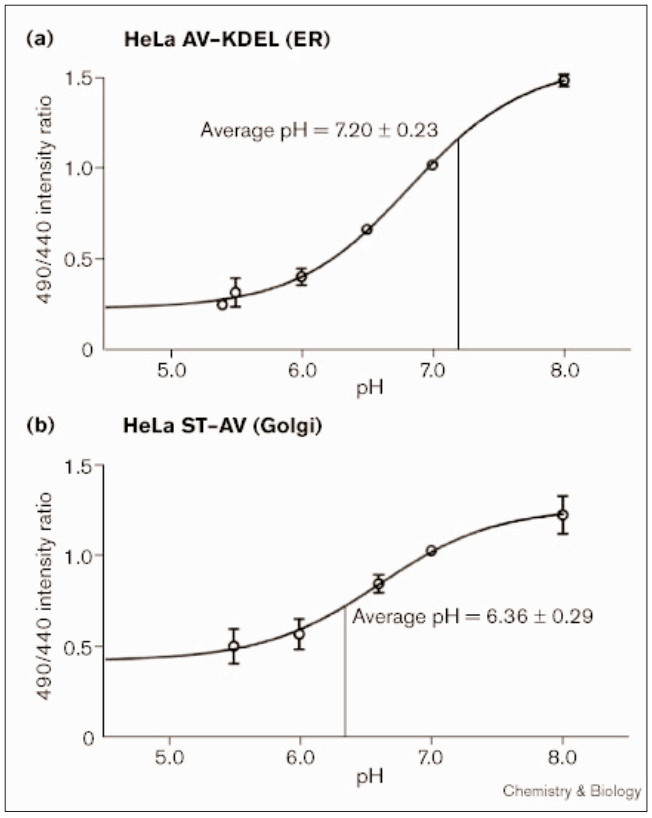
compartments to alkalinize because of rapid entry of membrane-permeable NH_3. Immediate changes in pH_C, pH_{ER} and pH_G during treatment of intact HeLa cells with either 30 mM or 40 mM NH_4^+-containing solution were used to calculate buffer capacities (β) of the cytosol, ER, and Golgi (see equation *2* in the supplementary material section). The following results were obtained from experiments similar to those in Figure 5: $\beta_C = 6.9 \pm 5.1$ mM/pH (n = 95), $\beta_{ER} = 5.9 \pm 6.3$ mM/pH (n = 33), and $\beta_G = 10.5 \pm 8.6$ mM/pH (n = 16). Because the cytosol, ER and Golgi had approximately the same buffer capacities in these experiments, it is unlikely that these organelles maintained different steady-state pHs by having different buffer capacities. The large standard deviations in our measurements might have been due to: different rates of mixing of NH_3/NH_4^+ solutions into the chamber, possible contributions of pH regulatory mechanisms in the organelle and plasma membranes, and/or finite permeability of membranes to NH_4^+. These factors, however, were similar for all the compartments, and therefore the variabilities were similar for all of them.

We also compared how the pH of different organelles recovered from NH_3/NH_4^+ treatments, to assess the NH_3/NH_4^+ permeabilities of the ER, Golgi and plasma membranes. After washout of the NH_3/NH_4^+-containing solution with Ringer's, pH_C and pH_{ER} both acidified past the baseline, probably as a result of the slow accumulation of NH_4^+ during treatment with the NH_3/NH_4^+-containing solution. In contrast, pH_G did not acidify but returned to the baseline after washout (Figure 5b), indicating that NH_4^+ had not entered the Golgi during the NH_3/NH_4^+ treatment. This could have been due to low NH_4^+ permeability of Golgi membranes, or the Golgi may have had a positive lumenal voltage.

Are Cl^- or K^+ conductances determinants of pH_G?

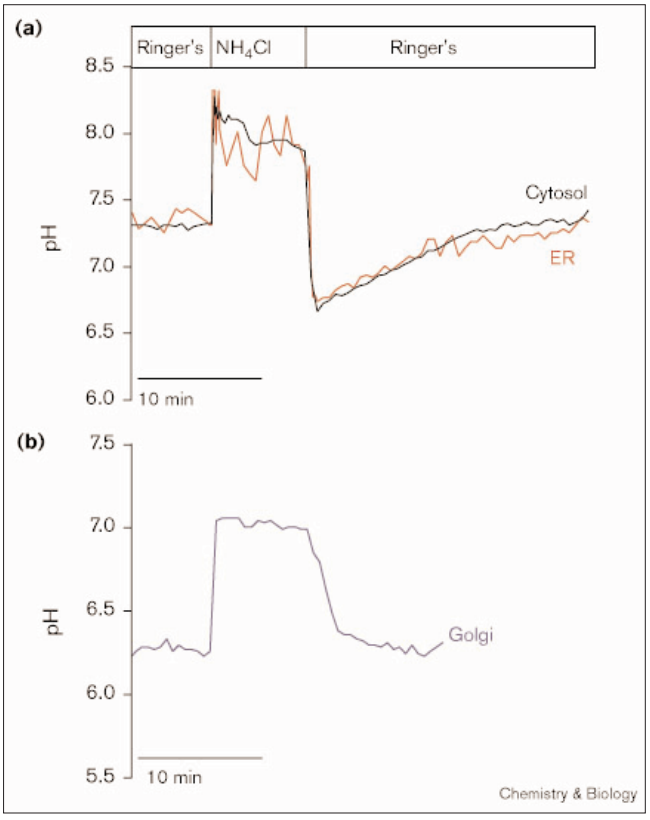
We next investigated the role of Cl^- and K^+ counterion conductances in controlling steady-state pH_G. Removing Cl^- (by replacing with gluconate) from the extracellular solution of intact cells did not affect steady-state pH_G (Figure 6a), indicating that Cl^- was not essential for maintaining an acidic pH_G. Experiments were also performed to test the role of K^+ permeability in regulating steady-state pH_G. Increasing the K^+ conductance by treatment with valinomycin, a K^+ ionophore, did not affect steady-state pH_G (data not shown).

Figure 4



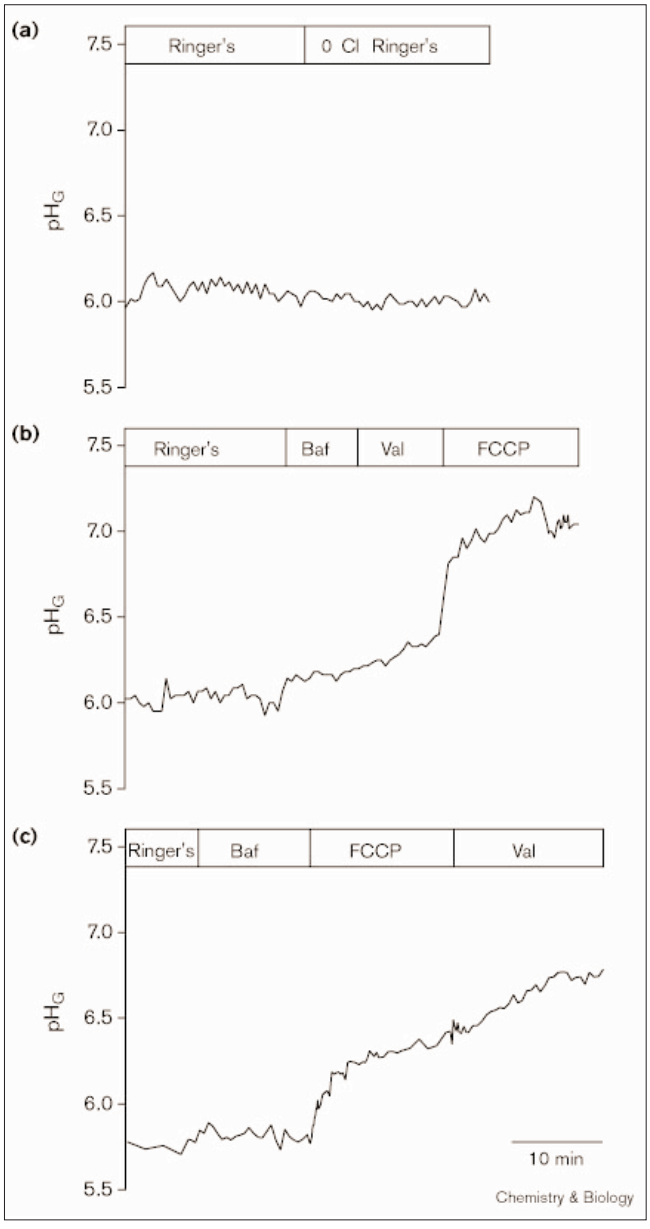
In situ characterization of Flubi-2. 490 nm/440 nm excitation ratio (emission = 520–560 nm) versus pH_{ER} and pH_G. Calibration solutions containing nigericin and monensin (10 μM each) were perfused onto cells at the end of each experiment. At least four solutions of different pHs (8.2, 7.0, 6.0, 5.5 and/or 5.0) were used. Calibration curves for **(a)** ER and **(b)** Golgi were used to convert ratio values to pH. Solid lines show averages for pH_{ER} and $pH_G \pm$ SEM obtained for 26 and 46 cells, respectively.

Figure 5



ER and Golgi have different pHs, and NH_3 and NH_4^+ permeabilities. pH_C (black curve, measured using BCECF-AM), pH_{ER} and pH_G (red and blue curves, respectively, measured using Flubi-2) were studied in intact HeLa cells. **(a)** Steady-state pH_{ER} and pH_C were about 7.4, whereas **(b)** pH_G was about 6.3. Treatment with 30 mM NH_4Cl caused all three compartments to alkalinize, probably because of rapid entry of NH_3. After washout of the NH_3/NH_4^+-containing solution with Ringer's, pH_C and pH_{ER} both overshot past baseline (a), whereas pH_G (b) returned to baseline without an overshoot.

Figure 6



Golgi pH (in intact cells) is not affected by magnitude of K^+ or Cl^- conductances. **(a)** Removal of Cl^- (0 Cl Ringer's, Cl replaced with gluconate) from the extracellular solution had no effect on pH_G in intact cells. **(b)** When cells were treated with bafilomycin (Baf; H^+ v-ATPase inhibitor, 500 nM), pH_G alkalinized at a finite rate. Further addition of valinomycin (Val; K^+ ionophore, 10 μM) did not change the rate of Golgi alkalinization. Final addition of FCCP (protonophore, 10 μM) caused pH_G to alkalinize rapidly. **(c)** Cells were treated with 500 nM bafilomycin to begin alkalinizing the Golgi, then treated with 20 μM FCCP, which caused pH_G to alkalinize rapidly and reach a plateau at around pH 6.5. Addition of 10 μM valinomycin after FCCP treatment caused further alkalinization, allowing pH_G to reach a new plateau at pH of about 7.

We also tested whether K^+ counterion conductance was required for H^+ leakage out of the Golgi. As shown in Figure 6b, cells were treated first with 500 nM bafilomycin to inhibit the H^+ v-ATPase, which caused pH_G to increase. Subsequent addition of 10 μM valinomycin did not affect the rate of Golgi alkalinization; the rate of alkalinization increased only upon addition of the protonophore carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP; 10 μM). When valinomycin was added to bafilomycin-treated cells after FCCP (Figure 6c), however, pH_G increased to a higher level than with FCCP alone. These results indicate that the permeability of the Golgi membrane to Cl^- and K^+ is greater than the permeability to H^+ (although K^+ permeability could be increased by valinomycin). The results further indicate that, in the steady state, pH_G is not limited by either Cl^- or K^+ conductances individually, but is limited by the number of H^+ leak pathways (shown by the FCCP response). We next compared the H^+ permeability across ER and Golgi membranes to establish whether H^+ leaks were a major determinant of steady-state pH_{ER} and pH_G.

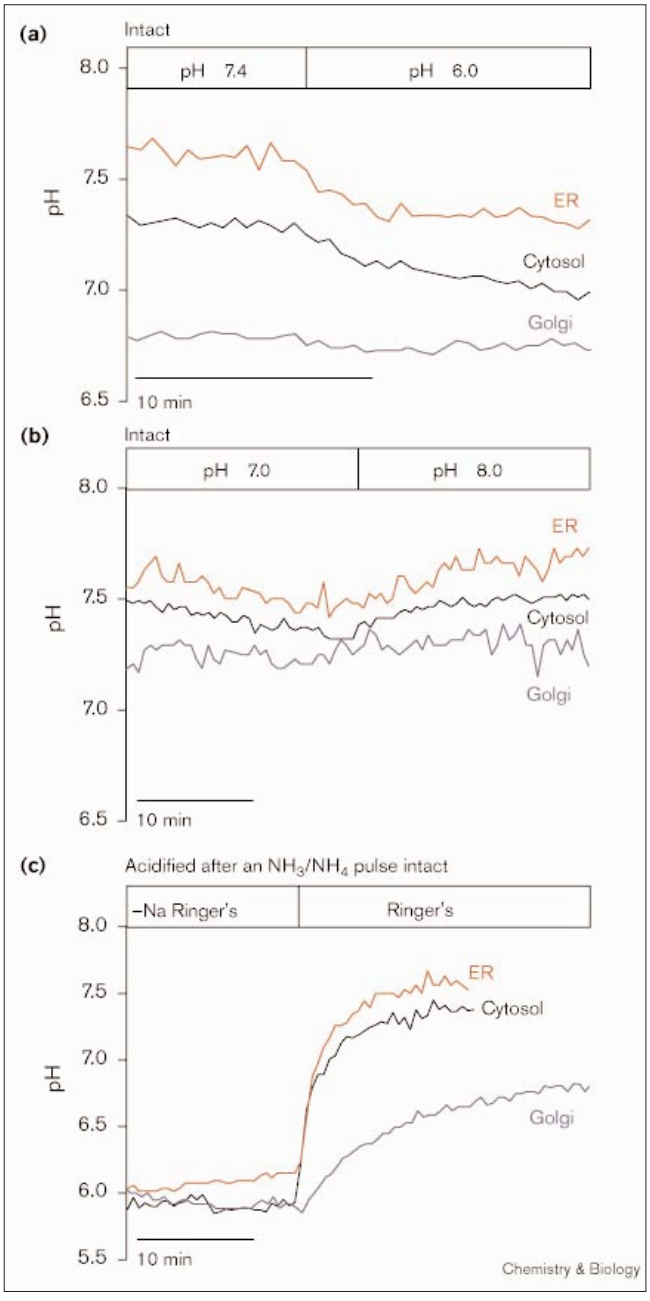
Responses of ER, Golgi and cytosol to altered transmembrane pH gradients

We measured the H^+ permeabilities across ER and Golgi membranes, as the different values and responses of pH_{ER} and pH_G to NH_3/NH_4^+ perturbations could have been caused at least in part by differences in the H^+ permeabilities of these membranes. Kim *et al.* [1] conducted experiments in which rates of acidification of pH_{ER} and pH_G were compared when the cytosol was acidified by treatment with Na-free, low-pH extracellular solution and found that the ER had a greater apparent H^+ permeability than the Golgi. The transmembrane pH gradients used in the ER, however, were different from those used in the Golgi, which could have contributed to the apparent differences found. We therefore tested for apparent H^+ leaks using protocols that assured similar pH gradients were employed by inhibiting the Golgi H^+ v-ATPase. We also tested whether apparent H^+ leaks were symmetrical, that is, whether rates of change of pH were similar when H^+ fluxed into and out of the organelle.

We first compared H^+ leak rates of the ER with those of the Golgi by measuring the responses of pH_{ER}, pH_G and pH_C to changes in extracellular pH (pH_O; Figure 7a,b). Measurements of pH_{ER} and pH_G were performed on cells pretreated with 500 nM bafilomycin (H^+ v-ATPase inhibitor) for 2 hours. Consistent with the previous work of Kim *et al.* [1], bafilomycin had no effect on pH_{ER} (data not shown) but caused pH_G to alkalinize (see Figure 6b), thus indicating that a H^+ v-ATPase is active in the Golgi but not in the ER. As shown in Figure 7a, when pH_O of intact, bafilomycin-treated HeLa cells was shifted from 7.4 to 6.0, pH_{ER} mirrored pH_C changes, whereas pH_G was less responsive. Similarly, when pH_O of intact HeLa cells was changed from 7.0 to 8.0, pH_{ER}, pH_C, and pH_G all changed similarly, although the pH_G response was slight (Figure 7b).

A second set of experiments was designed to study H^+ leak rates across the ER and Golgi under conditions in which larger *trans*-ER and Golgi membrane pH gradients were generated. This was done by pretreating cells with bafilomycin and then acid-loading the cytosol and the organelles with an NH_3/NH_4^+ pulse (pH_O remained constant). To acid-load the Golgi, both the concentration and duration of the NH_4Cl treatments were increased, because during our previous NH_4Cl treatments (30 mM, 8 minutes) pH_G did not acidify past the baseline (Figure 5b). H^+ leak rates measured from the acid-loading experiments are shown in Figure 7c. Cells were treated with 40 mM NH_4Cl for 20 minutes, followed by a washout of this solution with Na-free Ringer's. In the presence of Na-free Ringer's, pH_C remained acidic, which is likely to be due to the inability of the Na^+/H^+ exchanger in the plasma membrane to recover pH_C. In addition, both pH_{ER} and pH_G also acidified to less

Figure 7



Responses of the ER, Golgi, and cytosol to altered transmembrane pH gradients. Cells were pretreated with 500 nM bafilomycin before all experiments to inactivate the H^+ v-ATPase. **(a,b)** Extracellular pH (pH_O) was changed from either 7.4 to 6.0 or from 7.0 to 8.0 to study H^+ leak rates for ER (red), Golgi (blue) and plasma membranes (black). **(c)** The H^+ permeabilities of ER, Golgi and plasma membranes were studied in intact cells (in separate experiments) by acid-loading the cytosol with an NH_3/NH_4^+ pulse. Cells were treated with 40 mM NH_4Cl for 20 minutes, followed by washout with Na-free Ringer's (–Na Ringer's). After 20 minutes, Na-containing Ringer's was added, and the H^+ leak out of the ER and Golgi was monitored and compared with the rate of alkalinization of pH_C. pH_{ER} alkalinized at similar rates as pH_C, whereas pH_G recovered at a slower rate than pH_C and pH_{ER}.

than 6.4 (Figure 7c). When Na^+-containing Ringer's was returned to the chamber, pH_C recovered rapidly to pH 7.4, and the H^+ leak out of the ER and Golgi was monitored from the rates of increase of pH_{ER} and pH_G. As shown in Figure 7c, pH_{ER} alkalinized at a similar rate to that shown by the recovery of pH_C, whereas pH_G was slower and recovered only to pH 6.7 in 25 minutes. The faster cytosolic alkalinization shown in Figure 7c than that shown in Figure 7b was most likely caused by the pH-dependency of the Na^+/H^+ exchanger [18–20], which is stimulated when $pH_C = 6.5$ and $pH_O = 7.4$ (Figure 7c) and inhibited when pH_C is > 7.1 (Figure 7b).

The data for the pH recovery of cytosol, ER and Golgi were fit to a single exponential equation, from which we obtained rate constants (k). For each rate constant, the half-time ($t_{1/2}$) for pH recovery from the acid load was determined for each compartment. The results from these experiments are summarized in Table 1. The $t_{1/2}$ values for the pH recoveries of the cytosol and ER were nearly identical (99 and 105 seconds, respectively), indicating that the H^+ leak out of the ER occurred at the same rate as the H^+ were pumped out of the cytosol into the extracellular space by the Na^+/H^+ exchanger. As these measurements are limited by plasma membrane pH regulatory mechanisms, 105 seconds is the lowest estimate for the ER H^+ leak rate. Although the H^+ leak out of the Golgi was large, the $t_{1/2}$ for pH_G recovery (347 seconds) was more than three times slower than the recoveries of both pH_C and pH_{ER}. These results indicate that, in the absence of an active H^+ v-ATPase, both ER and Golgi membranes are quite permeable to H^+ equivalents, but the H^+ leak out of the ER is at least three times faster than the leak out of the Golgi.

Removal of K^+ or Cl^- does not affect ER or Golgi H^+ leak rates

Further experiments were performed to determine the relative magnitudes of counterion conductances in ER and Golgi membranes by measuring rates of apparent H^+ leak out of and into the organelles using constant transmembrane pH gradients. Because the buffer capacities of the ER and Golgi were approximately equal, different rates of change of pH were indicative of different rates of H^+ leak across the ER and Golgi membranes. We measured rates of apparent H^+ leak under control conditions and under conditions in which either Cl^- or K^+ conductances might be rate limiting. K-free solution would be expected to slow down passive H^+ leak out of the organelle lumen into the cytosol if K^+ were the major conductance of the membranes. Cl-free solution would be expected to slow down H^+ flux into the organelle lumen from the cytosol if Cl^- were the major conductance of the membranes. If neither Cl-free nor K-free solutions affected rates of apparent H^+ leak, however, then the most likely conclusion would have been that K^+ and Cl^- conductances were roughly equal to each other, and neither was rate limiting for determining rates of H^+ flux across the membrane. We measured the effect of K^+ or Cl^- removal on pH_{ER} and pH_G in bafilomycin-pretreated, plasma-membrane-permeabilized cells. We selectively permeabilized the plasma membranes of cells using streptolysin-O (SL-O), which did not significantly alter the properties of organelle membranes as Golgi membranes of permeabilized cells still responded to NH_3 and maintained an acidic pH of less than 6.5 when not pretreated with bafilomycin (data not shown).

A typical experiment monitoring pH_{ER} is shown in Figure 8. Permeabilized cells were first incubated in pH 7.4 intracellular buffer (IB) in which K^+ and Cl^- were the only major permeable ions; the cells were then shifted to pH 6.0 IB and returned to pH 7.4 IB to monitor the ER H^+ leak rates in the presence of counterions. We then repeated the pH changes (7.4 to 6.0 to 7.4) but this time with IB solutions lacking Cl^-. This experiment was carried out again to test the K^+ requirement for H^+ leak rates across the ER and to test K^+ and Cl^- requirements for the H^+ leak across the Golgi.

The data for the H^+ leak out of the ER and Golgi (when pH_C was changed from 6.0 to 7.4 in either K-containing or K-free IB solutions) were fit to single exponential curves from which rate constants and $t_{1/2}$ values were obtained. The data in Table 2 show that, in SL-O permeabilized cells, removal of K^+ had no effect on the half-times for the H^+ leak out of either the ER or Golgi. The data for the H^+ leak into ER and Golgi (when pH_C was changed from 7.4 to 6.0 in either Cl-containing or Cl-free IB solutions) fit double exponential decay equations; the two $t_{1/2}$ values ($t_{1/2a}$ and $t_{1/2b}$, corresponding to the two rate constants, k_a and k_b, obtained for each curve) for both ER and Golgi are listed in Table 3. The $t_{1/2a}$ and $t_{1/2b}$ values for both ER and Golgi were the same in the presence or absence of Cl^-, indicating that Cl^- counterion conductances were not required for the H^+ leak into either the ER or the Golgi.

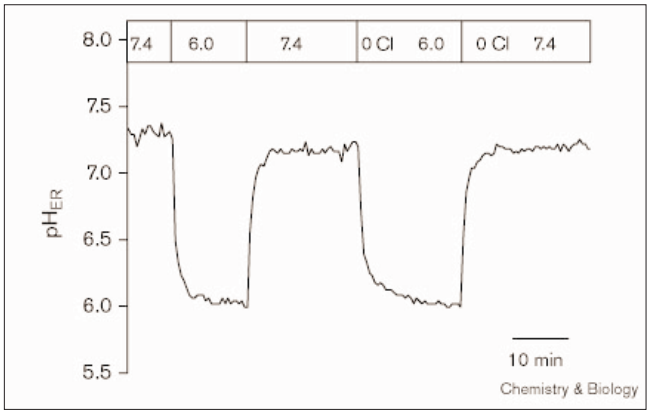
Table 1

Half-times for H^+ leak out of the plasma, ER and Golgi membranes of intact cells.

	Average $t_{1/2}$ (s)	Number of cells
Plasma membrane	99 ± 32	9
ER	105 ± 27	14
Golgi	347 ± 133	10

Data for the pH recoveries of the cytosol, ER and Golgi from Figure 7c were fit to the single exponential equation: $y = A(1-e^{-kt})$. From the curve fits, rate constants (k) and $t_{1/2}$ values, representing the half-time for H^+ leak out of the cytosol or organelle, were obtained. Shown are $t_{1/2}$ values averaged for all cells (± SD) whose cytosolic or organelle pH recoveries were measured.

Figure 8



Neither K^+ nor Cl^- conductance is a determinant of ER and Golgi H^+ leak rates. A typical experiment for measuring the effect of counterion (in this case, Cl^-) removal on ER or Golgi (in this case, ER) H^+ leak rates in permeabilized cells is shown. Flubi-2 loaded, bafilomycin pretreated (500 nM, 2 h) HeLa cells expressing either AV–KDEL or ST–AV were SL-O permeabilized before monitoring organelle pH. H^+ leak rates of ER and Golgi were studied ± counterions (K^+ or Cl^-) by shifting pH of the intracellular buffer (IB) solutions (6.0–7.4 or 7.4–6.0). 0 Cl 6.0 = Cl-free pH 6.0 IB solution; 0 Cl 7.4 = Cl-free pH 7.4 IB solution.

Discussion

Targeted avidin and Flubi dyes for measuring organelle pH

A major outcome of this research was the development of the targeted-avidin–Flubi method for measuring pH in specific intracellular organelles. Unlike other methods [1,3], the avidin–Flubi method can be used to measure pH in any organelle of the secretory pathway (including secretory granules; M.W., T.M. and H-P.M., unpublished observations) and in all transfectable cell types. Another advantage over other methods [2,4,5,15] is the Flubi dyes' ratiometric ability and capacity for chemical modification, thus providing different pH sensitivities suitable for making measurements in neutral organelles as well as in organelles that vary in acidity from pH 6.4 to pH 4.5. In addition to studying pH, the avidin–Flubi method could potentially be used to target other kinds of small-molecule indicators through biotin conjugation using the same avidin chimeras utilized in this study.

After careful consideration, we determined that expression of avidin-chimera proteins did not significantly perturb either the steady-state pH or H^+ leak rates of the ER or the Golgi. First, our pH measurements with avidin-chimeras yielded results that were entirely consistent with absolute ER and Golgi pH values obtained using several different methods [1–5,14,15]. It is unlikely that our method would have affected the organelle environment to produce artifactual results, yet still be able to obtain results similar to all the previous methods [1–5,14,15]. Second, the concentration of KDEL-expressing proteins in the ER is estimated to be in the millimolar range (P. Walter, personal communication). We estimate the concentration of Flubi-2 in the ER to be about 100 μM (M.W. and T.M., unpublished observations). Furthermore, the presence of 100 μM Flubi should not affect the buffer capacity of the ER (~10 mM). The amount of expressed AV–KDEL is therefore unlikely to have significantly affected the ER environment. Similar considerations and conclusions also hold true for the Golgi.

Although cytosolic streptavidin is toxic to bacteria unless the medium is heavily supplemented with biotin [21], chicken avidin in mammalian organelles did not affect either viability or survival, even in stably transfected cells (M.W., J.L. and H-P.M., unpublished observations) with

Table 2

Half-times for H^+ leak out of the ER and Golgi of SL-O permeabilized cells in the presence and absence of K^+.

	+K ave $t_{1/2}$ (s)	–K ave $t_{1/2}$ (s)	Number of cells
ER	110 ± 35	111 ± 16	3
Golgi	87 ± 22	112 ± 39	4

ER and Golgi pH were measured in bafilomycin-treated, SL-O permeabilized cells as the bathing solutions were changed from pH 7.4 to 6.0 to 7.4 in K-containing solutions, followed by pH 7.4 to 6.0 to 7.4 in K-free solutions. The data for the H^+ leak out of the ER and Golgi (as solutions were changed from IB pH 6.0 to 7.4) in the presence and absence of K^+ were fit to the single exponential equation: $y = A(1-e^{-kt})$. From the curve fits, rate constants (k) and $t_{1/2}$ values were obtained. All average $t_{1/2}$ values (shown ± SD) ± K^+ in the ER and Golgi were statistically the same (analysis of variance).

Table 3

Half-times for H^+ leak into the ER and Golgi of SL-O permeabilized cells in the presence and absence of Cl.

	+ Cl ave $t_{1/2a}$ (s)	–Cl ave $t_{1/2a}$ (s)	Number of cells
ER	10 ± 2	21 ± 5	3
Golgi	14 ± 4	14 ± 5	2
	+Cl ave $t_{1/2b}$ (s)	–Cl ave $t_{1/2b}$ (s)	Number of cells
ER	283 ± 199	283 ± 86	3
Golgi	716 ± 100	649 ± 130	2

ER and Golgi pH were measured in bafilomycin-treated, SL-O permeabilized cells as the bathing solutions were changed from IB pH 7.4 to 6.0 to 7.4 in Cl-containing solutions, followed by pH 7.4 to 6.0 to 7.4 in Cl-free solutions. See Figure 8 for a typical ER experiment. The data for the H^+ leak into the ER and Golgi (as solutions were changed from IB pH 7.4 to 6.0) in the presence and absence of Cl^- were fit to the double exponential decay equation: $y = A(e^{-k_a t}) + B(e^{-k_b t})+C$. From the curve fits, rate constants (k_a, k_b) and $t_{1/2a}$, $t_{1/2b}$ values were obtained. All average $t_{1/2}$ data are shown ± SD. Using analysis of variance, the average $t_{1/2a}$ values for ± Cl^- in both the ER and Golgi were statistically the same. The $+Cl^-$ and $-Cl^-$ $t_{1/2b}$ values for the ER were the same as determined by the *t*-test ($P < 0.05$), as were the $+Cl^-$ and $-Cl^-$ $t_{1/2b}$ values of the Golgi; however, the $t_{1/2b}$ values for the ER versus the Golgi were statistically

no extra biotin in the medium. These findings, like the ability of hens to survive while secreting large amounts of avidin into egg white, presumably result because organelles of the secretory pathway neither need nor take up underivatized biotin.

pH_G is not regulated by K^+ or Cl^- conductances alone

It has been proposed that membrane potential plays a significant role in controlling pH of the Golgi [12], endosomes and lysosomes [9,10,22]. In contrast, we observed that, in intact cells, removal of Cl^- had no effect on pH_G, and in permeabilized HeLa cells, rates of H^+ leak into the Golgi (and ER) were the same in Cl-containing and Cl-free solutions. The presence or absence of Cl^- therefore had little effect on pH regulatory properties of the Golgi (and ER). Furthermore, addition of valinomycin to bafilomycin-treated cells had no effect on pH_G dissipation (Figure 6a,b), although when valinomycin was added to bafilomycin-treated cells after FCCP (Figure 6c), pH_G increased to a higher level than with FCCP alone. In addition, in permeabilized cells, rates of H^+ leak out of the Golgi (and ER) were the same in the presence and the absence of K^+ (Tables 1–3).

The simplest explanation of these results is that there were large Cl^- and K^+ conductances in Golgi (and ER) membranes, and, in intact cells, removal of Cl^- from the solution had no effect on pH_G because the large K^+ conductance shunted any potentials that would affect the activity of the H^+ v-ATPase and/or H^+ leaks. Similarly, when H^+ fluxed across the organelle membranes in permeabilized cells, removal of either Cl^- or K^+ had no effect because the other counterion was available to flux and maintain electroneutrality. The K^+ conductance of the Golgi, however, was not as high as that exhibited in the presence of valinomycin. This was revealed in the experiment performed in Figure 6c, which showed that in the presence of bafilomycin, FCCP caused a rapid dissipation of pH_G, which was increased by further addition of valinomycin. In the presence of a very large H^+ permeability (presence of FCCP), K^+ conductance therefore became an important factor in determining steady-state pH_G.

Previous experiments, in which counterion dependence of Ca^{2+} flux across the ER membranes was measured, similarly concluded that both Cl^- and K^+ conductances of the ER were large [23]. Indirect considerations also indicate that the voltage across the sarcoplasmic reticulum is small (< 5 mV [24]). It is likely, therefore, that the voltage across the ER and Golgi membranes is close to zero, and the pH values of these organelles are not regulated by conductance of a single counterion.

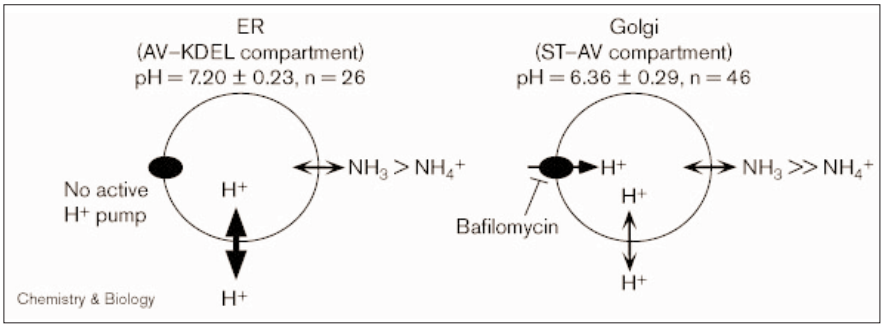
Our results showing that pH_G was unaffected by removal of Cl^- from the solutions were similar to those of Demaurex *et al.* [13] but different from Llopis *et al.* [4], who showed that Cl-free solution caused pH_G to increase rapidly. The explanation for this apparent contradiction probably results from the recent discovery that the fluorescence of enhanced yellow fluorescent protein (EYFP), which was used for the experiments of Llopis *et al.* [4], is sensitive to the concentration of Cl^- (increasing at low Cl^- concentration), in addition to being sensitive to H^+ [25]. This therefore emphasizes the value of using Flubi dyes for measurements of organelle pH. Golgi-targeted enhanced green fluorescent protein (EGFP; not sensitive to the Cl^- concentration) also showed no effect of Cl^- removal on the Golgi pH (J.L., unpublished observations).

ER and Golgi pHs are regulated by varying H^+ pump and leak activities

Theoretical models of organelle pH regulation have not considered H^+ leaks as major regulators of pH [9]. Kim *et al.* [1] concluded previously that the H^+ leak in the Golgi was much smaller than that of the ER, but they were unable to quantitate the relative H^+ leaks because they measured rates of organelle acidification using conditions in the ER (no active H^+ v-ATPase and $pH_{ER} = 7.2$) that were different from those in the Golgi (active H^+ v-ATPase and $pH_G = 6.4$). In contrast, we directly compared the rates of H^+ leak by using bafilomycin-treated cells (to inhibit the H^+ v-ATPase) with the same transmembrane pH gradients ($pH_C = 7.4$ and both pH_{ER} and $pH_G = 6.0$). Our data showed that the Golgi ($pH_G = 6.4$) was more acidic than the ER ($pH_{ER} = 7.2$) because the Golgi, but not the ER, contained an active H^+ v-ATPase to counter a H^+ leak that was smaller in the Golgi than in the ER (Figure 9).

The buffer capacities of the ER (6 mM/pH) and Golgi (11 mM/pH) were approximately equal (and also equal to that of the cytosol = 7 mM/pH). Farinas and Verkman [14] used methods similar to ours to measure the intrinsic buffer capacity of the Golgi of Chinese hamster ovary (CHO) cells, which they found to be 36 mM/pH — three to six times the values measured in this study. This discrepancy might have been due to differences between HeLa and CHO cells, or may also have been due to the fact that our measurements were made in cells in which the H^+ v-ATPase had been inhibited by bafilomycin (whereas it was unclear whether Farinas and Verkman [14] took this precaution). The presence of an active H^+ pump in the membranes will tend to lead to overestimations of buffer capacity. In any case, the buffer capacity of the ER and Golgi appeared to be roughly the same, which allowed us to determine relative rates of H^+ flux across ER and Golgi from rates of change of pH_{ER} and pH_G. Experiments on intact cells showed that, following an acid load, pH_C and pH_{ER} recovered to pH 7.5 at the same rates, whereas pH_G recovery was approximately three times slower than the pH_C and pH_{ER} recoveries, and recovered to only pH 6.7 in 25 minutes. This incomplete

Figure 9



Model for ER and Golgi pH regulation. The average pH_{ER} in HeLa cells was 7.20 ± 0.23 (n = 26). ER membranes had no active H^+ v-ATPase (bafilomycin had no effect on pH_{ER}), were slightly more permeable to NH_3 than to NH_4^+ and were highly permeable to H^+ (and also K^+ and Cl^-); therefore, pH_{ER} mirrored pH_C (7.2–7.6 in control cells). Average pH_G in HeLa cells was 6.36 ± 0.29 (n = 46) and was maintained by a constantly active, bafilomycin-sensitive, H^+ v-ATPase which opposed a substantial H^+ leak, which was one-third the magnitude of the ER H^+ leak. Golgi membranes were largely impermeable to NH_4^+ compared to NH_3 but had relatively large conductances to both Cl^- and K^+.

alkalinization is consistent with data from other studies showing incomplete dissipation of pH_G by bafilomycin [2,13]). These data indicated that firstly, the ER was extremely leaky to H^+, secondly, the control of pH_{ER} was through the action of plasma membrane mechanisms that regulated pH_C and thirdly, the apparent leak rate for H^+ across the Golgi membranes was large, but approximately one- third of that found for the ER. The large H^+ leak out of the Golgi is consistent with data showing that bafilomycin often causes pH_G to alkalinize rapidly [1–4,14]. The recent work of Farinas and Verkman [14] also showed that the Golgi had a large H^+ permeability and that the H^+ leak (with a constant pH gradient) was larger at pH 6.2 than at pH 7.0. This pH-dependent Golgi H^+ leak noted by Farinas and Verkman [14] was not specifically tested here. Future experiments will resolve the question of whether and how the H^+ leak (and also the H^+ v-ATPase) is regulated by pH and other factors.

Leakage of H^+ across the ER and Golgi occurred rapidly whether H^+ was leaking into or out of the organelle. There were, however, some unexplained differences in H^+ permeabilities of the ER and Golgi when measured in intact versus SL-O permeabilized cells. First, rates of change of pH_{ER} and pH_G were equal to each other in SL-O experiments in which H^+ leaked out of the organelles (Table 2), whereas experiments on intact cells (Table 1) showed H^+ leak rates across the Golgi were three times slower than leak rates across the ER and also resulted in incomplete dissipation of the pH gradient between the Golgi lumen and the cytosol. Second, rates of change of pH_{ER} and pH_G were fitted by a single exponential when H^+ was leaking out, but required two exponentials to fit the data when H^+ was leaking into the organelles (Table 3). These data indicate that H^+ might leak through several pathways across the organelles, and cytosolic regulatory factors (which may have been washed out in the SL-O permeabilization) might be important for maintaining the three times lower H^+ permeability of Golgi than that of ER observed in intact cells.

It is interesting, and still unknown, how pH_G appears to be so well regulated to a given pH. As discussed above, when the Golgi was acidified below its control level using NH_3/NH_4^+, the Golgi quickly leaked the accumulated H^+, and pH_G returned to the control level characteristic for that specific cell. The simplest explanation for these results is that the Golgi H^+ v-ATPase acidified the lumen to pH 6.4, a value at which the rate of H^+ being pumped into the lumen by the H^+ pump was equal to the H^+ moving out through the H^+ leak; this conclusion is supported by work from Farinas and Verkman [14]. The role of the H^+ leak in the Golgi remains unknown but the leak may provide a regulatory capability, both by second messengers [2] and for different organelles. For example, although the low pH of lysosomes and secretory granules could be generated by increasing the number or activity of H^+ pumps, a simpler mechanism would be to remove the H^+ leaks by selective retention, or the retrieval of leaks to 'earlier' organelles along the secretory pathway. Establishing a gradient of decreasing number (or regulated activity) of H^+ leaks from the ER to the Golgi to secretory granules would therefore be a possible mechanism for establishing the pH gradient (pH 7.0 to 6.4 to 5.5) between these organelles. Consistent with this idea, the apparent H^+ permeability of the plasma membrane of HeLa cells appeared to be much lower than that of either the ER or Golgi (compare pH_C in Figure 7a,b with pH_{ER} and pH_G in Figure 7c).

Significance

We describe a new method for studying organelle pH regulation that utilizes biotin-based ratiometric pH indicators targeted to avidin-containing organelles. Because our pH dyes have a wide range of pK_a values (4–7), we are able to measure pH in organelles of varying acidity. Using this

approach, we began a comprehensive study of secretory pathway organelle pH regulation by examining endoplasmic reticulum (ER) and Golgi pH regulatory mechanisms.

Our data indicate that ER pH (pH_{ER}) mirrors cytosolic pH (pH_C) because ER membranes lack an active H^+ v-ATPase and are highly conductive to H^+ (as well as to Cl^-, K^+ and NH_4^+). Because of these permeabilities, pH_{ER} is mainly controlled by plasma membrane pH regulatory mechanisms. Golgi pH (pH_G) is maintained by active H^+ v-ATPase(s), which acidified the Golgi even in the face of a large H^+ counter leak (at least three times smaller than the H^+ leak out of the ER) out of the Golgi lumen into the cytosol. Because ER and Golgi buffer capacities were nearly equal, and removing either K^+ or Cl^- counterions in both intact and permeabilized cells had no effect on either pH_{ER} or pH_G, it seems unlikely that different organelles of the secretory pathway maintain different pHs by varying single counterion conductances across their membranes. Instead, we favor a model in which organelles maintain their pHs primarily by regulating the number and/or magnitude of the H^+ pumps and H^+ leaks in their membranes. One way to regulate the distribution of H^+ pumps and H^+ leaks could be by retrieval or retention of H^+ leaks to earlier compartments in the secretory pathway.

Materials and methods

Perfusion and calibration solutions

Ringer's solution contained (in mM): 141 NaCl, 2 KCl, 1.5 K_2HPO_4, 1 $MgSO_4$, 10 Hepes, 2 $CaCl_2$, 10 glucose, buffered to pH7.4. NH_4Cl solution was the same as Ringer's except 30 mM (or 40 mM) NH_4Cl replaced 30 mM (or 40 mM) of the NaCl. In ion replacement experiments, all Cl^- was replaced with gluconate, Na^+ with N-methyl-D-glucamine (NMG), and K^+ with NMG^+ to make Cl-free, Na-free, and K-free Ringer's solutions, respectively. SL-O permeabilized cells were perfused with intracellular buffer (IB) containing (in mM): 110 K-gluconate, 20 KCl, 0.1 $CaCl_2$, 2.7 K_2HPO_4, 10 Hepes, 10 MES, 10 glucose, 5 Mg-ATP, 2 Na-GTP, 2 Mg-acetate, titrated to different pHs with NMG base. Calibration solutions contained (in mM): 70 NaCl, 70 KCl, 1.5 K_2HPO_4, 1 $MgSO_4$, 10 Hepes/MES, 2 $CaCl_2$, 10 glucose, titrated to different pHs (8.2, 7.0, 6.5, 6.0, 5.5) and supplemented with 10 μM each of nigericin and monensin.

Fluorescent labeling of cytosol, ER and Golgi, and permeabilization of plasma membrane with SL-O

Cell cytosol was labeled using two methods. In one, cells were loaded with 2 μM BCECF-AM in Ringer's for 30 min at room temperature and then chased with normal growth media for 1 h at 37°C. As membrane-permeable BCECF-AM can theoretically enter all cellular compartments, a second set of experiments to measure pH_C was performed (Figure 6c) on FITC-dextran scrape-loaded cells to eliminate contributions from organelle-localized dye. Cells were scrape-loaded in 10 mg/ml FITC-dextran (70,000 MW) in DME exactly as described [26].

To label the lumens of the ER and Golgi, the cell permeable Flubi-2 diacetate (Flubida-2, ~2 mM) was mixed 1:1 with Pluronic F-127 (20% w/v in dry DMSO) then diluted to the desired final concentration with serum-free medium. 30–48 h post-transfection with either AV–KDEL or ST–AV DNA, cells were rinsed with serum-free medium and incubated with 2–4 μM Flubida-2 overnight (10–15 h) at 37°C. Cells were then chased with normal growth medium for at least 2 h to wash excess dye from cytosol and the organelles not containing avidin. Biotin starvation of the cells was unnecessary before Flubi-2 loading, as staining was bright and stable.

For permeabilized cell experiments, 100 μl 'reconstitution buffer' (10 mM Hepes, pH 7.2; 10 mM DTT; 0.1% BSA) were added to lyophilized SL-O to give a 1 mg/ml stock solution. Cells were rinsed with ice-cold Ringer's, followed by incubation in cold Ringer's containing 5 μg/ml SL-O for 15 min on ice to allow SL-O binding. Excess SL-O was removed by washing twice with ice-cold Ringer's; cells were then incubated in IB for 15 min at 37°C to allow pore formation. SL-O permeabilization of the plasma membrane was confirmed at the end of each experiment by treating the cells with 1 μM propidium iodide, which bound to DNA and stained nuclei red only in permeabilized cells.

Fluorescence ratio imaging of cytosolic, ER and Golgi pH

Cytosol, ER and Golgi pH were studied in separate experiments using digitally processed fluorescence ratio imaging. Ratio imaging measurements were performed at room temperature as previously described [27].

pH-calibration and measurement of rates of change of pH

In vitro calibrations and spectroscopy were done on the complex avidin–Flubi-2. 0.8 mg avidin D (Vector Labs, Burlingame, CA) was incubated with excess Flubi-2 and the complex was separated from free Flubi-2 by gel filtration using a Sephadex G25 column. The fraction with retention time for avidin was fluorescent and was collected. Absorbance spectra were taken with a Cary 3E spectrophotometer (Varian, Australia). A pH titration of Flubi-2 and the complex avidin:Flubi-2 was performed using a fluorometer (Spex Industries, Edison, NJ). Buffers were prepared with pHs ranging from 4 to 10.5 in either 30 mM acetate, 2-(*N*-morpholino)ethanesulfonate, 3-(*N*-morpholino)propanesulfonate, Hepes, Bicine, or Tris in a saline solution composed of (in mM) 125 KCl, 20 NaCl, 0.5 $CaCl_2$, and 0.5 $MgCl_2$. Titration data were fit to a sigmoidal curve; the pK_a of avidin–Flubi-1 *in vitro* was 6.93, and the pK_a of avidin:Flubi-2 was 6.53.

In situ calibrations were performed as described [27]. All data have been presented as mean ± standard deviation (SD) unless otherwise indicated.

Rates of H^+ leak into and out of ER, Golgi, and plasma membranes were calculated by fitting the data to either a single exponential association curve (for leak out of membranes) or a double exponential decay curve (for leak into membranes) using GraphPad. Rate constants and half-times were determined from the curve fits.

Supplementary material

Supplementary material including information on materials, synthesis of Flubida-1 and Flubi-2, Flubida-2, Flubi-2, construction of avidin–KDEL and sialyltransferase-avidin plasmids, cell culture and transfection, immunofluorescence, immunoelectron microscopy and buffer capacities of the cytosol, ER and Golgi is available at http://current-biology.com/supmat/supmatin.htm.

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References

1. Kim, J.H., *et al.*, & Grinstein, S. (1998). Noninvasive measurement of the pH of the endoplasmic reticulum at rest and during calcium release. *Proc. Natl Acad. Sci. USA* **95**, 2997-3002.
2. Seksek, O., Biwersi, J. & Verkman, A.S. (1995). Direct measurement

of trans-Golgi pH in living cells and regulation by second messengers. *J. Biol. Chem.* **270**, 4967-4970.
3. Kim, J.H., Lingwood, C.A., Williams, D.B., Furuya, W., Manolson, M.F. & Grinstein, S. (1996). Dynamic measurement of the pH of the Golgi complex in living cells using retrograde transport of the verotoxin receptor. *J. Cell Biol.* **134**, 1387-1399.
4. Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G. & Tsien, R.Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl Acad. Sci. USA* **95**, 6803-6808.
5. Miesenböck, G., De Angelis, D.A. & Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**, 192-195.
6. Mellman, I., Fuchs, R. & Helenius, A. (1986). Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55**, 663-700.
7. Halban, P.A. & Irminger, J.C. (1994). Sorting and processing of secretory proteins. *Biochem. J.* **299**, 1-18.
8. Schmidt, W.K. & Moore, H.P. (1995). Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells. *Mol. Biol. Cell* **6**, 1271-1285.
9. Rybak, S.L., Lanni, F. & Murphy, R.F. (1997). Theoretical considerations on the role of membrane potential in the regulation of endosomal pH. *Biophys. J.* **73**, 674-687.
10. Fuchs, R., Schmid, S. & Mellman, I. (1989). A possible role for Na^+, K^+-ATPase in regulating ATP-dependent endosome acidification. *Proc. Natl Acad. Sci. USA* **86**, 539-543.
11. Glickman, J., Croen, K., Kelly, S. & Al-Awqati, Q. (1983). Golgi membranes contain an electrogenic H^+ pump in parallel to a chloride conductance. *J. Cell Biol.* **97**, 1303-1308.
12. Barasch, J., Gershon, M.D., Nunez, E.A., Tamir, H. & Al-Awqati, Q. (1988). Thyrotropin induces the acidification of the secretory granules of parafollicular cells by increasing the chloride conductance of the granular membrane. *J. Cell Biol.* **107**, 2137-2147.
13. Demaurex, N., Furuya, W., D'Souza, S., Bonifacino, J.S. & Grinstein, S. (1998). Mechanism of acidification of the trans-Golgi network (TGN). In situ measurements of pH using retrieval of TGN38 and furin from the cell surface. *J. Biol. Chem.* **273**, 2044-2051.
14. Farinas, J. & Verkman, A.S. (1999). Receptor-mediated targeting of fluorescent probes in living cells. *J. Biol. Chem.* **274**, 7603-7606.
15. Kneen, M., Farinas, J., Li, Y. & Verkman, A.S. (1998). Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* **74**, 1591-1599.
16. Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S. & Klausner, R.D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801-813.
17. Wong, S.H., Low, S.H. & Hong, W. (1992). The 17-residue transmembrane domain of beta-galactoside alpha 2,6-sialyltransferase is sufficient for Golgi retention. *J. Cell Biol.* **117**, 245-258.
18. Aronson, P.S., Nee, J. & Suhm, M.A. (1982). Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature* **299**, 161-163.
19. Aronson, P.S., Suhm, M.A. & Nee, J. (1983). Interaction of external H^+ with the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *J. Biol. Chem.* **258**, 6767-6771.
20. Grinstein, S. & Rothstein, A. (1986). Mechanisms of regulation of the Na^+/H^+ exchanger. *J. Membr. Biol.* **90**, 1-12.
21. Sano, T. & Cantor, C.R. (1990). Expression of a cloned streptavidin gene in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **87**, 142-146.
22. Cain, C.C., Sipe, D.M. & Murphy, R.F. (1989). Regulation of endocytic pH by the Na^+,K^+-ATPase in living cells. *Proc. Natl Acad. Sci. USA* **86**, 544-548.
23. Hofer, A.M., Schlue, W.R., Curci, S. & Machen, T.E. (1995). Spatial distribution and quantitation of free luminal [Ca] within the InsP3-sensitive internal store of individual BHK-21 cells: ion dependence of InsP3-induced Ca release and reloading. *FASEB J.* **9**, 788-798.
24. Garcia, A.M. & Miller, C. (1984). Channel-mediated monovalent cation fluxes in isolated sarcoplasmic reticulum vesicles. *J. Gen. Physiol.* **83**, 819-839.
25. Wachter, R. & Remington, J. (1999). Sensitivity of the yellow variant of green fluorescent protein to halides and nitrate. *Curr. Biol.* **9**, R628-629.
26. McNeil, P.L., Murphy, R.F., Lanni, F. & Taylor, D.L. (1984). A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* **98**, 1556-1564.
27. Teter, K., Chandy, G., Quiñones, B., Pereyra, K., Machen, T. & Moore, H.P. (1998). Cellubrevin-targeted fluorescence uncovers heterogeneity in the recycling endosomes. *J. Biol. Chem.* **273**, 19625-19633.