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pH of TGN and recycling endosomes of H⁺/K⁺-ATPase-transfected HEK-293 cells: implications for pH regulation in the secretory pathway

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Machen, Terry E., Mary Jae Leigh, Carmen Taylor, Tohru Kimura, Shinji Asano, and Hsiao-Ping H. Moore. pH of TGN and recycling endosomes of H⁺/K⁺-ATPase-transfected HEK-293 cells: implications for pH regulation in the secretory pathway. *Am J Physiol Cell Physiol* 285: C205–C214, 2003. First published March 26, 2003; 10.1152/ajpcell.00008.2003.—The influences of the gastric H⁺/K⁺ pump on organelle pH during trafficking to and from the plasma membrane were investigated using HEK-293 cells stably expressing the α - and β -subunits of human H⁺/K⁺-ATPase (H⁺/K⁺- α,β cells). The pH values of *trans*-Golgi network (pH_{TGN}) and recycling endosomes (pH_{RE}) were measured by transfecting H⁺/K⁺- α,β cells with the pH-sensitive GFP pHluorin fused to targeting sequences of either TGN38 or synaptobrevin, respectively. Immunofluorescence showed that H⁺/K⁺-ATPase was present in the plasma membrane, TGN, and RE. The pH_{TGN} was similar in both H⁺/K⁺- α,β cells (pH_{TGN} 6.36) and vector-transfected (“mock”) cells (pH_{TGN} 6.34); pH_{RE} was also similar in H⁺/K⁺- α,β (pH_{RE} 6.40) and mock cells (pH_{RE} 6.37). SCH28080 (inhibits H⁺/K⁺-ATPase) caused TGN to alkalinize by 0.12 pH units; subsequent addition of bafilomycin (inhibits H⁺ v-ATPase) caused TGN to alkalinize from pH 6.4 up to a new steady-state pH_{TGN} of 7.0–7.5, close to pH_{cytosol}. Similar results were observed in RE. Thus H⁺/K⁺-ATPases that trafficked to the plasma membrane were active but had small effects to acidify the TGN and RE compared with H⁺ v-ATPase. Mathematical modeling predicted a large number of H⁺ v-ATPases (8,000) active in the TGN to balance a large, passive H⁺ leak (with $P_H \sim 10^{-3}$ cm/s) via unidentified pathways out of the TGN. We propose that in the presence of this effective, though inefficient, buffer system in the Golgi and TGN, H⁺/K⁺-ATPases (estimated to be $\sim 4,000$ active in the TGN) and other transporters have little effect on luminal pH as they traffic to the plasma membrane.

pHluorin; H⁺ v-ATPase; *trans*-Golgi network; organelle pH; H⁺ permeability

LUMINAL PH OF ORGANELLES along the secretory pathway becomes more acidic as they progress from the endoplasmic reticulum (ER, pH_{ER} 7.2–7.5) (1, 40) to the Golgi (pH_G 6.2–6.6) (14, 21, 23, 34, 39, 40) to the organelles that cycle between the plasma membrane and the cytosol [*trans*-Golgi network, TGN, pH_{TGN} 5.9

(11)] and recycling endosomes [pH_{RE} 6.0–6.5 (13, 36, 41)] and finally to secretory granules (pH_{SG} 5) (39). Although the mechanisms that operate to assure these relatively consistent pH's continue to be explored, several general themes have emerged.

First, the Golgi is more acidic than the ER because the Golgi has an active H⁺ v-ATPase and a lower passive H⁺ permeability than the ER. This H⁺ permeability in the Golgi appears to be blocked by Zn²⁺ (33), a well-known blocker of H⁺ channels/conductance in the plasma membranes of multiple cell types (3, 6, 7, 9, 12, 15, 18, 38).

Second, the Golgi and TGN, like the ER, have large apparent permeabilities to both K⁺ and Cl⁻ so that these ions are likely to equilibrate rapidly with ions in the cytosol and membrane potential is likely to be small (<10 mV) (5, 11, 19, 21, 33, 40). Thus membrane potential seems not to play an important role in regulating pH in the Golgi and TGN, and the pH values of these compartments are likely to be maintained primarily by H⁺ pumping by the H⁺ v-ATPase balanced by a passive H⁺ leak driven solely by the differences in pH between the Golgi or TGN lumen and the cytosol.

Third, recycling endosomes appear to be a heterogeneous organelle with variable pH depending on the population in which measurements are made (4, 13, 16, 36, 41): endosomes that contain the transferrin receptor appear to be less acidic than cellubrevin-containing endosomes (pH 6.5 vs. pH 6.0), and this difference may arise because a subpopulation of endosomes (transferrin receptor-positive endosomes) contains the electrogenic Na⁺/K⁺-ATPase and generates a lumen-positive membrane voltage that inhibits H⁺ pumping by the electrogenic H⁺ v-ATPase (4, 16, 36).

An interesting and important question remains unanswered about pH regulation in these organelles. Different cells have different ion and acid-base transporters to accomplish different physiological functions. Because these transporters likely become active in the ER (28, 29, 31), do they affect the pH-regulatory properties of the Golgi and other organelles of the secretory

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pathway as the transporters are trafficked to the plasma membrane? A particularly dramatic example of this problem exists in the Golgi of parietal cells of the stomach. These cells express H⁺/K⁺-ATPase pumps in the apical plasma membrane. When these pumps and K⁺ and Cl⁻ channels are active in the apical membrane, the H⁺/K⁺-ATPases are capable of accumulating isotonic HCl, pH 0.8, in the gastric lumen (10, 26, 32). Because H⁺/K⁺-ATPases are continually synthesized to replace degraded pumps and the Golgi has large, inherent permeabilities to both K⁺ and Cl⁻ (thereby providing KCl needed for the operation of the pump), it might be expected that the H⁺/K⁺-ATPases present in the Golgi are functionally active. What effect do these pumps have on pH_G and other organelles of the secretory pathway in parietal cells? We attempted to answer this question for the TGN and recycling endosomes using the genetically targeted, pH-sensitive green fluorescent protein (GFP) derivatives TGN38-pHluorin and synaptobrevin (SV)-pHluorin (24).

Because parietal cells are difficult to transfect, we used HEK-293 cells that had been stably transfected with both the α - and β -subunits of human H⁺/K⁺-ATPase (H⁺/K⁺- α,β cells; see Ref. 22). These cells express functional H⁺/K⁺-ATPase in the plasma membrane, as shown by the fact that the cells exhibit ⁸⁶Rb⁺ (K⁺ substitute) uptake and Na⁺-independent H⁺ secretion that is blocked by SCH28080 (specific blocker of the H⁺/K⁺-ATPase). In addition, evidence from membrane fractionation indicated that a large fraction of the H⁺/K⁺-ATPase was expressed in organelles. Pulse-chase labeling studies showed that the ATPase had a half-life of ~12 h, so steady-state expression in the plasma membrane required continual synthesis (22). We confirmed the organelle localization of the pHluorins and the H⁺/K⁺-ATPase using immunofluorescence and then tested for the activity of the H⁺/K⁺-ATPase by measuring pH in the Golgi and recycling endosomes during treatments with SCH28080 (specific inhibitor of H⁺/K⁺-ATPase). Experiments were performed with normal Ringer's solutions bathing the cells to assure that conditions in the TGN would be as close to the *in vivo* state as possible. We used 50 μ M of this inhibitor to assure that all the H⁺/K⁺-ATPase pumps that were present in the TGN and recycling endosomes would be inhibited. For comparison, we also tested the effects of bafilomycin, the specific blocker of the H⁺ v-ATPase. Results from these experiments indicated that the H⁺/K⁺-ATPases that are trafficked to the plasma membrane are active in both the TGN and recycling endosomes, but their activity is much lower than that of endogenous H⁺ v-ATPase, so trafficking pumps have little effect on the pH of the organelles they traverse on their way to the plasma membrane.

METHODS

Materials. All salts, glucose, buffers, DMSO, bafilomycin, nigericin, and monensin were obtained from Sigma (St. Louis, MO); solvents were from Fisher Scientific (Pittsburgh, PA); and restriction enzymes were from New England Bio-

labs (Beverly, MA). Tissue culture reagents were obtained from GIBCO-BRL or Cellgro. Fetal bovine serum was obtained from Gemini Bio-products.

H/K- α,β and mock cells. cDNAs of α - and β -subunits of H/K-ATPase were prepared from rabbit gastric mucosa as described elsewhere (2). The α - and β -subunit cDNAs were digested with *EcoRI* and *XhoI*. The obtained fragments were each ligated into the pcDNA3 vector (for the β -subunit cDNA) or pcDNA3.1(+) (Zeo) (for the α -subunit cDNA) treated with *EcoRI* and *XhoI*. DNA sequencing was done by the dideoxy chain termination method using an ABI Prism 377 DNA sequencer (Applied Biosystems, Tokyo, Japan).

Stable cell lines expressing α - and β -subunits of gastric H⁺/K⁺-ATPase were generated as follows. HEK-293 cells were cultured as described previously (1, 2). HEK-293 cells were transfected with pcDNA3-H⁺/K⁺ β cDNA by lipofection using an Effectene transfection reagent, and stable cell lines were selected in the presence of 1 mg/ml Geneticin (G-418 sulfate). Single colonies were isolated, expanded, and maintained in the presence of 0.5 mg/ml Geneticin. The expression of the β -subunit was confirmed by immunofluorescence and Western blot. The α + β -expressing cells (H⁺/K⁺ α,β cells) were then generated by transfecting HEK cells stably expressing the β -subunit with pcDNA3.1(+) (Zeo)-H/K- α cDNA construct. Stable cell lines were selected in the presence of 0.2 mg/ml Zeocin plus 0.5 mg/ml Geneticin. Single colonies were isolated, expanded, and maintained in the presence of 0.5 mg/ml Geneticin and 0.1 mg/ml Zeocin. The expression of the α + β subunits in H⁺/K⁺- α,β cells was confirmed by immunofluorescence and Western blot. H⁺/K⁺-mock cells were transfected with empty vectors and then treated identically to the H⁺/K⁺- α,β cells.

Cell culture. Cells were plated onto plastic culture dishes or collagen-coated coverslips and maintained in a 37°C incubator with 5% CO₂. All media were supplemented with penicillin, streptomycin, and glutamine. All HEK-293 cells were maintained in DMEM supplemented with 10% FBS.

Transfections of H⁺/K⁺- α,β and mock cells with TGN38-pHluorin or synaptobrevin-pHluorin. HEK-293-mock and H⁺/K⁺- α,β cells were transiently transfected with TGN38-pHluorin or synaptobrevin-pHluorin plasmids using the Effectene transfection reagent (Qiagen, Germany). Cells were split onto a collagen-coated 10-cm culture dish at 50% confluency 6–8 h before transfection. They were then transfected with 2 μ g of DNA complexed with 16 μ l of enhancer and 60 μ l of Effectene reagent. After 24 h, cells were split onto collagen-coated glass coverslips and incubated with growth medium containing 6 mM Na-butyrate for 13–16 h to induce gene expression. Cells were returned to normal growth medium lacking Na-butyrate 1 h before imaging experiments.

Solutions. Ringer's solution contained (in mM) 141 NaCl, 2 KCl, 1.5 K₂HPO₄, 1 MgSO₄, 10 HEPES, 2 CaCl₂, and 10 glucose brought to pH 7.4 with NaOH. Calibration solutions contained (in mM) 70 NaCl, 70 KCl, 1.5 K₂HPO₄, 1 MgSO₄, 10 HEPES, 10 MES, 2 CaCl₂, 10 glucose, adjusted to various pH values (5.5, 6.0, 6.5, 7.0, 7.5, or 8.2) with KOH, 0.01 nigericin, and 0.01 monensin. In experiments in which cells were acidified using a brief NH₄ treatment, 30 mM NH₄Cl was substituted for 30 mM NaCl. Bafilomycin was used at 100–500 nM (yielding equivalent effects) and SCH28080 at 50 μ M.

Fluorescence ratio imaging of pH in the cytosol, TGN, and recycling endosomes of HEK-293 cells. General methods used in this laboratory for measuring organelle pH values have been described previously (5, 39). Briefly, TGN labeled with TGN38-pHluorin, recycling endosomes labeled with SV-pHluorin, and cytosol labeled with 10 μ M BCECF-AM (Mo-

lecular Probes, Eugene, OR) were monitored in separate experiments using digitally processed fluorescence ratio imaging. Labeled cells were placed in an open perfusion chamber on an inverted microscope (Zeiss IM35 or Nikon Diaphot). The solutions used for perfusing the cells, the chamber holding the cells, and the objective were all heated to 37°C. A ×40 oil-immersion objective (1.3 NA; Nikon) was used to collect fluorescence from 1 to 30 cells during each experiment. A lens was used to focus the image through a phototube (Diagnostic Instruments) onto a low-light-level DAGE 68 silicon-intensified tube camera. Emission images of the cells were collected through a 530-nm long-pass filter during sequential excitation at either 410 and 470 or 380 and 440 ± 10 nm (Omega Optical, Brattleboro, VT). Filters were changed with a Lambda 10-2 filter wheel (Sutter Instruments, Novato, CA). Separate images for each wavelength were averaged over eight frames by a digital image processor (Axon Image Lightning, Axon Instruments, Foster City, CA) and subsequently converted pixel by pixel to a ratio image. Data collection rate (one ratio image every 5–180 s), filter wheel position, and shutter opening/closing were controlled by a 133-MHz Pentium computer (Gateway 2000) running the version 2.x of Axon's Imaging Workbench.

Data were collected by electronically selecting regions of the image for quantitation. Cytosolic measurements were made from entire cells. When making measurements on TGN and recycling endosomes, only the brightest perinuclear regions were selected. Intensities were balanced with neutral density filters. Photobleaching was negligible.

Methods describing calibration of cytosolic and organelle pH measurements have been reported previously (36, 39). Calibrations of TGN- and SV-pHluorin showed pH-ratio relationships that were nearly identical to those in Miesenbock et al. (24).

Immunofluorescence. HEK-mock and H⁺/K⁺-α,β cells were transiently transfected with TGN38-pHluorin or synaptobrevin-pHluorin plasmids as described above. Two days after

transfection, they were fixed with 3% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. H⁺/K⁺-ATPase was detected by incubation with monoclonal antibody 2G11 (courtesy of Dr. John Forte, Univ. of California, Berkeley) against the cytoplasmic domain of β-subunit (1:1,000 dilution), followed by rhodamine-conjugated goat anti-mouse antibodies (1:25 dilution). For some coverslips, the recycling endosomes were stained by incubating cells in serum-free medium for 30 min at 37°C followed by incubation with 50 μg/ml rhodamine-conjugated human transferrin (Molecular Probes) in the same medium for 1 h at 37°C. GFP and rhodamine fluorescence was visualized using a Zeiss Axiophot fluorescence microscope.

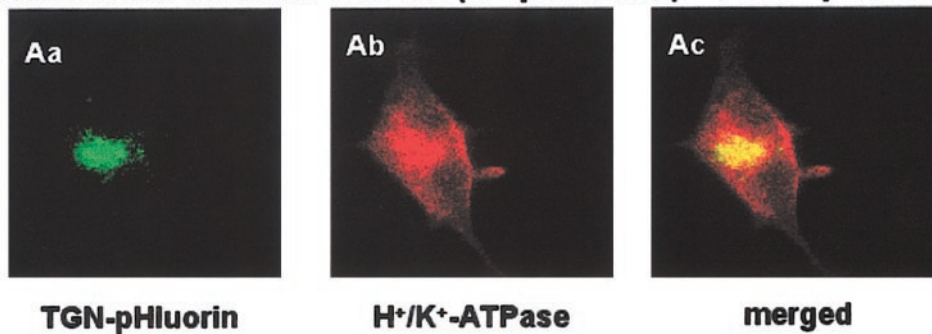
Statistics. Unless otherwise specified, data are presented as means ± SE. Experimental data were compared using unpaired Student's *t*-test (two-tailed). Differences were considered significant if *P* < 0.05.

RESULTS

TGN-pHluorin, SV-pHluorin, and H⁺/K⁺-ATPase expression in HEK-293 cells. TGN-pHluorin localized to a subcellular organelle near the nucleus, likely the TGN, in both H⁺/K⁺-α,β (Fig. 1Aa) and mock (Fig. 1Ba) cells, consistent with results presented previously (24).

Antibody to the β-subunit of the H⁺/K⁺-ATPase was used to test for the subcellular localization of the pump in H⁺/K⁺-α,β and mock cells transiently transfected with TGN38-pHluorin. In H⁺/K⁺-α,β (Fig. 1Ab), but not in mock cells (Fig. 1Bb), H⁺/K⁺-ATPase staining was observed in both the plasma membrane and also in intracellular organelles. Some internal ATPase colocalized to the same compartment where TGN38-pHluorin was localized (Figs. 1Ab and 1Ac).

Transfection with H⁺/K⁺-ATPase (α + β subunits) + TGN38-pHluorin



Transfection with TGN38-pHluorin alone

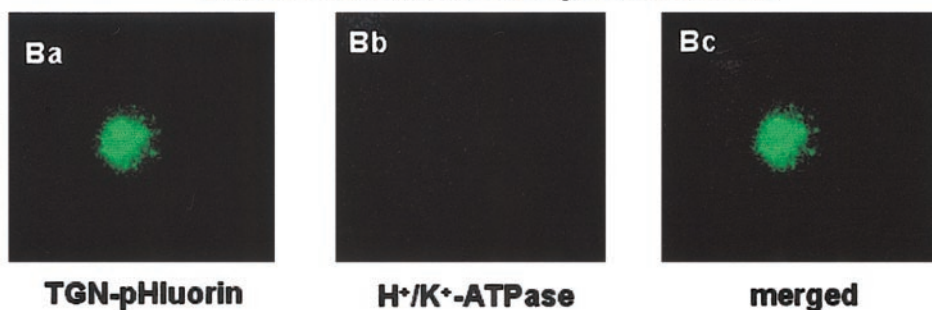
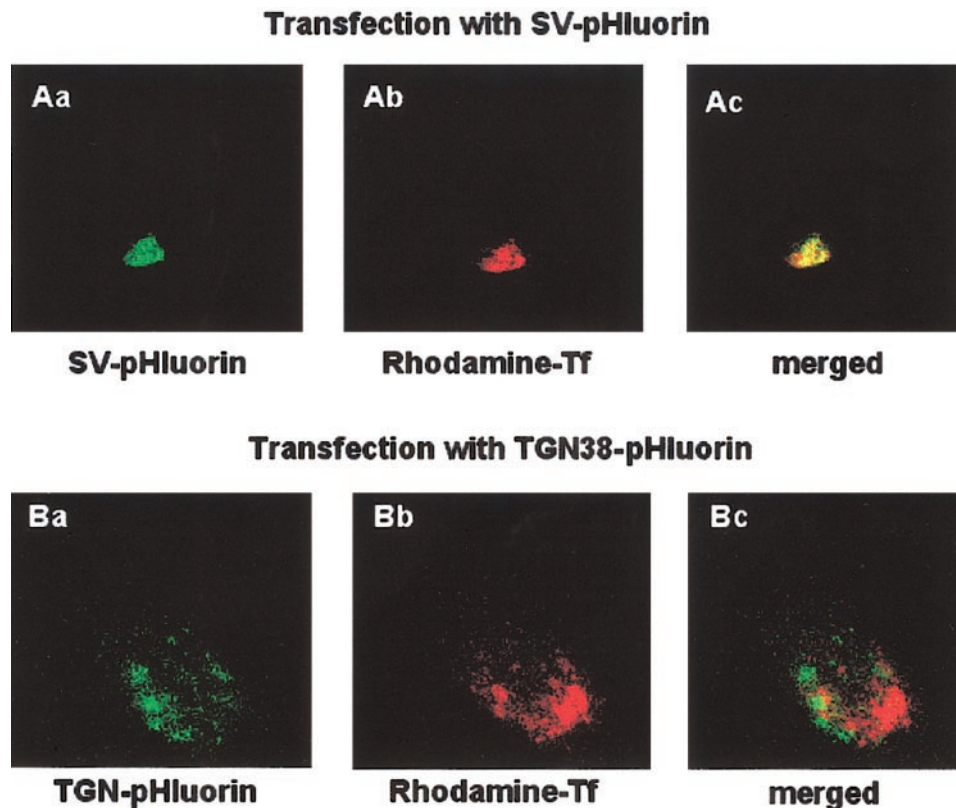


Fig. 1. Localization of H⁺/K⁺ ATPase and *trans*-Golgi network (TGN)-pHluorin in HEK cells. HEK-H⁺/K⁺-α,β (Aa–Ac) or mock (Ba–Bc) cells were transiently transfected with TGN38-pHluorin. Fixed and permeabilized cells were stained with anti-H⁺/K⁺ ATPase β-subunit, followed by rhodamine-conjugated goat anti-mouse antibodies. The patterns of green fluorescent protein (GFP) fluorescence from TGN38-pHluorin (Aa and Ba) were compared with rhodamine fluorescence from H⁺/K⁺-ATPase (Ab and Bb). Merged images are shown in Ac and Bc.

Fig. 2. SV-pHluorin localizes to recycling endosomes. HEK-mock cells were transiently transfected with synaptobrevin (SV)-pHluorin (*Aa–Ac*) or TGN38-pHluorin (*Ba–Bc*). Transfected cells were labeled with rhodamine-transferrin and fixed. The patterns of GFP fluorescence from the pHluorins (*Aa* and *Ba*) were compared with rhodamine fluorescence from rhodamine-transferrin (*Ab* and *Bb*). Merged images are shown in *Ac* and *Bc*. Similar results were obtained with H⁺/K⁺- α,β cells (data not shown).



Similar experiments were performed on H⁺/K⁺- α,β and mock cells that had been transiently transfected with SV-pHluorin to compare the cellular localization of SV-pHluorin and that of the TGN (identified using TGN38) and recycling endosomes (identified using rhodamine-transferrin). SV-pHluorin is a marker of synaptic vesicles in neuronal cells. It shares similar subcellular trafficking patterns as cellubrevin (8), and like cellubrevin it is targeted to endosomes in HEK-293 cells. Rhodamine-transferrin colocalized to the same compartment as SV-pHluorin (Fig. 2, *Aa–Ac*) but not to the compartment where TGN38-pHluorin was localized (Fig. 2, *Ba–Bc*). In addition, some H⁺/K⁺-ATPase appeared to colocalize with SV-pHluorin in the H⁺/K⁺- α,β cells (Fig. 3, *Aa–Ac*), whereas there was no H⁺/K⁺-ATPase staining in the SV-pHluorin-transfected mock cells (Fig. 3, *Ba–Bc*). These data showed that the H⁺/K⁺-ATPase was expressed in the plasma membrane and also in both the TGN and recycling endosomes. The next set of experiments was designed to test for H⁺/K⁺-ATPase pumping by testing for SCH28080-induced changes in cytosolic pH and in luminal pH of the TGN and recycling endosomes.

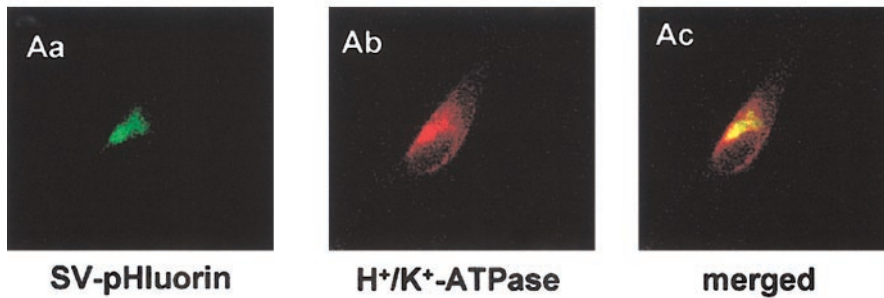
H⁺/K⁺-ATPase is active in the plasma membrane of H⁺/K⁺- α,β cells. Previous work (22) showed that H⁺/K⁺- α,β cells exhibited Rb⁺ influx and H⁺ efflux consistent with the presence of active H⁺/K⁺-ATPase in the plasma membrane. SCH28080-sensitive Rb⁺ uptake amounted to $\sim 0.3 \text{ nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{min}^{-1}$. Assuming that the H⁺/K⁺-ATPase is a neutral one-for-one pump with a turnover rate of 300/s, this rate of Rb⁺ uptake

indicated that there were 10,000 active H⁺/K⁺-ATPase pumps in the plasma membrane of each cell.

Further measurements of cytosolic pH (pH_C) by Kimura et al. (22) showed Na⁺-independent (i.e., occurred in absence of Na⁺ in the medium), SCH28080-sensitive pH_C recovery from an acid load occurred at a rate of 0.06 pH/min. We repeated these experiments here using procedures similar to those of Kimura et al. (22). H⁺/K⁺- α,β cells were acid-loaded by a 5-min treatment with 30 mM NH₄-containing Ringer's solution followed by incubation in Na⁺-free Ringer's solution (*N*-methyl-D-glucamine replaced Na⁺). Under these Na⁺-free conditions, pH_C recovered toward baseline at rates of 0.05 and 0.07 pH unit/min ($n = 2$ experiments), and this recovery was totally blocked by 50 μM SCH28080. This Na⁺-independent, SCH28080-blockable pH_C recovery was absent in two similar experiments on mock cells (data not shown). Assuming that the buffer capacity of H⁺/K⁺- α,β cells was 25 mmol·liter⁻¹·pH unit⁻¹ (35) and cell volume was 10⁻¹² liter, the pH_C recovery rate of 0.06 pH/min (22) was used to calculate that each H⁺/K⁺- α,β cell expressed 50,000 active pumps in the plasma membrane.¹ Thus these previous experiments indicated

¹Number of H⁺/K⁺-ATPases in the plasma membrane was calculated as follows: number of pumps = (pH recovery rate) \times (cell buffer capacity) \times (cell volume) \times (Avogadro's number) \times (pump turnover number)⁻¹ = (0.06 pH/min \times 1 min/60 s) \times (25 \times 10⁻³ mol·l cell water⁻¹·pH unit⁻¹) \times (10⁻¹² liter) \times (6.02 \times 10²³ H⁺/mol) \times (300 H⁺/s)⁻¹ = 5.02 \times 10⁴ H⁺/K⁺ ATPase pumps in the plasma membrane.

Transfection with H⁺/K⁺-ATPase (αβ) + SV-pHluorin



Transfection with SV-pHluorin alone

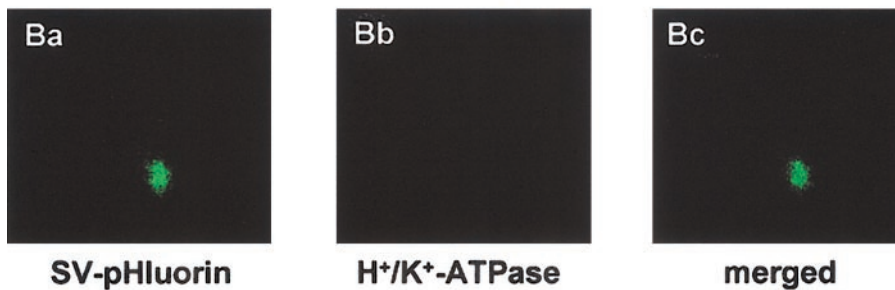


Fig. 3. Localization of H⁺/K⁺ ATPase and SV-pHluorin in HEK cells. H⁺/K⁺-α,β (Aa–Ac) or mock (Ba–Bc) cells were transiently transfected with SV-pHluorin. Fixed and permeabilized cells were stained with anti-H⁺/K⁺ ATPase β-subunit, followed by rhodamine-conjugated goat anti-mouse antibodies. The patterns of GFP fluorescence from SV-pHluorin (Aa and Ba) were compared with rhodamine fluorescence from H⁺/K⁺-ATPase (Ab and Bb). Merged images are shown in Ac and Bc.

that H⁺/K⁺-α,β cells expressed between 10,000 and 50,000 H⁺/K⁺-ATPases in the plasma membrane. Despite the presence of these “extra” acid extruders in the plasma membranes of H⁺/K⁺-α,β as compared with mock cells, Kimura et al. (22) found that H⁺/K⁺-α,β cells were not significantly more alkaline than mock cells, indicating that the activity of other pH-regulatory mechanisms dominated over the H⁺/K⁺-ATPase in maintenance of steady-state pH_C. Similarly, parietal cells that have been stimulated to secrete HCl also exhibit only small changes in pH_C due to the fact that anion- and cation-coupled acid-base transporters in the basolateral membrane are able to acidify the cytosol at the same rate that the H⁺/K⁺-ATPase alkalinizes the cytosol (25, 27, 37).

H⁺/K⁺-ATPase in the TGN: pH measurements using TGN-pHluorin. It was expected that if the H⁺/K⁺-ATPase was active in the TGN, then the TGNs of the H⁺/K⁺-α,β cells would be more acidic than the mock cells. However, similar to the cytosol of H⁺/K⁺-α,β vs mock cells, the TGNs of H⁺/K⁺-α,β cells and mock cells were nearly identical: average pH_{TGN} was 6.36 in H/K-α,β cells and 6.34 in mock cells (Table 1). These values of pH_{TGN} were similar to pH_{TGN} 6.21 measured by Miesenbock et al. (24) and 6.0–6.7 measured by Poschet et al. (30) but somewhat more alkaline than pH_{TGN} 5.9 measured by Kim et al. (20). We concluded that the TGN was roughly one pH unit more acidic than the cytosol in both H⁺/K⁺-α,β cells and mock cells, although pH_{TGN} in individual cells varied between pH 5.8 and pH 6.8.

One possible explanation for the fact that the TGNs of H⁺/K⁺-α,β cells and mock cells had very similar average pH values was that the H⁺/K⁺-ATPase was

not active in the TGN. Alternatively, there could be an SCH28080-inhibitable acidification mechanism in H⁺/K⁺-α,β cells, but pH_{TGN} was variable enough that small differences could not be detected using different sets of cells. We therefore tested for the effects of

Table 1. SCH28080 and bafilomycin on pH_{TGN} in H⁺/K⁺-α,β vs. mock cells

	H ⁺ /K ⁺ -α,β	Mock
Control		
pH _{TGN} (initial)	6.36 ± 0.04 (9,36)	6.34 ± 0.04 (9,38)
[H ⁺], nM	551 ± 67 (9,36)	540 ± 50 (9,38)
+ SCH28080		
ΔpH _{TGN}	0.12 ± 0.03 (5,20)	0.02 ± 0.01 (5,22)
Δ[H ⁺], nM	141 ± 32 (5,20)	24 ± 10 (5,22)
+ bafilomycin		
ΔpH _{TGN}	0.65 ± 0.05 (5,20)	0.55 ± 0.03 (5,22)
Δ[H ⁺], nM	297 ± 29 (5,20)	328 ± 48 (5,22)
pH (final)	7.20 ± 0.05 (9,36)	7.03 ± 0.04 (9,38)
Δ[H ⁺](baf)/Δ[H ⁺](SCH)	2.1	

H⁺/K⁺-α,β cells and mock cells were transfected with *trans*-Golgi network (TGN)38-pHluorin and treated first with 50 μM SCH28080 and then 100–500 nM bafilomycin. Values are means ± SE for pH_{TGN} before addition of inhibitors (initial) and final pH_{TGN} after both inhibitors were present are shown at *top* and *bottom*. The changes of pH_{TGN} and of [H⁺] due to the two inhibitors are also shown for experiments in which effects of SCH28080 and bafilomycin were compared in the same cells. Numbers in parentheses represent the number of different experiments, number of cells for each treatment. Note that average values for pH and [H⁺] were calculated from individual values for every cell, and, due to this fact, average [H⁺] did not equal [anti-log(average pH)]⁻¹. The last line shows the average Δ[H⁺] due to bafilomycin treatment divided by the Δ[H⁺] due to SCH28080 treatment for the same experiments. As described in the text, this ratio is equal to the ratio of number of H⁺ v-ATPase pumps to H⁺/K⁺-ATPase pumps in the TGN.

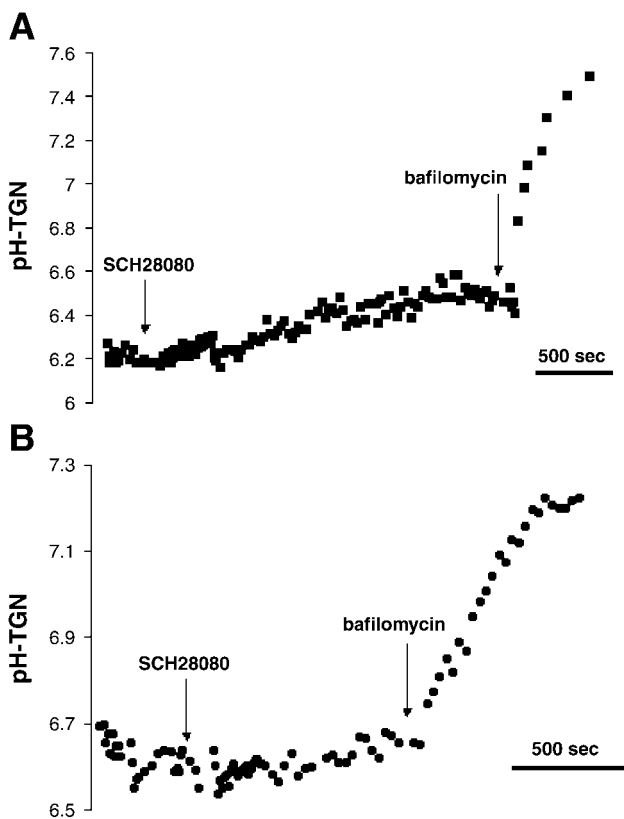


Fig. 4. Effects of SCH28080 and bafilomycin on pH_{TGN} in $\text{H}^+/\text{K}^+-\alpha,\beta$ cells (A) and mock (B) cells. SCH28080 (50 μM) and bafilomycin (500 nM) were added (arrows) to Ringer's solution bathing $\text{H}^+/\text{K}^+-\alpha,\beta$ (A) and mock (B) cells that had been transfected with TGN-pHluorin. SCH28080 caused pH_{TGN} to increase slowly by about 0.20 pH units in the $\text{H}^+/\text{K}^+-\alpha,\beta$ cells but had no effect on pH_{TGN} in mock cells. Bafilomycin caused pH_{TGN} to increase rapidly by >0.6 pH units in both $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells.

SCH28080 on steady-state pH_{TGN} . This H^+/K^+ -pump blocker caused a slow, but consistent alkalinization of pH_{TGN} in $\text{H}^+/\text{K}^+-\alpha,\beta$ cells (Fig. 4A) but not in mock cells (Fig. 4B). Subsequent addition of bafilomycin caused a more rapid and larger increase in pH_{TGN} to pH 7.0–7.5 (Fig. 4, A and B), similar to values that were observed in the cytosol in both $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells (22).

The effects of these inhibitors on steady-state pH_{TGN} of $\text{H}^+/\text{K}^+-\alpha,\beta$ and mock cells are summarized in Table 1. SCH28080 caused pH_{TGN} to increase (at an average rate of $0.20 \pm 0.03 \times 10^{-3}$ pH/s) to a new steady-state value that was on average 0.12 pH units more alkaline than the initial pH_{TGN} in $\text{H}^+/\text{K}^+-\alpha,\beta$ cells, whereas there was no significant effect of SCH28080 on pH_{TGN} in mock cells. Further addition of bafilomycin caused pH_{TGN} to increase by ~ 0.6 pH units (at initial rates of $1.95 \pm 0.07 \times 10^{-3}$ pH/s and $1.61 \pm 0.13 \times 10^{-3}$ pH/s, respectively) in $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells. Similar results were obtained when $\text{H}^+/\text{K}^+-\alpha,\beta$ cells were treated first with bafilomycin followed by SCH28080: initial pH was 6.34 ± 0.13 (1, 6); bafilomycin caused pH_{TGN} to increase by 1.21 ± 0.16 (1, 6) pH units, and subsequent addition of SCH28080 caused pH to in-

crease a further 0.23 ± 0.08 (1, 6) pH units; final pH_{TGN} was 7.62 ± 0.08 (1, 6). Thus bafilomycin caused large, rapid increases and SCH28080 caused smaller and slower increases in pH_{TGN} . These results were consistent with the idea that the H^+/K^+ -ATPase was active in the TGN and contributed to a small but significant acidification of this organelle in the steady state.

H⁺/K⁺-ATPase in recycling endosomes: pH_{RE} measurements using SV-pHluorin. As shown in Figs. 5, A and B, experiments were also performed on SV-pHluorin-transfected $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells to test for the effects of SCH28080 and bafilomycin. Similar to the results obtained for the TGN, average pH_{RE} was similar in both $\text{H}^+/\text{K}^+-\alpha,\beta$ cells (average pH_{RE} 6.47) and mock cells (average pH_{RE} 6.37; see Table 2). SCH28080 caused pH_{RE} to alkalinize slowly but consistently in $\text{H}^+/\text{K}^+-\alpha,\beta$ cells (Fig. 5A) but not in mock cells (Fig. 5B). Subsequent addition of bafilomycin caused a more rapid and larger increase in pH_{RE} to pH 7.0–7.5, similar to effects on pH_{TGN} .

The effects of these inhibitors on steady-state pH_{RE} of $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells are summarized in

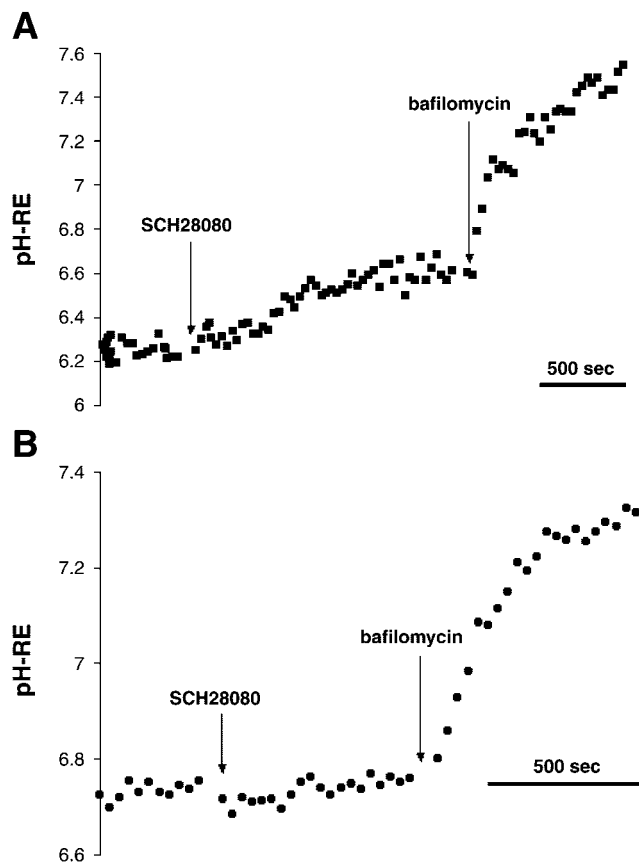


Fig. 5. Effects of SCH28080 and bafilomycin on pH_{RE} in $\text{H}^+/\text{K}^+-\alpha,\beta$ (A) and mock (B) cells. SCH28080 (50 μM) and bafilomycin (500 nM) were added (arrows) to Ringer's solution bathing $\text{H}^+/\text{K}^+-\alpha,\beta$ cells (A) and mock cells (B) that had been transfected with SV-pHluorin. SCH28080 caused pH_{RE} (recycling endosomes) to increase by about 0.25 pH units in the $\text{H}^+/\text{K}^+-\alpha,\beta$ cells but had no effect on pH_{RE} in mock cells. Bafilomycin caused pH_{RE} to increase rapidly by >0.6 pH units in both $\text{H}^+/\text{K}^+-\alpha,\beta$ cells and mock cells.

Table 2. SCH28080 and bafilomycin on pH_{RE} in H⁺/K⁺-α,β vs. mock cells

Control	H ⁺ /K ⁺ -α,β	Mock
Control		
pH _{RE} (initial)	6.40 ± 0.04 (2,20)	6.37 ± 0.04 (3,22)
[H ⁺], nM	439 ± 45 (2,20)	486 ± 77
+ SCH28080		
ΔpH _{RE}	0.22 ± 0.02 (2,15)	-0.003 ± 0.008 (1,9)
Δ[H ⁺], nM	144 ± 23 (2,15)	2 ± 7 (1,9)
+ bafilomycin		
ΔpH _{RE}	0.42 ± 0.02 (2,15)	0.51 ± 0.09 (3,16)
Δ[H ⁺], nM	140 ± 16 (2,15)	233 ± 27 (3,16)
ΔpH _{RE} (final)	7.14 ± 0.04 (2,20)	6.96 ± 0.08 (3,16)
Δ[H ⁺](baf)/Δ[H ⁺](SCH)	0.80	

H⁺/K⁺-α,β cells and mock cells were transfected with SV-pHluorin and treated first with 50 μM SCH28080 and then 100–500 nM bafilomycin. Initial pH_{RE} (recovery interval) before addition of inhibitors and final pH_{RE} after both inhibitors were present are shown at the top and bottom. The magnitude of change of pH and of [H⁺] due to the two inhibitors are also shown for experiments in which effects of SCH28080 and bafilomycin on pH_{RE} were compared in the same cells. Numbers in parentheses represent the number of different experiments, number of cells. Note that average values for pH and [H⁺] were calculated from individual values for every cell, and, due to this fact, average [H⁺] did not equal [anti-log(average pH)]⁻¹. The last line shows the average steady-state Δ[H⁺] due to bafilomycin treatment divided by the Δ[H⁺] due to SCH28080 treatment. See text for details.

Table 2. SCH28080 caused pH_{RE} to increase by an average of 0.22 pH units (at an average rate of 0.28 ± 0.24 × 10⁻³ pH/s) in H⁺/K⁺-α,β cells, and further addition of bafilomycin caused pH_{RE} to increase by 0.42 pH units (at a rate of 1.61 ± 0.40 × 10⁻³ pH/s) in H⁺/K⁺-α,β cells. There were no significant effects of SCH28080 on pH_{RE} in mock cells, whereas bafilomycin caused pH_{RE} to alkalinize as expected (Fig. 5B and Table 2).

These experiments indicated that the H⁺/K⁺-ATPase was also active in recycling endosomes, although, like the situation in the TGN, inhibition of this pump had smaller effects on pH_{RE} compared with the effects of bafilomycin. The H⁺/K⁺-ATPase requires the presence of K⁺ at its lumen-facing aspect to exchange one-for-one with H⁺ that are transported from the cytosol into the lumen. Because the K⁺ permeability properties of recycling endosomes have not been tested, it was possible that the recycling endosomes (unlike the TGN, which has large K⁺ and Cl⁻ permeability; see Refs. 11 and 33) may not have had enough K⁺ in the lumen to permit full activity of the H⁺/K⁺-pumps. We therefore tested whether valinomycin treatment would enhance H⁺/K⁺-ATPase activity by allowing K⁺ to permeate from the cytosol into the lumen of the endosomes, thereby increasing availability of K⁺ at the luminal aspect of the pump. Cells were treated first with bafilomycin and then valinomycin, and pH_{RE} attained a new steady state of 6.92 ± 0.08 (3, 26). Further addition of SCH28080 caused pH_{RE} to increase (at a rate of 0.25 ± 0.17 × 10⁻³ pH/s) up to a new steady state that was more alkaline by 0.23 ± 0.04 pH units (3, 26), similar to the effects observed when SCH28080 was added before bafilomycin (Table 2). There were no

significant effects of SCH28080 on pH_{RE} in mock cells. These results indicated that the H⁺/K⁺-ATPase that was active in recycling endosomes was not limited in activity by the availability of K⁺ in the lumen of this organelle.

Relative rates of H⁺ pumping by H⁺ v-ATPase and H⁺/K⁺-ATPase in the TGN and recycling endosomes. Data presented in the previous sections showed that the H⁺/K⁺ ATPase pump was present and active in both the TGN and recycling endosomes in H⁺/K⁺-α,β cells, but the activity of this pump was less than that of the H⁺ v-ATPase that was present endogenously in these organelles. The relative pumping activities due to these two pumps can be estimated from the alkalinization that occurred after treatment with SCH28080 and bafilomycin because the two inhibitors were specific to the two H⁺ pumps. We reasoned that blocking the H⁺ v-ATPase with bafilomycin would lead to an alkalinization due to the loss of activity of this transporter, and similarly blocking the H⁺/K⁺-ATPase with SCH28080 would lead to an alkalinization due to the loss of activity of this transporter. Furthermore, because the TGN appears to have large permeabilities to both K⁺ and Cl⁻ and likely a small membrane potential (33; also see Ref. 39), the relative numbers of H⁺ v-ATPases and H⁺/K⁺-ATPases can be estimated (assuming their turnover rates are approximately equal) from the relative changes in [H⁺] that occurred after each treatment, i.e., steady-state [H⁺] will be directly proportional to the number of H⁺ ATPases present in the membrane (see Ref. 17). As shown in the last line of Table 1, when the Δ[H⁺] due to bafilomycin treatment was compared with Δ[H⁺] due to SCH28080 treatment in the same experiments, the ratio was 2.1, indicating that there were 2.1 times as many bafilomycin-sensitive H⁺ v-ATPases as H⁺/K⁺-ATPases in the TGN of H⁺/K⁺-α,β cells.

Using similar reasoning and the data in Table 2 for the recycling endosomes, it can be concluded that the ratio of H⁺ v-ATPase/H⁺/K⁺-ATPase ratio was 0.8, i.e., that there were approximately equal numbers of H⁺ v-ATPases and H⁺/K⁺-ATPases in the recycling endosomes of H⁺/K⁺-α,β cells. Although this conclusion is much less secure because the permeability properties for K⁺ and Cl⁻ of recycling endosomes have not been determined and the role of membrane potential in this compartment remains unknown, the results clearly showed that the H⁺/K⁺-ATPase contributed to pH regulation in recycling endosomes.

An "inefficient" H⁺ pump/leak system stabilizes the pH of trafficking organelles. It might have been expected that steady-state pH_{TGN} in H/K α,β cells would have been much more acidic than the values of pH 6.0–6.5 observed in mock cells (Table 1) and in other cell types (11, 24, 30): the H⁺/K⁺-ATPase pump has the capability to generate a highly acidic fluid with pH 1.0 or less, the TGN has the required K⁺ and Cl⁻ conductances required for the H⁺/K⁺-ATPase to transport H⁺, and this pump was active in the TGN during its trafficking to the plasma membrane. Instead, based on the effects of SCH28080 on pH_{TGN}, the H⁺/K⁺-ATPase

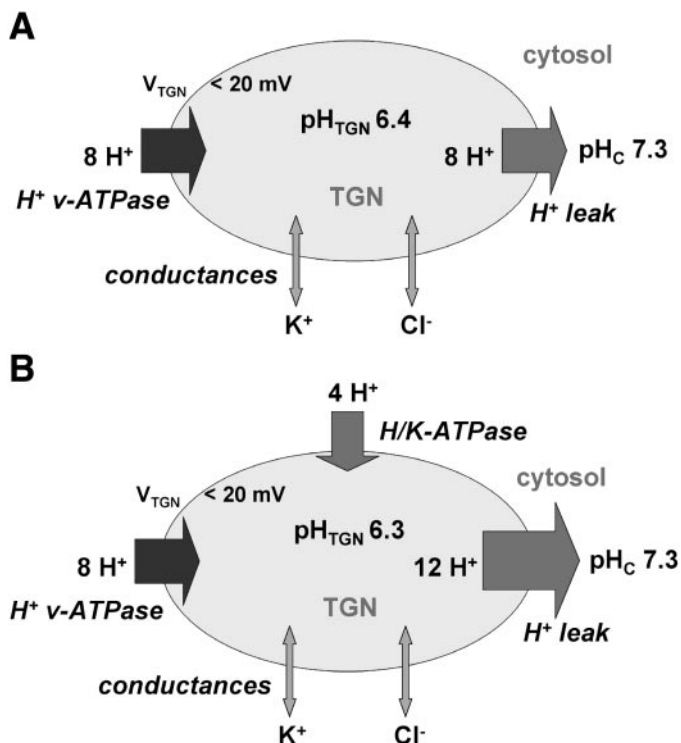


Fig. 6. Models for steady-state pH regulation in TGN in H⁺/K⁺ α,β cells and mock cells. Relative rates of H⁺ pumping by the H⁺ v-ATPase and the H⁺/K⁺-ATPase and rate of H⁺ leak through the passive H⁺ permeability are shown by the numbers associated with the arrows. TGN's are in a steady state, so the total H⁺ pumped into the lumen of the TGN per second equals the H⁺ that leak out of the lumen into the cytosol. A: mock cells have a large rate of H⁺ pumping (due to H⁺ v-ATPase) and leaking (through H⁺ permeability) with steady-state pH_{TGN} = 6.4. As described in the text and shown in Fig. 7, we estimate there to be 8,000 H⁺ v-ATPases in the TGN of mock cells. Therefore, the value "8" was chosen arbitrarily to represent the rate of H⁺ accumulation by the H⁺ v-ATPase and leak by the passive H⁺ permeability in the control state. B: H⁺/K⁺ α,β cells have the same H⁺ v-ATPase and H⁺ permeability, but in addition they have the H⁺/K⁺-ATPase, which also acidifies the TGN lumen, but ~2.1 times less than the H⁺ v-ATPase (see text for details). The additional H⁺ pumping into the TGN by the H⁺/K⁺-ATPase (shown as 4 to be in the ratio of H⁺ v-ATPase/H⁺/K⁺-ATPase = 2 as calculated in the text and Table 1) leads to the following: an ~0.10 pH unit acidification of the TGN, no change in cytosolic pH, and an increase in H⁺ leak into the cytosol such that pH_{TGN} is maintained in a steady state at this new, slightly more acidic level. In the steady state, the sum of the H⁺ pumping by the H⁺/K⁺-ATPase (4) plus that of the H⁺ v-ATPase (8) from the cytosol into the TGN lumen must equal the rate of H⁺ leak (12) from the TGN lumen into the cytosol.

appeared to acidify the TGN of H/K α,β cells by only 0.12 pH units compared with the TGN in mock cells (Table 1).

An explanation for the similar values of pH_{TGN} in the H⁺/K⁺ α,β and mock cells comes from a consideration of the overall H⁺ pump and leak characteristics of the TGN and Golgi in mock cells (Fig. 6A) and in H⁺/K⁺ α,β cells (Fig. 6B). Previous experiments have shown that the Golgi has large passive H⁺ permeability equal to $\sim 10^{-3}$ cm/s (5, 39), which assures that H⁺ leaks rapidly across the membrane. The rapid alkalization of pH_{TGN} when bafilomycin is added to cells shows that the TGN has a similarly large H⁺ permeability. Be-

cause the H⁺ permeability is large, the TGN and Golgi require a large number of H⁺ v-ATPase pumps to maintain an acidic lumen. Thus, in the steady state, the Golgi and TGN operate in an inefficient mode in which H⁺ are constantly being recycled (pumped and leaked) across the membrane. In this condition, trafficking H⁺/K⁺ pumps through the Golgi and TGN has only a small effect on the steady-state pH of these organelles because the number of H⁺/K⁺ pumps that are trafficking to the plasma membrane through these organelles is, in the steady state, small relative to the number of H⁺ v-ATPase pumps that are already operating.

This effect can be better appreciated by consideration of Fig. 7, which shows results of calculations of pH in the Golgi as a function of the number of H⁺ v-ATPase pumps. Details of the model and its use in calculating H⁺ permeabilities of organelles have been presented previously (5, 17, 39). Here, we assumed that the TGN and Golgi behaved similarly and used the model to determine the effect of trafficking different numbers of H⁺/K⁺-ATPases through the Golgi using previously published data regarding H⁺ pumping rate of the H⁺ v-ATPase, anion and cation permeabilities of the Golgi and the membrane voltage, buffer capacity, and surface area and volume (see Refs. 5, 17, 40). We then calculated pH_G with H⁺ permeability set to various values between 3×10^{-3} and 7×10^{-5} cm/s.

As shown in Fig. 7, we predict for a Golgi with H⁺ permeability of 10^{-3} cm/s that ~8,000 H⁺ v-ATPase pumps are required to generate a Golgi pH of 6.4. Because the ratio of H⁺ v-ATPases to H⁺/K⁺-ATPases in the Golgi of H⁺/K⁺ α,β cells was calculated to be 2.1, we further estimate that there are 8,000/2.1 = 3,800 H⁺/K⁺-ATPases in the Golgi of the H⁺/K⁺ α,β cells. As can be seen from Fig. 7, it is predicted that these 3,800 H⁺/K⁺-ATPases in the Golgi would have caused the Golgi to acidify to about pH 6.2, i.e., by only 0.20 pH

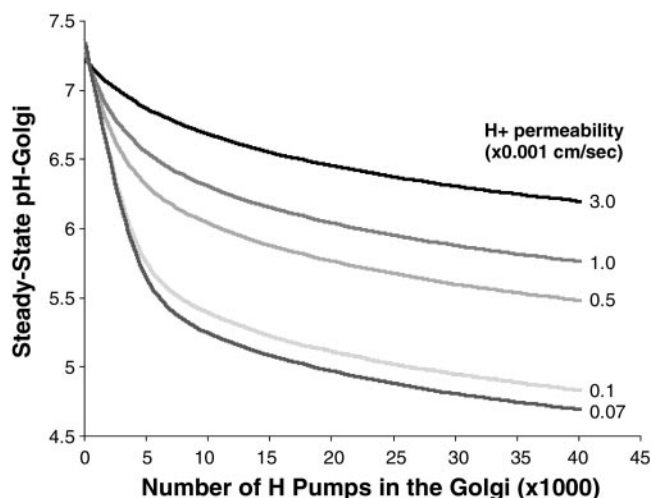


Fig. 7. Effects of H⁺-ATPase density on pH of Golgi with different H⁺ permeabilities. Model calculations show effect of increasing numbers of H⁺ pumps on pH in the Golgi, assuming H⁺ permeability of the Golgi was equal to the values shown at the ends of each line (in cm/s $\times 10^{-3}$). See text for details.

units compared with the value observed in mock cells, similar to the 0.12 pH unit acidification observed in the present experiments (Table 1). This small acidification is predicted, though, to increase the rate of H⁺ leak out of the Golgi or TGN in the steady state, as shown in comparing A and B of Fig. 6. Thus pH in the Golgi remains stable because there are so many endogenous H⁺ v-ATPase pumps and H⁺ leaks that any trafficking pH regulators have only small effects on pH_G.

Calculations shown in Fig. 7 also show that the H⁺ permeability of the Golgi cannot be too high; otherwise, an excessive number of H⁺ v-ATPases would be required to acidify the Golgi to pH 6.3. In contrast, if the Golgi had a H⁺ permeability of 10⁻⁴ cm/s (i.e., 10 times lower than what has been previously calculated) or lower, then only 2,500 H⁺ v-ATPases (or fewer) would be required to generate pH 6.4 (Fig. 7). However, in this circumstance, the trafficking of 3,800 H⁺/K⁺-ATPases through the Golgi would cause pH_G to acidify to pH 5.3.

DISCUSSION

It should be stressed that these calculations of numbers of H⁺ v-ATPases and H⁺/K⁺-ATPases in the Golgi are only rough estimates that require assumptions of the buffer capacity, surface/volume ratio, H⁺ permeability, and membrane potential of the Golgi/TGN, as well as the turnover rates of the H⁺ v-ATPase (150/s; Ref. 17) and H⁺/K⁺-ATPase (300/s; J. G. Forte, personal communication) in these compartments. Although values for these different parameters have been reported previously for other cells (5, 11, 39, 40), they have never been determined for HEK-293 cells, so our estimates for pump numbers may be off due to cell type differences. However, such potential inaccuracies should not overshadow the general principle that the Golgi and TGN likely have similar pH values in all cells, despite their needs for trafficking different transporters to the plasma membrane.

It might be expected that stability of Golgi pH to trafficking of H⁺/K⁺-ATPases would be particularly important in the parietal cells of the stomach, which have 0.5 million pumps in their plasma membranes (J. G. Forte, unpublished observations), compared with the transfected HEK-293 cells used for the present studies in which we estimated there were only between 10 and 50,000 H⁺/K⁺-ATPases active in the plasma membrane and ~3,800 H⁺/K⁺-ATPases in the TGN. However, due to the relatively slow rate of turnover of H⁺/K⁺-ATPase in parietal cells ($t_{1/2}$ ~24 h; see Ref. 26; also, J. G. Forte, personal communication), it is expected (based on approximate residence time of 15 min in the Golgi) that there would be only ~2,600 H⁺/K⁺-ATPase pumps in the Golgi of parietal cells. As shown in Fig. 7, this number of H⁺/K⁺-ATPase pumps would be expected to acidify the Golgi only to pH 6.3, which is within the range of reported values in different cells (5, 11, 14, 39, 40). Thus it seems that the Golgi and TGN (and, using similar reasoning, the recycling endosomes) likely have similar pH values in all cells (both

in vivo and in transfected cells in culture) despite trafficking different types and numbers of transporters to the plasma membrane. This is due to the fact that the Golgi (and possibly endosomes) has relatively large H⁺ permeabilities that require large numbers of H⁺ pumps to keep the organelles even mildly acidic; the pumps and other transporters trafficked to the plasma membranes, therefore, have only small effects on pH of organelles along the secretory pathway. Thus trafficking organelles seem to sacrifice efficiency to generate pH stability.

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