Mechanisms of pH Regulation in the Regulated Secretory Pathway*

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A precise pH gradient between organelles of the regulated secretory pathway is required for sorting and processing of prohormones. We studied pH regulation in live endocrine cells by targeting biotin-based pH indicators to cellular organelles expressing avidin-chimera proteins. In AtT-20 cells, we found that steady-state pH decreased from the endoplasmic reticulum (ER) (pHER = 7.4 ± 0.2, mean ± S.D.) to Golgi (pH0 = 6.2 ± 0.4) to mature secretory granules (MSGs) (pHMSG = 5.5 ± 0.4). Golgi and MSGs required active H⁺ v-ATPases for acidification. ER, Golgi, and MSG steady-state pH values were also dependent upon the different H⁺ leak rates across each membrane. However, neither steady-state pHMSG nor rates of passive H⁺ leak were affected by Cl⁻-free solutions or valinomycin, indicating that MSG membrane potential was small and not a determinant of pHMSG. Therefore, our data do not support earlier suggestions that organelle acidification is primarily regulated by Cl⁻ conductances. Measurements of H⁺ leak rates, buffer capacities, and estimates of surface areas and volumes of these organelles were applied to a mathematical model to determine the H⁺ permeability (PfH⁺) of each organelle membrane. We found that PfH⁺ decreased progressively from ER to Golgi to MSGs, and proper acidification of Golgi and MSGs required gradual decreases in PfH⁺ and successive increases in the active H⁺ pump density.

Maintenance of luminal pH in organelles of the secretory pathway is required for proper sorting and proteolytic processing of prohormones. Even small pH differences between organelles can be critical in separating cellular events. For example, a difference of <0.5 pH can determine whether a prohormone is processed (1). In professional secretory cells (e.g. endocrine and neuroendocrine), “regulated” secretory proteins have been hypothesized to be sorted from constitutively secreted proteins by a process of pH- and calcium-dependent selective aggregation (2–4). The exact site where aggregation occurs is controversial, largely due to the lack of direct measurements of organelle pH along the regulated secretory pathway. The “sorting for entry” model postulates that sorting occurs when proteins encounter the ionic milieu of the trans-Golgi network (3). In contrast, the “sorting by retention” model asserts that aggregation serves to retain regulated proteins in granules and does not occur until prohormones have entered acidic immature secretory granules (ISGs) and become proteolytically processed (5).

Work by several groups (1, 6–11) using a variety of techniques indicates that organelles of the secretory pathway, from ER to Golgi to secretory granules, become increasingly acidic. Indirect measurements of pH in isolated secretory granules of endocrine and neuroendocrine cells using either electron microscopy (measuring acidity based on accumulation of the weak base DAMP or biochemical reactions (measuring acidity based on the extent of processing) (1, 6–11) suggested that ISGs and MSGs were both acidic (pHMSG = 6.3–5.7; pHMSG = 5.5–5.0). In live cell pH measurements using the green fluorescent protein derivative pHlorin (12), secretory granules of mast cells were also acidic (pH 5.2). In “non-regulated” cells (Chinese hamster ovary, HeLa, HepG2, and Vero), pH experiments performed using both DAMP on fixed cells (13, 14) and fluorescent probes in live cells showed that ER pH (pHER = 7.1–7.2) was similar to cytosolic pH (pHc), whereas Golgi pH was acidic (pH0 = 6.5–6.2). In contrast, in “regulated” cells, experiments using DAMP detected no acidification of Golgi in pancreatic islet cells (7, 8). It is unclear whether the conflicting Golgi pH data are due to differences in cell type or techniques used. Therefore, there is general agreement regarding the acidity of MSGs, the exact pH of Golgi versus ISGs (and hence the site of sorting) in regulated secretory cells remains in question.

Furthermore, although the dynamics of pH regulation in the Golgi and ER of non-regulated cells has been extensively studied, there has been no examination of the pH regulatory mechanisms of Golgi and MSGs in live, intact cells with regulated secretory pathways. pH studies of these organelles have been limited to either fixed cells or isolated organelles in vitro. Data from these studies suggested that Golgi and secretory granules controlled their acidic pH values by altering their conductances to Cl⁻, which served as a counterion for the H⁺ v-ATPase (6, 9).

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1 The abbreviations used are: ISGs, immature secretory granules; ER, endoplasmic reticulum; MSGs, mature secretory granules; v-ATPase, vacuolar ATPase; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyleneester; AV-POMC, avidin-pro-opiomelanocortin; DMEM, Dulbecco’s modified Eagle’s medium; ODE, ordinary differential equations; DAMP, 3-(2,4-dinitroanilino)-3’-amino-N-methyl dipropylamine.
critical role for Cl\(^{-}\) in organelle acidification (22, 23). These results led to the general hypothesis that acidic organelles maintain distinct luminal pH values by maintaining different permeabilities to Cl\(^{-}\), the primary counterion (24). However, experiments in non-regulated cells demonstrated that although Cl\(^{-}\) and K\(^{+}\) did serve as counterions for H\(^{+}\) pumping, Cl\(^{-}\) and K\(^{+}\) conductances were large compared with the passive H\(^{+}\) conductance, arguing against modulation of Cl\(^{-}\) and K\(^{+}\) conductances as a mechanism for trans-Golgi network or Golgi pH regulation (18, 21). In addition, the pH-dependent processing of secretoratatin II measured in vitro was not stimulated by increasing the outside Cl\(^{-}\) concentration (11). Thus, the exact mechanisms regulating acidification along the regulated secretory pathway are unknown.

To begin to address the controversies and the gaps in our understanding of pH regulation in organelles of the regulated secretory pathway, we targeted pH indicators to the ER, Golgi, and MSGs to study the pH regulatory mechanisms in live, intact endocilve. We chose the AtT-20 mouse pituitary cell line because it has a well characterized regulated secretory pathway. The data presented in this paper represent the first line because it has a well characterized regulated secretory and MSGs to study the pH regulatory mechanisms in live, intact, and intact endoc威尼斯. We chose the AtT-20 mouse pituitary cell line because it has a well characterized regulated secretory pathway. The data presented in this paper represent the first study of pH regulation in MSGs of live, intact cells, and the first systematic comparison of the pH regulatory mechanisms between the major organelles of the regulated secretory pathway. We tested for the importance of Cl\(^{-}\) and K\(^{+}\) conductances in determining pHe and pH\(_{\text{MSG}}\) by eliminating Cl\(^{-}\) from the solutions and by using the K\(^{+}\) ionophore valinomycin. In addition, since H\(^{+}\) leaks appear to be crucial determinants of organelle pH (18, 21, 25), we measured H\(^{+}\) leak rates and buffer capacities of the ER, Golgi, and MSGs and applied the results to a mathematical model (26) to calculate H\(^{+}\) permeabilities for each organelle. From our experimental data and mathematical modeling, we found that the acidification step between the ER and Golgi in AtT-20 cells was similar to that in non-regulated cells (21, 25) in that it required both an increase in active H\(^{+}\) pump density and a reduction of H\(^{+}\) permeability from the ER to the Golgi. Meanwhile, the acidification step between Golgi and MSGs required a decrease in H\(^{+}\) permeability in Golgi versus MSG membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Salts, amiloride, FCCP, fluorescein isothiocyanate-dextran, monensin, sodium butyrate, nigericin, puromycin, and valinomycin were from Sigma; all other organic chemicals were from Aldrich; solvents were from Fisher; and restriction enzymes were from New England Biolabs (Beverly, MA). Bafaloxycin was from Calbiochem; mouse laminin was from Life Technologies, Inc.; BACEF-AM and Phuronic F-127 were from Molecular Probes, Inc. (Eugene, OR).

**Construction of ER, Golgi, and MSG-targeted Avidin-Chimera Proteins**—The avidin-KDEL (AV-KDEL) encoding plasmid was constructed as described (21). An avidin-pro-opiomelanocortin (AV-POMC)-encoding plasmid was constructed by polymerase chain reaction amplification of avidin (Dr. Markku Kulomaa, University of Jyväskyla, Finland) using primers (5'-ggggagagacagtcacctgag-cgaac-3' and 5'-cggggagagctctcctcctgccgacgc-3'), which allowed iso- lation of avidin by HindIII and BamHI digestion. A signal sequence-lacking POMC was polymerase chain reaction-amplified from mouse POMC (gift from Dr. Edward Herbert) using primers 5'-cgccgagatctccgagagcc-3' and 5'-cggccggagacagtcacctgagc-3', which allowed iso- lation by BamHI and NotI digestion. Avidin and POMC were ligated into sfg7-poly vector (Dr. Brian Seed, Massachusetts General Hospital) using HindIII, BamHI, and NotI sites (avidin replaced IgG).

**Cell Culture and Transfection of AtT-20 Cells**—AtT-20 cells were grown as described previously (1). Golgi and MSG pH experiments were performed on AtT-20 cells that were either transiently transfectected (by electroporation, see below) or stably transfectected (27) with AV-POMC DNA. ER pH experiments were performed on AtT-20 cells transiently transfected with AV-KDEL DNA by electroporation.

24 h before electroporation, AtT-20 cells were passaged to obtain a 50% confluent 15-cm dish. On the day of electroporation, cells were trypsinized, rinsed with normal growth medium, and pelleted. The cell pellet was rinsed with cold DMEM, re-pelleted, and resuspended in 300 μl of cold DMEM. 100 μg of AV-POMC or AV-KDEL DNA and 50 μg of a puromycin resistance gene-encoding plasmid (pSFFACEBv, Dr. Brian Seed) were combined, phenol/chloroform-extracted, ethanol-precipitated, and resuspended in 100 μl of DMEM. To electroporate, the 300 μl mixture was mixed with the same volume of DNA, incubated for 5 min on ice, and transferred to a cold 0.4-cm gap cuvette. Cells were electroporated at 250 V, 0 ohm resistance, and 960 microfarads using a Bio-Rad Gene Pulser. After electroporation, cells recovered for 10 min on ice before being replated onto a 15-cm plate. 24 h post-transfection, 0.75 μg/ml of puromycin (Sigma) was added to the cell medium to select for positive transfectants. After 48 h of puromycin treatment, cells were replated onto laminin-covered coverslips and allowed to recover 24 h before imaging experiments were performed.

**Butyrate Induction of AV-POMC Expression**—Because the expression level of AV-POMC in AtT-20 stable cell lines was too low for fluorescence imaging experiments, we used a butyrate incubation protocol, described previously (28), to boost expression of AV-POMC. Butyrate prevents histone deacetylation, inducing expression from viral promoters. AV-POMC-stably expressing AtT-20 cells were plated on laminin-covered coverslips; 24 h after plating, cells were incubated in 6 mM sodium butyrate in normal growth medium for 15 h. After butyrate induction, cells were rinsed once in a large volume of DMEM and chased in normal growth medium for at least 34 h before loading with Flubi and measuring pH. The butyrate incubation had no effect on endogenous POMC or AV-POMC processing, indicating that MSG pH was unaffected by this protocol (data not shown).

**Fluorescent Labeling of Cytosol, ER, Golgi, and MSGs**—Cytosol, ER, Golgi, and MSG pH values were measured in separate experiments using digitally processed fluorescence ratio imaging. Ratio imaging measurements were performed at room temperature as described previously (21). For each of the bright Flubin-stained cell body of AtT-20 cells represents both AV-POMC-containing Golgi and some AV-POMC-containing MSGs. MSG pH was measured by collecting data from only the brightly labeled tips of cell processes of AtT-20 cells.

**Perfusion and Calibration Solutions and pH Calibration**—Ringer’s, NH\(_4\)Cl, Ringer’s, and Cl−-free, and sodium-free Ringer’s solutions were prepared as described (21). Calibration solutions were also prepared as described previously (21), with the exceptions being titrated to the following pH values: 8.2, 7.0, 6.5, 6.0, 5.4, and 4.9. In situ calibration were performed and the data fit to calibration curves, and the calibration curves were used to convert ratio values to pH values exactly as described (21).

**Determination of H\(^{+}\) Leak Rates, Buffer Capacities, and H\(^{+}\) Permeabilities**—Rates of H\(^{+}\) leak out of ER, Golgi, and MSG plasma membranes were calculated by fitting the data to the single exponential equation: \[y = A(1 - e^{-kx})\] using GraphPad InPlot (Kelvin Gee, Irvine, CA). Rate constants (k) and waiting times (t\(_{0}\)) were determined from the curve fits.

Since H\(^{+}\) leak rates across organelle membranes are affected by buffer capacity (β) and surface area-to-volume (SVV) ratio, differences in each compartment during perfusion with solutions containing 20, 30, or 40 mM NH\(_4\)Cl or from rapid decreases in pH of compartments during perfusion with solutions containing 20 or 30 mM sodium acetate. Values of β for ER, Golgi, and MSGs were determined using bafloxicin-generated experiments in normal growth medium for 0–2 h (0 h of chase for Golgi measurements; >2 h of chase for MSG measurements) at 37°C.

**Fluorescence Ratio Imaging of Cytosolic, ER, Golgi, and MSG pH**—Cytosol, ER, Golgi, and MSG pH values were measured in separate experiments using digitally processed fluorescence ratio imaging. Ratio imaging measurements were performed at room temperature as described previously (21). For each of the bright Flubin-stained cell body of AtT-20 cells represents both AV-POMC-containing Golgi and some AV-POMC-containing MSGs. MSG pH was measured by collecting data from only the brightly labeled tips of cell processes of AtT-20 cells.
NH$_4$ reaction and assuming that NH$_4$ equilibrates equally across all the membranes, $\beta$ was calculated from the change in pH (extrapolated to time 0) during the switch from Ringer’s to NH$_4$Cl according to (31) Equation 1.

$$\beta = (\text{NH}_4)^+ \Delta pH$$

(Eq. 1)

For sodium acetate experiments, using $pK = 4.7$ for the CH$_3$CO$_2$H $\rightarrow$ H$^+$ + CH$_3$CO$_2$H reaction and assuming that CH$_3$CO$_2$H equilibrates equally across all the membranes, $\beta$ was calculated from the change in pH (extrapolated to time 0) during the switch from Ringer’s to sodium acetate according to Equation 3.

$$\beta = -(\text{CH}_3\text{CO}_2)^- \Delta pH$$

(Eq. 2)

After determining key physical characteristics for each organelle, such as $\beta$, a mathematical model for the movement of H$^+$ across the organelle was required in order to predict the H$^+$ permeability of the membrane. We employed a model that has been shown to be in quantitative agreement with a diverse range of organelle pH and membrane potential data (26). Our present modeling effort is a subset of this more complete model in that we ignored H$^+$ pumping by the H$^+$-v-ATPase, since all the permeability measurements were performed in the presence of bafilomycin. Given this, the measured rate of change of luminal pH is related to the passive flux of H$^+$ and the physical characteristics of the organelle as shown in Equation 3.

$$\frac{dpH}{dt} = \frac{1}{2} S \frac{V}{p_e} J_{\text{leak}}$$

(Eq. 3)

where $J_{\text{leak}}$ is the total passive flux of H$^+$ across a unit area of membrane and $p_e$ is the organelle buffer capacity. We used surface areas, $S$, and volumes, $V$, of ER and Golgi as determined from terminal tubule and acinar cells of the rat submandibular gland (32); $S$ and $V$ of MSGs were based on a 200-nm diameter sphere (33). Although our H$^+$ permeability calculations were determined for A2T-20 (mouse anterior pituitary) cells, we used the $S$ and $V$ values from Taga et al. (32) because this was the only published report where $S$ and $V$ for both ER and Golgi membranes were measured in the same cell type.

We modeled the H$^+$ leak, $J_{\text{leak}}$, as simple passive diffusion. With this assumption, the leak depends on the membrane potential and the concentration gradient as shown in Equation 4.

$$J_{\text{leak}} = -\frac{1}{e} \frac{S}{p_e} \frac{\psi}{V}$$

(Eq. 4)

where $F_{\text{H}^+}$ is the H$^+$ permeability of the membrane; $C_{\text{H}^+}$ is the concentration of H$^+$ in the organelle (or cytosol); $C_{\text{o}}$ is the valence of the H$^+$; and $U = \nabla\psi/\nabla R$, where $\psi$ is the organelle membrane potential.

We computed $\psi$ in terms of the excess charge inside the organelle membrane, which was treated as a parallel plate capacitor (Equation 5),

$$\psi = \frac{V}{C_m} \left[ \left[ K^+ \right]_o - \left[ C_{\text{H}^+} \right]_o \right] + \int_{p_{K^+}}^{p_{C_{\text{H}^+}}} \beta dpH$$

(Eq. 5)

where $C_m$ is the total capacitance of the membrane (calculated assuming capacitance = 1 microfarad/cm$^2$); $[K^+]_o$ and $[C_{\text{H}^+}]_o$ are the concentrations of $K^+$ and $C_{\text{H}^+}$ in the organelle (based on previous experiments, we assumed that $K^+$ and $C_{\text{H}^+}$ diffuse across organelle membranes according to equations of the form (4)); the integral term represents the total H$^+$ in the organelle lumen (both buffered and free), and $B$ (a constant) is the concentration of charged species trapped in the organelle. In the absence of any membrane-energizing enzymes, one expects H$^+$ to diffuse across the organelle membrane until the luminal pH and the cytosolic pH are equal. However, this is often not the case for organelles that have been treated with bafilomycin (18, 21); furthermore, at rest, the distribution of H$^+$ across a membrane can be manipulated by the distribution of other ionic species (34). This discrepancy can be attributed to trapped, negatively charged species inside the organelle that, in the absence of an active H$^+$-v-ATPase, lead to an accumulation of H$^+$. Our model accounts for the difference in steady-state pH between organelle lumen and cytosol only through changes in the parameter $B$. Average values of $B$ varied between ~50 and 200 mV. Average $B$ values determined from the model for ER, Golgi, and MSGs did not significantly contribute to the different steady-state pH values of these compartments.

From our buffer capacity measurements, we know that a significant number of H$^+$ are transported into/out of the organelle during acidification/alkalinization. If this change in charge is not offset by the subsequent movement of counterions, a large membrane potential will build up across the organelle membrane and quickly limit further changes in pH. This physical insight suggests that organelles that undergo acidification must be permeable to some counterions. However, it is unclear which ions are permeable and how fast their movement is coupled to movement of the pH gradient. Studies on Secreptin (22) have convincingly shown that the Golgi is permeable to K$^+$; they also concluded that the fast dissipation of H$^+$ gradients in the Golgi by protonophores implied that the counterion movement must be much faster than the endogenous H$^+$ leak. As discussed under “Results,” our present data support these ideas. Therefore, in predicting $F_{\text{H}^+}$, we assumed that both K$^+$ and Cl$^-$ were free to diffuse across the organelle membranes. We chose permeabilities for these ions based upon plasma membrane measurements (35). Our numeric results remain unaffected for counterion permeability values down to 10$^{-9}$ cm/s. Below this point, counterion movement becomes the rate-limiting step during alkalinization, which affects predicted $F_{\text{H}^+}$ values.

Equation 3 and the two additional equations for the passive flux of K$^+$ and Cl$^-$ combine to form a set of ordinary differential equations (ODEs). The ODEs are coupled (pH, $p_{\text{H}^+}$) in the bafilomycin-induced alkalinization experiments was chosen to be the average steady-state pH of the organelle before the addition of bafilomycin. All other parameters were recorded experimentally or determined from equilibrium conditions. As mentioned above, the value $B$ was constrained by the data since it was the only free parameter in the model that accounted for equilibrium differences between pH and $p_{\text{H}^+}$.

The free parameters, $F_{\text{H}^+}$, $P_{\text{H}^+}$, and $B$, were determined by using the model to fit the experimentally measured changes of pH (Figs. 5 and 6, data points). For every data set, the model ODEs were solved to find which values of $F_{\text{H}^+}$, $P_{\text{H}^+}$, and $B$ gave the best fit to the experimental data. Example model fits, from which $F_{\text{H}^+}$ values were determined, are shown in Figs. 5 and 6 (solid curves). The search for the best model fit was performed with a Nelder-Mead algorithm, and the ODEs were solved with a stiff method in both Matlab (Mathworks, Natick, MA) and Berkeley Madonna (George Oster and Robert Macey, University of California, Berkeley).

In experiments in which $F_{\text{H}^+}$ was calculated from rates of bafilomycin-induced alkalinization (e.g., Figs. 3C and 6), we assumed pH was constant, since bafilomycin had no effect on pH (data not shown). In experiments in which $F_{\text{H}^+}$ was calculated from rates of recovery of pH, we assumed that following an acid-load, the pH recovery was dependent upon the recovery of $p_{\text{H}^+}$ (see Figs. 4 and 5). To determine $F_{\text{H}^+}$, using these experimental data, knowledge of the instantaneous H$^+$ gradient across the organelle membrane was required. Because our experimental system did not permit us to measure pH$_o$ and pH$_c$ in the same cells simultaneously, we undertook the computationally intensive task of fitting all pH recovery data against all separately recorded pH$_c$ recovery calculations. For each data set, the combination of MSG data sets fit against corresponding measurements of pH$_c$ in Cl$^-$-free solutions. In each of the data runs (e.g., 782 runs in the case of the ER data; 17 pH$_c$ data sets $\times$ 46 pH$_o$ data sets), a fitness parameter was used in order to determine the likelihood that the pH$_o$ data were a result of a particular pH$_c$ data set. A root mean square fit of the model to the pH$_o$ data was used as a measure of fitness, and its inverse, $w$, was used as a weight for computing averages and S.E. (Equation 6),
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Fig. 1. Avidin-POMC follows the regulated secretory pathway in AtT-20 cells. A, an AV-POMC chimera protein was constructed by fusing amino acids 1–132 of chicken avidin (signal peptide intact, stop codon removed) with amino acids 27–235 of mouse POMC (signal peptide removed). sp, signal peptide. AtT-20 cells expressing AV-POMC were loaded with 3 μM Flubi-2 (4.5 h) and chased for either 1.5 (B) or 3 h (C) before viewing. After the longer chase (C), Flubi-2 was chased out of the Golgi (arrow in B) and into ISGs and MSGs (punctate cell body and tip staining). Arrow, cell body staining (Golgi and some ISGs); arrowheads, tips of cell processes where MSGs accumulate. Scale bars, 10 μm.

\[
R = \frac{1}{\sqrt{N}} \sum_{i=1}^{N} \left| \frac{\text{pH}_{\text{observed}} - \text{pH}_{\text{model}}}{\text{pH}_{\text{model}}} \right|^2 \text{ or } w = \frac{1}{R} \quad \text{(Eq. 6)}
\]

where the sum was over all \( N \) experimentally measured time points. Chauvenet's criteria were used to exclude three highly improbable predicted permeability values (40). Two sets were rejected because the organelle pH was more alkaline than any cytosolic pH values, and one additional set was rejected due to technical difficulties in finding a reliable best fit.

RESULTS AND DISCUSSION

Targeting Avidin-Chimera Proteins and Flubi Dyes to the ER, Golgi, and MSGs of AtT-20 Cells—ER pH measurements in AtT-20 cells were performed exactly as in HeLa cells (21), where Flubi-2 was loaded into cells expressing avidin-KDEL (AV-KDEL). For AtT-20 Golgi and MSG pH measurements, an avidin-POMC (AV-POMC) chimera protein was expressed, and cells were loaded with Flubi dye to monitor pH. In AtT-20 cells, POMC is a regulated secretory protein that is transported from the ER to the Golgi and packaged into ISGs that mature into secretory granules of living AtT-20 cells in situ within secretory granules of living AtT-20 cells is shown in Fig. 2. Secretory granules were labeled with Flubi-2, and calibration solutions containing nigericin and monensin were perfused onto cells at the end of each experiment. An average calibration curve was generated by plotting 490/440 nm fluorescence ratio versus pH of the external solution. \( \text{pH}_{\text{MSG}} \) was determined by averaging the steady-state pH of MSGs of cells bathed in pH 7.4 Ringer's solution. \( \text{pH}_{\text{MSG}} \) (mean ± S.D.) was 5.5 ± 0.4 (Fig. 2, solid line).

We used similar methods to determine ER and Golgi pH values in AtT-20 cells by averaging the steady-state pH values of each compartment of cells bathed in pH 7.4 Ringer's solution. \( \text{pH}_{\text{ER}} \) of AtT-20 cells (7.4 ± 0.2) was similar to \( \text{pH}_{\text{ER}} \) in HeLa cells (7.2 ± 0.2) (21). In AtT-20 cells, as in HeLa cells, \( \text{pH}_{\text{ER}} \) was slightly lower than cytosolic pH (AtT-20 \( \text{pH}_c = 7.6 ± 0.2; \) HeLa \( \text{pH}_c = 7.4 ± 0.2 \)). In AtT-20 cells, \( \text{pH}_c \) was 6.2 ± 0.4, similar to values measured for the Golgi in non-regulated cells (15, 16, 21). Thus, the pH of the Golgi compartments in live, intact endocrine cells appears acidic and similar to the Golgi pH in non-regulated cells. Although the Flubi-2 staining in the cell body (Fig. 1B) may consist of signals from both Golgi and ISGs, the majority of these signals come from the Golgi compartments and not the ISGs. This latter conclusion is based on the observation that brefeldin A, which causes Golgi but not trans-Golgi network and post-Golgi organelles to redistribute to the ER (36), causes complete dispersal of the perinuclear POMC staining to the ER (data not shown). Future experiments using avidin constructs targeted specifically to individual cisternae of the Golgi complex and the ISGs will be necessary to resolve the likely small pH differences between these compartments.

Are Cl⁻ or K⁺ Conductances Determinants of Steady-state \( \text{pH}_{\text{MSG}} \) or the H⁺ Leak Out of MSGs?—Early models of pH regulation in secretory granules suggested that the assumed inside positive organelle membrane potential limited H⁺ accumulation by the H⁺ v-ATPase and that a Cl⁻ conductance was required to shunt this potential to allow adequate acidiﬁcation of organelles (9). We examined the potential role of Cl⁻ conductance in regulating \( \text{pH}_{\text{MSG}} \) in AtT-20 cells by replacing all Cl⁻ with gluconate in the extracellular solution of intact Flubi-2-loaded, AV-POMC-expressing AtT-20 cells. We assumed that cytosolic Cl⁻ would be depleted after a long (>20 min) incubation in Cl⁻-free solution, since for plasma membrane Cl⁻ permeabilities between 10⁻⁵ and 10⁻⁶ cm/s (35), the half-time for cytosolic Cl⁻ depletion from a 1000-μm² cell is 100–200 s. This calculation follows from Equation 4 (see “Experimental Procedures”) but neglects the inside negative membrane potential.
that is likely to exist in AtT-20 cells. In all likelihood, the membrane potential and also the presence of neutral transporters (e.g. NaKCl cotransport) will cause the half-time for Cl− depletion to be shorter than 100−200 s, ensuring that cell [Cl−] will probably be lower than 1 mM after about 10 min. We expected that if Cl− were required to provide a counterion for the accumulation of luminal H+, then incubation in Cl−-free solution for 30 min would reduce the acidity of MSGs. Similar to previous experiments on the Golgi of Chinese hamster ovary and HeLa cells (18, 21, 25), treatment with Cl−-free solution did not affect steady-state pHMSG over the course of >30 min (Fig. 3A), indicating that Cl− was not required to maintain acidic MSGs. A summary of results from experiments such as the one in Fig. 3A is shown in Fig. 3D. In Cl−-containing solutions pHMSG (mean ± S.D.) was 5.5 ± 0.4, nearly the same as pHMSG in Cl−-free solutions, 5.6 ± 0.4; pHG was 6.3 ± 0.3 in Cl−-free solutions, insignificantly different from pHG obtained in Cl−-containing solutions, 6.2 ± 0.4.

We also tested for the role of K+ and Cl− conductances in determining the acidity in MSGs and Golgi using a different approach. As shown in Fig. 3C, cells were treated first with 500 nM bafilomycin (H+ v-ATPase inhibitor) which caused pHMSG to alkalinize. This result showed that pHMSG was maintained by constantly active, bafilomycin-sensitive, H+ v-ATPases opposing H+ leaks. The rate of H+ leak out of the acidic MSGs into the pH 7.4 cytosol is a function of the H+ permeability, transmembrane pH gradient, and membrane potential (which, in the presence of bafilomycin, is a function of the conductances to the major ionic constituents of the cytosol, K+, and Cl−). If the K+ conductance were limiting the rate of H+ leak through effects on membrane potential, then addition of valinomycin (K+ ionophore) should increase both the K+ conductance and also the rate of H+ leak out of MSGs. As shown in Fig. 3C, addition of 10 μM valinomycin did not affect the rate of bafilomycin-induced MSG alkalinization; the rate of alkalinization increased only upon treatment with the protonophore FCCP (20 μM). This result indicated that in the presence of valinomycin, the H+ conductance was limiting the rate of H+ leak out of the MSGs. Similar experiments were performed on cells bathed in Cl−-free solutions for >30 min to reduce cell [Cl−] (Fig. 3D). By incubating the cells in Cl−-free solution, we expected that the major counterion conductance (for H+) across MSG membranes would now be due solely to K+. If the K+ conductance were smaller than the H+ conductance, then during bafilomycin treatment H+ would leak out of the MSGs only as fast as K+ leaked into the MSGs, and the rate of alkalinization would be increased by valinomycin. However, valinomycin had no effect on the rate of H+ leak out of MSGs in the absence of Cl− (Fig. 3D). Again, the H+ leak out of the MSGs increased only when the cells were treated with FCCP.

The simplest conclusion from these experiments was that the conductances to both Cl− and K+ were larger than the H+ conductance for both MSGs and Golgi. Because the conductance to K+ was large, removing Cl− (eliminating Cl− as a counterion) had no effect on either the ability of MSGs to acidify their lumens in control cells or on the rates of passive H+ flux out of the lumen in bafilomycin-treated cells. The K+ conductance appeared to be larger than the H+ conductance because valinomycin did not affect H+ flux in Cl−-free solutions (when the rate of H+ exit from the MSGs is determined by the H+ permeability and the rate of K+ entry). Thus, it appeared that the Golgi compartments in both regulated AtT-20 cells and non-regulated HeLa cells (21, 25) had similar pH values and were also highly conductive to both K+ and Cl−.

Our conclusion that MSGs did not require Cl− to generate normal luminal acidity contradicts previous conclusions (9, 22). However, it should be noted that these previous experiments were non-quantitative pH measurements using acridine orange in isolated synaptic vesicles in vitro; furthermore, the dose-dependent requirement for Cl− in acidification of the secretory vesicles was accomplished by changing the concentrations of both K+ and Cl−, and the role of Cl− alone or K+ alone was not determined. The contradictory Cl− results may also have been due to different pH regulatory mechanisms present in organelles of the endocytic pathway versus organelles of the secretory pathway. Early studies of pH regulation in the endocytic pathway indicated that endosome acidification could be regulated by altering the membrane potential by changing the Cl− conductance or the NaK-ATPase activity (37, 38). Perhaps the conflicting Cl− data reflect two sets of organelles with distinct pH regulatory mechanisms as follows: (i) organelles of the endocytic recycling pathway (including synaptic vesicles), where membrane potential and thus Cl− are important regulators of pH, and (ii) organelles of the biosynthetic secretory pathway (ER, Golgi, secretory granules), where organelle pH is
not regulated by Cl− or K+ conductances.

Measuring H+ Leak Rates and Intrinsic H+ Permeabilities across ER, Golgi, and MSG Membranes in AtT-20 Cells—The data presented above were consistent with the hypothesis that the MSG membrane was likely to have large conductances to both K+ and Cl− but lower conductance/permeability to H+. Under these conditions, pH_MSG was limited by the magnitude of the H+ “leak” or “permeability” pathway. Based on our previous work on ER and Golgi pH regulation in HeLa cells (21) and the finite H+ permeability of phospholipid bilayers (10−4 cm/s, 39), we hypothesized that the pH differences between the ER, Golgi, and secretory granules in AtT-20 cells might be generated by gradually decreasing the H+ leak or permeability along the secretory pathway. To gain insights into H+ leak rates across AtT-20 ER, Golgi, and MSG membranes, we followed the same acid-loading protocol we used to measure rates of pH recoveries for organelles of HeLa cells (21). AtT-20 pH measurements were performed on cells loaded with the well-known lysosomatic pH dye, BCECF-AM (2 μM loaded 1 h, 1-h chase). AtT-20 cells expressing either AV-KDEL or AV-POMC were loaded with Flubi-2 (at least 4 h), chased (0–2 h), and treated with 500 nM bafilomycin (at least 2 h) before measuring pH_cyto, pH_ER, pH_Golgi, and pH_MSG. AtT-20 cells were acid-loaded by incubating in 40 mM NH4Cl for 20 min. When the NH4Cl solution was replaced with sodium-free Ringer’s, pH_cyto, pH_ER, pH_Golgi, and pH_MSG remained acidic until sodium-containing Ringer’s was returned to the chamber, at which point pH_cyto recovered rapidly to pH 7.4, and the ER, Golgi, and MSGs leaked out H+ to recover their pH values. This protocol is illustrated in Fig. 4A, which shows typical pH traces for cytosol (black) and MSG (green) compartments. Fig. 4B shows the representative pH recoveries for each compartment, cytosol (black), ER (red), Golgi (blue), and MSGs (green), as the outside solution was switched from sodium-free to sodium-containing Ringer’s.

The data for the pH recoveries of AtT-20 cytosol, ER, Golgi, and MSGs were fit to single exponential equations to obtain half-times ($t_{1/2}$) for the pH recovery of each compartment. The results from these calculations have been summarized in Table I. ER membranes had the fastest pH recovery ($t_{1/2} = 59$ s), indicating a large ER H+ leak that quickly equilibrated pH_ER and pH_cyto ($t_{1/2} = 40$ s). This result is very similar to results obtained on HeLa cells (21). In AtT-20 cells, just as in HeLa cells, more distal organelles had slower pH recoveries compared with ER and cytosol. The pH recovery out of the Golgi ($t_{1/2} = 283$ s) was almost 5 times slower than the ER; the pH recovery of MSGs ($t_{1/2} = 734$ s) was 2.5 times slower than that of the Golgi and 12 times slower than that of the ER.

These data suggested that organelles in the regulated secretory pathway exhibited decreasing H+ leak rates, which may be a major contributor to the decreasing steady-state pH values of these organelles. However, the decreasing H+ leak rates we measured could also have been the result of increasing buffer capacities of organelles along the secretory pathway, or these results could have been due to differences in the surface area-to-volume (S/V) ratios of secretory pathway organelles. Therefore, to account for buffer capacities and S/V ratios, we determined the intrinsic H+ permeability, $P_{H,1+}$, for each organelle membrane (ER, Golgi, and MSG).

Buffer capacities (mean ± S.E. mmol/pH) were determined as described under “Experimental Procedures” for the cytosol (23 ± 3, n = 38), ER (17 ± 3, n = 21), Golgi (26 ± 6, n = 10), and MSG (20 ± 6, n = 10) compartments. By using these mean buffer capacity values and previously estimated surface areas and volumes (32, 33), the data from acid-load recovery experiments (Fig. 4B) were fit (Fig. 5) using the mathematical or-ganelle pH model described under “Experimental Procedures” to determine $P_{H,1+}$ for the ER, Golgi, and MSG membranes. Fig. 5 shows typical organelle pH recovery data (colored data points), the corresponding best pH recovery (black), and the model curve fits (colored solid curves, determined using Equations 3–5). $P_{H,1+}$ values are listed in Table II, which shows that $P_{H,1+}$ decreased progressively from the ER ($P_{H,1+} = 51 \times 10^{-4}$ cm/s) to Golgi ($P_{H,1+} = 21 \times 10^{-4}$ cm/s) to MSGs ($P_{H,1+} = 3 \times 10^{-4}$ cm/s). The $P_{H,1+}$ that we estimated for ER ($51 \times 10^{-4}$ cm/s) should be considered a lower estimate since these measurements may have been limited by the rates of pH recovery, which were controlled by plasma membrane pH regulatory mechanisms. The $P_{H,1+}$ value for each organelle was statistically different ($p < 0.07$) from the other organelles.

In addition to using pH recovery data from acid-loaded cells and organelles, we also estimated $P_{H,1+}$ for Golgi and MSG membranes using H+ leak data obtained from bafilomycin-treated cells. By using H+ leak data from experiments such as the one shown in Fig. 3C, we fit the bafilomycin-induced alkalinization rate using the mathematical organelle pH model as shown in Fig. 6. The main difference between the bafilomycin-induced alkalinization experiments and the acid-load pH recovery experiments was that using the former protocol pH remained constant (data not shown) since bafilomycin had no effect on pH_cyto, whereas using the latter protocol pH_acidified and changed as pH_cyto changed (e.g. Fig. 4). For both protocols,
area-to-volume ratios (see Table II), were incorporated to determine the
suggested as mean
$P$ values are presented as mean ± S.E. $P$, $H$ values for each organelle were compared using the Welch test: ER versus Golgi
$p<0.07$, Golgi versus MSG ($p<0.07$), and ER versus MSG ($p<0.02$).

### Table III
$H^+$-permeabilities determined from rates of bafilomycin-induced
alkalinization of the ER, Golgi, and MSGs of AtT-20 cells

Data from bafilomycin-induced alkalization rates measured in Cl$^-$
containing (e.g. Fig. 3C) and Cl$^-$-free (e.g. Fig. 3D) solutions, together
with measured buffer capacities and previously determined surface
area-to-volume ratios (see Table II), were incorporated to determine the
intrinsic $H^+$ permeabilities ($P$) of AtT-20 Golgi and MSG membranes
using the Berkeley Madonna modeling program. $P$ values are presented
as mean ± S.E. Golgi $P$ values determined in the presence and
absence of Cl$^-$ were statistically the same according to the Student’s t
-test. MSG $P$ values determined in the presence and absence of Cl$^-$
were also statistically the same according to the Student’s t test.

<table>
<thead>
<tr>
<th>AtT-20 membrane</th>
<th>$P$</th>
<th>CI$^-$-free $P$ $H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER (n = 16)</td>
<td>17 ± 3</td>
<td>1110 ± 35.4</td>
</tr>
<tr>
<td>Golgi (n = 10)</td>
<td>26 ± 6</td>
<td>514/26.4</td>
</tr>
<tr>
<td>MSG (n = 10)</td>
<td>20 ± 6</td>
<td>0.1260/0.00419</td>
</tr>
</tbody>
</table>

The data from Fig. 3 indicated that Cl$^-$ was not required to
maintain an acidic steady-state pH$_{MSG}$. We further tested for
the potential role of Cl$^-$ conductance in controlling Golgi and
MSG membrane potential by determining the $H^+$ permeability in
Cl$^-$-free solutions. In the presence of bafilomycin, the $H^+$
leak across MSG membranes will be a function of membrane
potential and pH gradient, and if Cl⁻ conductance were important for shunting membrane potential, then we would expect that the calculated \( P_{H+} \) would be smaller for experiments in Cl⁻-free versus Cl⁻-containing solutions. To determine whether \( P_{H+} \) of MSGs was Cl⁻-dependent, we used data from Cl⁻-free bafilomycin-induced alkalinization experiments (e.g. Fig. 3D and Fig. 6) to determine the \( P_{H+} \) of MSG membranes in the absence of Cl⁻ (Table III). The \( P_{H+} \) in Cl⁻-free solutions was \( 1.6 \times 10^{-4} \) cm/s for Golgi membranes and \( 0.6 \times 10^{-4} \) cm/s for MSG membranes, in both cases greater than the H⁺ permeabilities measured in Cl⁻-containing solutions (Golgi = \( 1.3 \times 10^{-4} \) cm/s MSG = \( 0.35 \times 10^{-4} \) cm/s), indicating that Cl⁻ was not required for the H⁺ leak out of either the Golgi or MSGs in AtT-20 cells.

Summary, A Model for pH Regulation in the Regulated Secretory Pathway—This study constitutes the first investigation into the dynamics of pH regulation in organelles of the secretory pathway of live, intact endocrine cells, where the organelle acidification process is crucial for the sorting and processing of regulated secretory hormones. We determined that, just as for the ER and Golgi of HeLa cells, the steady-state pH values of AtT-20 ER, Golgi, and MSGs appeared to be controlled primarily by rates of H⁺ v-ATPase pumping and by the magnitude of H⁺ leaks. Our data and mathematical modeling showed that the membrane potential in Golgi and MSGs of AtT-20 cells was small and not perturbed by large changes in Cl⁻ and K⁺ conductances; these results indicated that membrane potential was not a determinant of steady-state Golgi and MSG pH values.

We previously found in HeLa cells that the rate of H⁺ leak out of the Golgi was three times slower than the H⁺ leak out of the ER (21). In AtT-20 cells, the rates of H⁺ leak steadily decreased from ER to Golgi to MSGs, with MSGs having a 12 times slower leak rate than ER membranes. After accounting for buffer capacities and S/V ratios, and with the realization that the calculated \( P_{H+} \) of the ER was likely to be somewhat underestimated (see above), we conclude that \( P_{H+} \) of the ER was twice as large as that of the Golgi which was 4–7 times greater than MSGs. The variability of our calculated \( P_{H+} \) values stemmed in part from the variability in the experimentally determined buffer capacity (\( \beta \)) values. The experimental determination of \( \beta \) is prone to large variabilities due to different rates of mixing of NH₄⁺/NH₃⁺ solutions into the chamber, possible contributions of pH regulatory mechanisms in the organelle and plasma membranes, and the finite permeability of membranes to NH₃⁺. We tried to limit these factors as much as possible by performing experiments in sodium-free solutions following bafilomycin treatment, but it is nearly impossible to eliminate these complicating factors completely. Furthermore, the buffer capacity experiments were performed separately from the experiments used to determine \( P_{H+} \). It is likely that our error bars for \( P_{H+} \) would have been smaller had we been able to perform the buffer capacity experiments and H⁺ leak experiments in the same cells and then use the \( \beta \) value for a particular cell to calculate the \( P_{H+} \) for that cell.

Are the differences in H⁺ permeabilities sufficient to account for the different steady-state pH values of the ER, Golgi, and MSGs? Alternatively, are concomitant increases in H⁺ pumping activity required to establish the stepwise decreases in pH between these organelles? To answer these questions, we used the previously published organelle pH model (26) that describes both H⁺ pumping and H⁺ leaking. By using this model, along with our experimentally determined Golgi and MSG buffer capacities and estimated Golgi and MSG \( P_{H+} \) and S/V values (32, 33), we found that generating the >1.0 unit pH drop from the ER (pH 7.4) to Golgi (pH 6.2) required either a 10-fold increase in the active H⁺ pump density (assuming the H⁺ pump activity of each H⁺ v-ATPase is constant) or a 10-fold decrease in \( P_{H+} \) between the ER and Golgi. Generating the 0.7 unit pH drop between Golgi (pH 6.2) and MSGs (pH 5.5) required either a 5-fold increase in active H⁺ pump density or a 5-fold decrease in \( P_{H+} \) between Golgi and MSGs.

Since our calculated \( P_{H+} \) values (Table II) for the ER and Golgi differed by 2-fold rather than 10-fold, an increase in H⁺ pump activity must have accompanied the 2-fold decrease in \( P_{H+} \) to generate the lower pH of the Golgi compared with the ER. These calculations, together with previous data showing that bafilomycin treatment had no effect on steady-state pHER of Vero and HeLa cells (17, 21), indicate that for AtT-20 cells, the Golgi had a higher density of active H⁺ v-ATPases compared with the ER. Based on the acid-load pH recovery data (Table II), our estimated \( P_{H+} \) values for Golgi and MSGs differed by 7-fold. When we used the rate of alkalinization due to bafilomycin treatment (Table III) to calculate \( P_{H+} \), for Golgi and MSGs differed by only 4-fold. It is unclear why the different protocols (pH recovery after an acid-load versus bafilomycin-induced alkalinization) for measuring the H⁺ leak out of organelles produced different H⁺ permeability values for Golgi and MSGs. The main difference between the two protocols was the effect on pH. In the bafilomycin-induced H⁺ leak protocol, pH₇ was ~7.4 and remained constant throughout the protocol. In the NH₄⁺/NH₃⁺ acid-load protocol, pH₇ acidified to ~6.5 and then alkalized throughout most of the protocol. In the acid-load protocol, pH₇ recovery was directly dependent upon the recovery of pH₇. The different \( P_{H+} \) values measured using the different protocols could be explained by a pH-dependent H⁺ leak across the organelle membrane which would increase as pH decreased. In the acid-load protocol, the acidic initial pH₇ would result in a much faster \( P_{H+} \) than in the bafilomycin protocol, where pH₇ is neutral. Since both protocols had their advantages and disadvantages, we chose to present the data obtained by both protocols rather than select one data set over the other. Most likely, the difference in \( P_{H+} \) between Golgi and MSGs in AtT-20 cells lies somewhere between 7- and 4-fold. Both data sets are consistent with a gradual decrease in organelle H⁺ permeability from ER to Golgi to secretory granules. Our working model for organelle pH regulation along the regulated secretory pathway is illustrated in Fig. 7. Based on our experimental and modeling results, we conclude that the decreasing pH values of organelles of the regulated secretory pathway is established by gradually increasing the density of active H⁺ pumps from ER to Golgi while concomitantly decreasing the H⁺ permeability from ER to Golgi to MSGs.

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