Determinants of the pH of the Golgi complex*

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SUMMARY

The factors contributing to the establishment of the steady state Golgi pH (pH\textsubscript{G})\textsuperscript{1} were studied in intact and permeabilized mammalian cells by fluorescence ratio imaging. Retrograde transport of the non-toxic B subunit of verotoxin 1 was used to deliver pH-sensitive probes to the Golgi complex. To evaluate whether counterion permeability limited the activity of the electrogenic V-ATPase, we determined the concentration of K\textsuperscript{+} in the lumen of the Golgi using a null-point titration method. The [K\textsuperscript{+}] inside the Golgi was found to be close to that of the cytosol, and increasing its permeability had no effect on pH\textsubscript{G}. Moreover, the capacity of the endogenous counterion permeability exceeded the rate of H\textsuperscript{+} pumping, implying that the potential across the Golgi membrane is negligible and has little influence on pH\textsubscript{G}. The V-ATPase does not reach thermodynamic equilibrium nor does it seem to be allosterically inactivated at the steady state pH\textsubscript{G}. In fact, active H\textsuperscript{+} pumping was detectable even below the resting pH\textsubscript{G}. A steady-state pH was attained when the rate of pumping was matched by the passive backflux of H\textsuperscript{+} (equivalents), or "leak". The nature of this leak pathway was investigated in detail. Neither vesicular traffic nor H\textsuperscript{+}/cation antiporters or symporters were found to contribute to the net loss of H\textsuperscript{+} from the Golgi. Instead, the leak was sensitive to voltage changes and was inhibited by Zn\textsuperscript{2+}, resembling the H\textsuperscript{+} conductive pathway of the plasma membrane. We conclude that a balance between an endogenous leak, which includes a conductive component, and the H\textsuperscript{+} pump determines the pH at which the Golgi lumen attains a steady state.
INTRODUCTION

Stringent regulation of the internal pH of endomembrane compartments is required for their optimal function. In mitochondria, alkalinization of the matrix contributes to the generation of the transmembrane proton-motive force used to generate ATP (1). By contrast, luminal acidification is essential for the distribution and degradation of internalized ligands in the endocytic pathway (2,3,4), while it regulates post-translational modification and sorting of proteins along the secretory pathway (5,6,7). The pH varies in different sub-compartments of the endocytic pathway, with acidification increasing progressively from the endocytic vesicles and early endosomes, to late endosomes and ultimately lysosomes (8). Conversely, the pH of the secretory pathway becomes more acidic as the cargo travels towards the cell surface. While the pH of the endoplasmic reticulum is thought to be near neutral (9,10), acidification develops along the Golgi complex and is maximal at the trans-Golgi network (11,12,13). The development of such gradients appears to be important in targeting and retrieving components to and from individual subcompartments (6,15). However, little is known about the determinants of pH in each subcompartment and particularly about the source of their differential acidification. In the case of the secretory pathway, this paucity of information is attributable, in part, to methodological limitations. Until very recently, determination of the luminal pH of the endoplasmic reticulum, Golgi or trans-Golgi network was limited to the use of static, immunoelectron microscopy-based methods (16,17). In the past couple of years, however, a variety of ingenious techniques have been introduced which allow the continuous and comparatively non-invasive detection of pH in intact cells. These include the microinjection of pH-responsive probes trapped in size-fractionated liposomes (14,18), the transfection of pH-sensitive variants of green fluorescent protein linked to organelle-specific targeting sequences (10,13), and the expression of targetted chimeric constructs that can be used to trap soluble probes in specific organelles (12,19). In addition, bacterial proteins have been used to measure the pH of the Golgi complex (11). Toxins produced by Shigella and by enteropathogenic strains of E. coli have been shown to bind to surface glycolipids and to be subsequently transported.
along an endogenous retrograde pathway, accumulating in the Golgi complex (20,21). We used these toxins earlier to estimate the pH of the Golgi in cultured cells and, in agreement with other recent studies, found this compartment to be acidic as a result of active proton pumping by a vacuolar (V-type)\(^1\) ATPase (11).

The stoichiometry of the V-ATPase is generally believed to be 2-3 protons per ATP hydrolyzed (22). On a thermodynamic basis, a maximum transmembrane pH gradient of 4.2 units could therefore be generated by the V-ATPase at 37\(^\circ\)C. Because in most cells the cytosolic pH approximates 7.2 (23,24), an intra-Golgi pH of 3 could be reached if the ATPase attained chemical equilibrium\(^2\). This value differs markedly from the reported values, that range between 6.25 and 6.58 (11,19,13). The apparent discrepancy could in principle be attributed to the development of an electrical potential (inside positive) across the Golgi membrane. An electrical potential would develop if the rate of proton pumping exceeds the rate of permeation of counterions (see 25 for model). Alternatively, "leak" pathways could facilitate the escape of protons from within the organelle, thereby partially dissipating the gradient generated by the ATPase. The contribution of these mechanisms to the establishment of the steady-state pH of the Golgi was assessed in the present study, where we used fluorescence ratio imaging to monitor signals emitted by recombinant B subunit of verotoxin 1 (VT1B) that was covalently coupled to a pH-sensitive probe.
EXPERIMENTAL PROCEDURES

Materials and solutions. GTPγS, α,α’-dipyridyl, TPEN (N,n,n’,n’-tetrakis(2-pyridylmethyl) ethylenediamine) and valinomycin were from Sigma. Nigericin was from Molecular Probes, Inc. (Eugene, OR) and concanamycin A from Kamiya Biochemical Company (Thousand Oaks, CA). The polyclonal antibody to α-mannosidase II was the kind gift of Dr. M. Farquhar (University of California at San Diego, CA). Cy3-labeled donkey anti-rabbit antibodies were from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). Lyophilized streptolysin O (SLO) was provided by Dr. S. Bhakdi (Johannes-Gutenberg Universitat, Mainz, Germany), dissolved in Dulbecco’s phosphate buffered saline solution (PBS; Pierce, Rockford, IL) at 1 mg/ml and stored at -80°C. Immediately before use, SLO was diluted in permeabilization buffer containing 2 mM dithiothreitol to a final concentration of 0.25 µg/mL. Vero cells were from the American Tissue Culture Collection (Rockville, MD). Cell culture media and antibiotics were from GIBCO. All other chemicals and reagents were of the highest purity available.

The calibration KCl-rich solution contained (in mM): 140 KCl, 10 glucose, 10 HEPES, 10 MES, 1 CaCl2 and 1 MgCl2 (pH ranged from 6 to 7). The permeabilization buffer consisted of (in mM) 90 K-glutamate, 50 KCl, 10 NaCl, 1 MgCl2, 2 CaCl2, 4 EGTA, 2 K2HPO4, 20 HEPES, 4 ATP, 3 Na-pyruvate and 1 mg/ml bovine serum albumin, pH 7.2. EGTA was omitted in the experiments where ZnCl2 was used. Where indicated, the concentration of K+ was altered by isoosmolar replacement with NMG+. EGTA was removed from the permeabilization buffer in the experiments where ZnCl2 was used, EGTA was removed from the permeabilization buffer.

Toxin Purification and Labelling. Recombinant B subunit of verotoxin 1 (VT1B) was purified as described earlier by affinity chromatography (26). Purified VT1B was fluoresceinated by the addition of FITC directly to VT1B (1:1 (w/w) ratio) in 0.5 M Na2CO3/NaHCO3, pH 9.5. The mixture was gently rotated for 1-2 h at room temperature after which free FITC was removed. The resulting labeled toxin is called FITC-VT1B hereafter.
Cell culture, labeling and permeabilization. Vero cells were cultured at 37°C in minimal essential medium (MEM) containing glutamine, vitamins, 5% fetal calf serum, 40 µg/ml gentamicin and 1% penicillin-streptomycin under 5% CO2. To label the Golgi, cells were washed three times in cold PBS containing 1 mM CaCl2 and 1 mM MgCl2, pH 7.4 and then preincubated with 10 µg/ml of FITC-labeled VT1B in PBS for 1 h at 4°C in order to promote binding to the plasmalemmal receptors without endocytosis. After washing twice with PBS, internalization was initiated by incubating the cells at 37°C for 1 h in MEM.

Where indicated, cells were permeabilized by incubation for 5 min at 37°C in permeabilization buffer containing 0.25 µg/ml of SLO. The effectiveness of the permeabilization protocol was assessed by incubating the cells for 5 min in the presence of the impermeant dye FM143 (1 µg/ml) and analyzing its distribution by fluorescence imaging. The kinetics of equilibration of small solutes in permeabilized cells were also quantified by fluorescence microscopy. Following permeabilization, cells were pre-equilibrated with Lucifer Yellow (1 mM) and then abruptly switched to permeabilization buffer without dye. The rate of loss of internal fluorescence was continuously monitored by digital imaging.

Other methods. Staining and mounting of samples for immunofluorescence, acquisition of images and fluorescence ratio imaging of intra-Golgi pH were performed as described in 11. Quantification of cell-associated fluorescence was performed using Metamorph/Metafluor software (Universal Imaging, West Chester, PA). Calibration was performed in situ at the end of each experiment by sequentially perfusing the cells with KCl-rich media (125 mM KCl, 20 mM NaCl, 10 mM Hapes, 10 mM MES, 0.5 mM CaCl2, 0.5 mM MgCl2) containing 5 µg/ml of the K+/H+ ionophore nigericin and buffered to pH values ranging from 5.5 to 7.5. Data were plotted using the Origin software (MicroCal Software Inc., Northhampton, MA) and are representative of at least three separate experiments of each type. Unless otherwise indicated, experiments were performed at 37°C.
RESULTS

Golgi pH measurements using FITC-VT1B. The fluorescence of FITC-VT1B was used to measure Golgi pH (pHG). FITC-VT1B (10 µg/ml) was initially bound to the surface of Vero cells by incubation for 1 hr at 4°C, followed by washing and incubation for another hr at 37°C to allow internalization of the probe. The intracellular localization of FITC-VT1B was confirmed by fluorescence microscopy. As illustrated in Fig. 1A, the toxin accumulates in a compact juxtanuclear compartment reported earlier to coincide with the Golgi complex (11). Accordingly, the distribution of the toxin overlaps precisely with that of α-mannosidase II (Fig. 1B), a well established marker of the medial and trans cisternae of the Golgi (27,28).

Fluorescence ratio imaging was used to estimate pHG in otherwise untreated Vero cells. pHG averaged 6.47 ± 0.4 (mean ± SE) in 4 separate experiments and increased rapidly upon addition of 500 nM concanamycin, an inhibitor of the V-ATPase, approaching the cytosolic pH within 5 to 10 min (see below).

Estimation of intra-Golgi [K⁺]. Calibration of pHG using nigericin rests on the assumption that the luminal Golgi [K⁺] is comparable to that of the cytosol. To our knowledge, however, the [K⁺] of the Golgi complex has not been determined. To validate the calibration method, and to define the potential counterion composition of the Golgi, we implemented a null-point procedure to estimate [K⁺] (29). The method is based on the mechanism of action of nigericin, which carries out a one-for-one, electroneutral exchange of K⁺ for H⁺ (30). As a result, the exchange process will cease when [K⁺]i/[K⁺]o = [H⁺]i/[H⁺]o. Because cytosolic pH is known, the intra-Golgi [K⁺] can be estimated by assessing the effect of nigericin on pHG when the cytosolic compartment is bathed at varying concentrations of K⁺.

This experimental paradigm requires manipulation of the [K⁺] of the extra-Golgi (cytosolic) compartment. This could in principle be performed by inhibition of the Na⁺/K⁺-ATPase combined with judicious choice of the composition of the extracellular medium. However, the resulting cytosolic [K⁺] changes would be slow, and parallel changes in the Golgi [K⁺] are likely
to occur. Ideally, the change in cytosolic [K+] must be imposed abruptly prior to addition of nigericin, to preclude spurious changes in Golgi [K+].

Rapid changes in extra-Golgi ionic composition were imposed by permeabilizing cells with SLO, a bacterial toxin that binds to cholesterol, generating pores on the plasma membrane without necessarily altering the integrity of intracellular compartments (31). Effective permeabilization of the plasma membrane by SLO was shown by labelling the cells with FM143, a membrane impermeant amphiphilic dye that becomes fluorescent upon insertion in lipid bilayers. As shown in Fig. 2A, when added to intact cells the fluorescence of FM143 is restricted to the plasma membrane and forming endosomes. When added following treatment with SLO, FM143 rapidly stains intracellular membranes with a resulting 10-fold increase in cell-associated fluorescence (note that a much longer exposure time was used in Fig. 2 for panel A than for B). Importantly, no changes in the resting pHG were detected for up to 10 min in cells treated with SLO (Fig. 3 and 6 and Table 1), confirming that this organelle remains intact after selective permeabilization of the plasma membrane.

To estimate the time required for equilibration of the extracellular solution with the cytosolic compartment following SLO permeabilization, cells were first equilibrated with Lucifer Yellow and the dye was suddenly removed from the perfusing medium. The rate of fluorescence decay was used as an index of the permeation rate of Lucifer Yellow through the pores generated by SLO. Typical results are illustrated in Fig. 2C. In 3 similar experiments, the time required for loss of 50% of fluorescence was 2 sec. This value is likely an overestimate of the t1/2 for equilibration of K+ added externally because the molecular size and therefore the rate of diffusion of Lucifer Yellow differ from that of K+.

We next proceeded to estimate Golgi [K+] by the null-point method (Fig. 3). Cells were bathed in medium containing 140 mM K+, to approximate the cytosolic [K+]. They were then permeabilized with SLO and perfused with media of varying [K+], osmotically balanced with NMG+. Three min were allowed for equilibration of the cytosolic compartment with external solution, a time chosen based on the results of Fig. 2, and nigericin was immediately added. As
shown in Fig. 3A, the ionophore induced a rapid alkalinization when added in medium with 140 mM K+. The alkalosis was considerably slower at 70 mM K+ and only marginal at 35 mM. The null-point was more precisely calculated by plotting the rate of nigericin-induced change in pHG vs. [K+] (Fig. 3B). Using the averages of 3 experiments, the null-point was attained at •20 mM [K+]. Because under the conditions chosen the transmembrane pH = 0.73, we estimated the intra-Golgi [K+] to approximate 107 mM, which is only slightly lower than the cytosolic concentration. This finding validates the use of nigericin for the in situ calibration of pHG. Moreover, it identifies intraluminal K+ as a possible counterion to neutralize the electrogenic action of the V-ATPase.

Are V-ATPases quiescent at the resting pHG? As mentioned in the Introduction, it has been suggested that organellar pH may be dictated by the level at which the V-ATPase attains thermodynamic equilibrium (25). Alternatively, by analogy with Na+/H+ exchangers (32,33), V-ATPases may be allosterically controlled by H+, reaching quiescence prior to thermodynamic equilibration. We therefore tested the prediction that the V-ATPases are quiescent at resting pHG and that they are unable to pump H+ at more acidic luminal pH. This analysis required acidification of pHG below its resting level. We had found earlier that incubation with NH4+ elicited a rapid alkalosis of the Golgi, followed by a gradual re-acidification that likely reflected accelerated H+ pumping (11). Subsequent removal of NH4+ results in a pH "undershoot" that can be explained by rapid efflux of NH3 (see Fig. 4).

This paradigm was used to establish the pH dependence of the V-ATPase at and below the resting pHG. To distinguish between passive H+ (equivalent) fluxes and active pumping by the V-ATPase, experiments were performed in the presence and absence of fully inhibitory doses of concanamycin. Typical results are shown in Fig. 4. In the absence of the inhibitor, pHG returned towards the steady state comparatively slowly, requiring upwards of 5 min to re-equilibrate. By contrast, in concanamycin-treated cells pHG reached and exceeded the original baseline much more rapidly. The difference in the rate of re-alkalinization can be attributed to the V-ATPase.
Rates of recovery at varying pH$_G$ were calculated in 3 similar experiments and are summarized in the inset to Fig. 4. It is clear that the rate of alkalosis is greater in the presence of concanamycin at and below the resting pH$_G$, implying that the pump is active under these conditions. We conclude that the resting pH$_G$ is not equal to and therefore not dictated by the pH at which the V-ATPase attains quiescence. Other factors must therefore contribute to the establishment of the resting pH$_G$.

**Characterization of the counterion conductance.** The observation that the V-ATPase is measurably active at or below the resting pH$_G$ implies that its activity is not limited by the permeability to counterions. However, earlier reports have implied that pH$_G$ is indeed dictated by a limiting counterion conductance (34,35). To resolve this apparent discrepancy, we studied further the role of counterions in the establishment of pH$_G$.

Because the preceding findings indicated that intra-Golgi [K$^+$] is high, we used the conductive ionophore valinomycin to provide a path for K$^+$ efflux, thereby increasing the total counterion conductance. As summarized in Table 1, addition of 1 µM valinomycin did not decrease pH$_G$, as would have been expected if counterion conductance were rate-limiting. Moreover, addition of the lipid-soluble anion SCN$^-$ similarly failed to enhance Golgi acidification (Table 1). It could be argued that little SCN$^-$ accumulates in the cytosol of intact cells, as it is excluded by the plasma membrane potential. To circumvent this potential problem, SCN$^-$ was also added to cells previously permeabilized with SLO. As shown in Table 1, pH$_G$ under these conditions was similarly unaffected. It is noteworthy that, when added together with concanamycin, neither valinomycin nor SCN$^-$ enhanced the rate of dissipation of the luminal acidification (Table 1). This indicates that the permeability to H$^+$ (equivalents) was unaffected by these agents and remained the limiting factor in the dissipation process.

Another indication of the relative magnitude of the counterion conductance was obtained comparing the rates of H$^+$ pumping and leakage. Because the pump is active in the steady state, its tendency to acidify must be balanced by a backward "leak" of H$^+$ of equal magnitude. The
leak can be easily revealed by addition of concanamycin (Fig. 5). Addition of CCCP, an exogenous conductive H\(^+\) ionophore to the cells further increases the rate of dissipation of the pH gradient. The latter observation implies that the intrinsic H\(^+\) conductance was limiting the rate of H\(^+\) efflux from the Golgi, and more importantly, that the counterion conductance is greater than the endogenous H\(^+\) conductance. Because at steady state the rates of the pump and leak are identical, the counterion conductance must also be greater than the rate of pumping and cannot therefore be rate-limiting.

The above experiments rest on the assumption that CCCP selectively increases H\(^+\) permeability, without affecting the conductance to other ions. This premise was verified in the experiment shown in Fig. 5B. Permeabilized cells were treated with CCCP in medium with 140 mM K\(^+\) and pH 7.2, leading to rapid dissipation of the luminal acidification. The concentration of K\(^+\) was then rapidly reduced, by isoosmotic substitution with NMG\(^+\). Despite the large change in the K\(^+\) concentration gradient, pH\(_G\) remained unaltered, indicating that the membrane potential is not significantly altered by this manipulation. This implies that the H\(^+\) permeability induced by CCCP is the predominant contributor to the transmembrane conductance and that the ionophore does not appreciably increase K\(^+\) permeability under these conditions.

Jointly, these observations confirm that, at least in Vero cells, the development of a potential across the Golgi membrane as a result of limited counterion permeability cannot explain the observed level of pH\(_G\).

**Characterization of the H\(^+\) leak.** The existence of a large counterion conductance, together with the evidence that the V-ATPase is active in the steady state suggest that pH\(_G\) is maintained by a dynamic balance between the rates of H\(^+\) pumping and leakage. Indeed, the existence of a robust leak was documented above in cells treated with concanamycin (Fig. 5). The remainder of this study was dedicated to the characterization of the leak pathway.

We initially tried to differentiate between physiological transport pathways and imperfections in the continuity of the membrane. The latter would be expected to allow passive diffusion of
H\(^+\) (equivalents), a process that would have a low temperature coefficient. We therefore tested the temperature dependence of the leak. Reduction of the temperature from 37\(^\circ\)C to 10\(^\circ\)C immediately before the addition of concanamycin greatly depressed the rate of alkalinization, from 0.16 ± 0.04 to 0.03 ± 0.01 (means ± SE of 3 determinations). This considerable temperature sensitivity suggests that specific transport systems mediate the efflux of H\(^+\) from the Golgi complex.

i) Role of vesicular traffic. While pH\(_G\) is acidic, the luminal pH of the ER was shown to be neutral (10, 11). Because vesicular flux into and out of the Golgi complex is rapid (36) it was conceivable that delivery of neutral luminal contents of the ER into the Golgi, coupled with loss of acidic contents from the \textit{trans} side of the Golgi, would contribute to the net loss of H\(^+\), \textit{i. e.} to the leak. This possibility was evaluated by comparing the rates of H\(^+\) leakage under normal conditions and when vesicular traffic was interrupted. Initial indications were obtained in cells permeabilized with SLO. Loss of cytosolic components through the large (10-30 nm) pores generated by SLO would be anticipated to reduce or eliminate vesicular traffic (37,38). However, the rate of H\(^+\) leakage, measured as the rate of dissipation of the acidification upon addition of concanamycin, was not significantly decreased by washout of cytosolic constituents (Table 1). To ascertain that vesicular traffic was impaired, we also permeabilized cells in media containing 25 \(\mu\)M GTP\(_{\gamma}\)S. This non-hydrolyzable nucleotide has been reported to prevent uncoating of ER and Golgi-derived vesicles, thereby terminating flow of membranes and cargo through the Golgi complex. The rate of concanamycin-induced dissipation of the pH gradient was also unaffected by the nucleotide (Table 1). These observations suggest that vesicular traffic does not contribute significantly to the efflux of H\(^+\) from the Golgi.

ii) Role of divalent metal-H\(^+\) transporters. A system that co-transports divalent cations with H\(^+\) was recently described to mediate Fe\(^{2+}\) uptake across intestinal and erythroid cell membranes (39, 40). This transporter, called Nramp2, is present also in endomembranes (41, 42), where it likely accounts for the uptake of Fe\(^{2+}\) internalized by transferrin. Nramp1, an isoform of Nramp2, was also shown to be present in endomembranes (43). We therefore
considered the possibility that an Nramp homolog may exist in the Golgi, where it could mediate metal-H+ cotransport, contributing to the H+ leak pathway. The efflux of H+ from the Golgi was measured as before in cells treated with two different lipid-soluble heavy metal chelators: α,α′-dipyridyl, which has high affinity for Cu2+ and Ni2+, and TPEN, that binds Fe2+ and Cd2+ with high affinity. As summarized in Table 1, neither one of these chelators altered the rate of H+ leakage, suggesting that Nramp-like metal cotransporters contribute little to the steady state efflux of H+ from the Golgi.

iii) Role of Na+/H+ exchange. Virtually all mammalian cells express Na+/H+ exchangers in their plasma membrane and mitochondrial membranes (44,45). It is not clear whether Na+/H+ exchangers are also active in the Golgi complex, either as resident transporters or while en route to other organelles following biosynthesis. We explored the possibility that exchange of cytosolic Na+ for luminal H+ accounted for at least part of the Golgi leak pathway. This was accomplished using amiloride, which is a potent inhibitor of several isoforms of the Na+/H+ exchanger family (46). As reported in Table I, amiloride did not affect the resting pH of the Golgi and exerted no inhibition of the leak unmasked by concanamycin.

Because not all members of the Na+/H+ exchanger family are equally sensitive to amiloride, we also analyzed the effect of increasing cytosolic Na+ on the rate of H+ efflux from the Golgi. In cells permeabilized with SLO, a sudden elevation of cytosolic (extracellular) [Na+] from 13 mM to 103 mM also had little impact on the rate of leakage (Table I). Together with the ineffectiveness of amiloride, these findings argue against a role of Na+/H+ exchangers in the steady-state efflux of H+ from the Golgi.

iii) Voltage dependence of the leak. Since bicarbonate was nominally absent from all the solutions used, it appeared as if neither Cl-/HCO3- exchange nor Na+/H+ exchange were responsible for the leak. As an alternative to electroneutral exchangers, we considered the involvement of conductive pathways. To this end, the voltage sensitivity of the rate of leakage was assessed. We compared the rate of dissipation of •pH under conditions where the potential
across the Golgi membrane was either • 0 mV, or highly negative inside. These conditions were met by permeabilizing the cells with SLO, setting the cytosolic [K+] to 140 or 0 mM, respectively, and then making the Golgi membrane preferentially permeable to K+ by addition of valinomycin. The results of such experiments are illustrated in Fig. 6. Addition of concanamycin elicited a faster and greater alkalinization in media containing 140 mM K+ than in K+-free medium. This observation is consistent with a conductive H+ (equivalent) efflux, that would have been retarded by intraluminal negativity.

iv) Insensitivity to DCCD and NEM. Transport of protons through some systems, including the channel moiety of H+-ATPases can be blocked by dicyclohexyl carbodiimide (DCCD), a carboxyl group reagent. However, we detected no inhibition of the H+ leak pathway of the Golgi complex in cells treated with 150 µM DCCD (Table 1). Similarly, H+ leakage was not significantly affected by 100 µM of the alkylating agent N-ethylmaleimide (NEM). Because they are known to inhibit the V-ATPase, these agents were added immediately before the addition of concanamycin and had only limited time to reach and react with Golgi proteins. We therefore cannot rule out that these agents may inhibit the leakage pathway(s) if allowed to react more extensively.

v) Inhibition of the leak by Zn2+. A highly H+-selective conductive pathway has been described in the membranes of neuronal, epithelial and myeloid cells (see 47 and 48 for reviews). Though the molecular entities responsible for the conductance have not been identified, they share similar pharmacological properties in all systems tested. Specifically, permeation of H+ through this pathway is effectively blocked by Zn2+ and Cd2+ (49). These cations were used to test the possible involvement of this or a similar pathway in H+ leakage from the Golgi. To allow access of the cations to the Golgi membrane, the cells were porated with SLO as above and, finally, the leak was unmasked by addition of concanamycin. In the presence of 200 µM Zn2+ the initial rate of alkalinization was reduced, from 0.16 ± 0.04 to 0.04 ± 0.02 pH/min (Fig. 7 and Table 1). A more detailed concentration dependence of the effect of Zn2+ is shown in Fig. 7.
7B. Notice that a residual fraction of the leak was not blocked by higher concentrations of Zn$^{2+}$, suggesting the existence of multiple components.
DISCUSSION

K⁺ concentration inside the Golgi complex. To our knowledge, the concentration of free K⁺ within the Golgi complex had not been determined earlier. Using a null-point approach, based on the stoichiometric exchange of K⁺ for H⁺ catalyzed by nigericin, we estimated Golgi [K⁺] to be approximately 107 mM, slightly lower than the concentration of the cytosol. The concentration of the latter is established by the balance between inward pumping of K⁺ via the Na⁺/K⁺ ATPase and the passive leak which occurs primarily via K⁺ channels, which in turn dictate the membrane potential. The high [K⁺] of the Golgi can be accounted for by simply assuming that the Golgi membrane possesses a significant permeability to K⁺ while lacking active K⁺ transport mechanisms and that the transmembrane potential is almost negligible. The absence of a measurable potential is consistent with the effects of ionophores and lipid-soluble anions on pH (see below).

The high intra-Golgi [K⁺], together with the presence of K⁺ permeation pathways suggest that efflux of this cation could serve to neutralize the inward pumping of H⁺ by the V-ATPase. The physiological significance of the high intra-Golgi K⁺ is otherwise not clear but it is conceivable that, as described for some cytosolic pathways (50), some of the enzymes within the Golgi perform optimally at high concentrations of this cation.

Role of membrane potential as a determinant of pH₆. In vitro determinations using isolated Golgi fractions had shown earlier that increasing counterion conductance by means of ionophores enhanced luminal acidification (51). Moreover, Llopis et al. (13) showed more recently that removal of extracellular Cl⁻, which presumably was accompanied by depletion of cytosolic Cl⁻, rapidly dissipated the acidification of the Golgi lumen. These observations indicate that the rate of pumping by the electrogenic V-ATPase can be limited by the generation of a transmembrane voltage. In fact, such a limitation has been repeatedly hypothesized to be the primary determinant of intraorganellar pH (25,34).

In the case of the Golgi, however, several findings in this and other reports argue convincingly against this possibility. First, flux of counterions through the endogenous
conductance was shown to be greater than the rate of H⁺ pumping at the steady state. This was concluded from the ability of the conductance to support a rapid dissipation of pH upon addition of a conductive protonophore (Fig. 5). In these experiments, the rate of dissipation was greater than that observed upon addition of concanamycin, which was used as a measure of the rate of pumping (see Results). Secondly, pHᵢ failed to become more acidic when putatively membrane-permeant anions were present, or when valinomycin was added. As discussed above, the substrate transported by this conductive ionophore, namely K⁺, was plentiful within the Golgi. The inability of valinomycin to alter pHᵢ, which had been noted earlier by Llopis et al. (13), also implies that the electrical potential across the Golgi membrane is insignificant. To the extent that the V-ATPase is electrogenic, any modification in the prevailing voltage would be anticipated to alter the rate of pumping. Because valinomycin is expected to clamp the potential near zero, given the similarity of the cytosolic and intra-Golgi [K⁺], the constancy of pHᵢ suggests that the resting voltage is negligible. Thirdly, while this manuscript was in preparation, Farinas and Verkman (19) reported that the V-ATPase is not quiescent at the steady state pHᵢ. The results in our Fig. 4 are consistent with this interpretation, demonstrating the presence of bafilomycin-sensitive changes in pHᵢ below the resting level. The cumulative evidence suggests that counterion conductance is not rate limiting to H⁺ pumping, at least near the steady state pH, and therefore that membrane potential is not an important determinant of pHᵢ.

**Allosteric control of the V-ATPase.** Other transporters, like the family of Na⁺/H⁺ antiporters, attain near-quiescence despite the persistence of large thermodynamic. The reduced activity has been attributed to an allosteric site that upon protonation exerts an inhibitory effect on the exchangers gradients (33). Conceivably, a similar effect could contribute to the establishment of the pH set point of the Golgi. However, both our results and those of Farinas and Verkman (19) revealed an approximately linear relationship between pHᵢ and the rate of H⁺ pumping in the range studied. This contrasts with the sharp decline in Na⁺/H⁺ exchanger activity as the cytosolic pH approaches the steady state. Thus, while the occurrence of allosteric
control of the V-ATPase has not been ruled out, it does not appear to be the primary determinant of the steady-state pHG.

**Contribution and nature of H+ leak pathways.** The existence of a sizable H+ (equivalent) backflux or "leak" when pHG is at equilibrium can be readily revealed by inhibition of the pump (Fig. 5 and refs. 11,19,13). Because by definition the magnitude of this leak must equal the rate of pumping at the steady state, passive H+ backflux is an essential contributor to the establishment of pHG. Indeed, it is likely that the differential luminal pH of organelles within the endocytic and secretory pathways is dictated as much by the magnitude of their leak, as by the density and activity of their pumps.

Despite the importance of the passive H+ conduction pathways, little is known about their nature. Several insights were provided by the results in Figs. 5-7 and in Table 1. Briefly, the net loss of H+ is not likely a consequence of import of alkaline solution from the ER via vesicular traffic, nor of delivery of acidic vesicles towards the trans-Golgi network. Similarly, we found no evidence that either Na+/H+ exchange or Nramp-related molecules transport H+ out of the Golgi. Bicarbonate transport via an anion exchanger is also deemed unlikely, since all our measurements were carried out in nominally bicarbonate-free media. Instead, at least part of the flux occurs via a conductive pathway, inasmuch as it is altered by manipulation of the electrical potential across the Golgi membrane (Fig. 6). Like the H+-selective conductance found in the plasma membrane of several cell types (47,48), the backflux of H+ from the Golgi was inhibited by micromolar concentrations of Zn2+. Therefore, the pathways involved may bear some similarity. The plasmalemmal system displays very small unitary conductance (48), arguing against a continuous large pore across the membrane. The alternative possibilities, involving intimate association of the ions with the channel or a carrier mechanism that requires conformational changes are consistent with the large temperature coefficient of the leak pathway of the Golgi. Direct electrophysiological analysis will be required to more thoroughly characterize the Golgi leak pathway.
In summary, we propose that $\text{pH}_G$ is dictated by a compromise between the rates of $\text{H}^+$ pumping and leakage. If $\text{pH}_G$ is indeed established by the balance between pump and leak, blockade of the latter would be predicted to accentuate the luminal acidification. Unfortunately, this premise cannot be tested at present because $\text{Zn}^{2+}$, the only identified inhibitor of the leak, is also a powerful inhibitor of the V-ATPase (52-53). Finally, while the $\text{H}^+$ backflux pathway(s) include a $\text{Zn}^{2+}$-sensitive conductive system, the contribution of substrate-$\text{H}^+$ symporters or antiporters has not been excluded. Such systems could take advantage of the transmembrane $\text{pH}$ gradient to accumulate substrates or eliminate metabolic products from the Golgi. Clearly, the rate of the V-ATPase and the magnitude of the leakage need not be invariant and modulation of one or both of these systems could result in alteration of $\text{pH}_G$. A better understanding of the regulation of the pump and leak is therefore essential.
THE abbreviations used are: DCCD, dicyclohexyl carbodiimide; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; NEM, N-ethylmaleimide; NMG⁺, N-methyl-D-glucammonium; PBS, phosphate-buffered saline; pHG, Golgi pH; SLO, streptolysin O; TPEN, N,n,n',n'-tetrakis(2-pyridyldimethyl)ethylenediamine; V-ATPase, vacuolar-type ATP hydrolase; VT1B, recombinant B subunit of verotoxin 1.

2 Calculated using a stoichiometry of 3 and 50 kJ/mol as the free energy of ATP hydrolysis by the V-ATPase (see ref. 54). A minimum pH of 4.4 is calculated using a stoichiometry of 2.

3 The error in the pHG calculations introduced by the difference between the extracellular/cytosolic [K⁺] and the intra-Golgi [K⁺] would be 0.11 units.
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FIGURE LEGENDS

**Fig. 1. Localization of FITC-VT1B to the Golgi complex.** FITC-VT1B (10 μg/ml) was allowed to bind to the surface of Vero cells for 1 hr at 4°C. Internalization was induced by incubation for an additional hr at 37°C (A). The cells were then fixed, permeabilized and labeled with antibody to the Golgi marker α-mannosidase II, followed by Cy3-conjugated secondary antibody (B). Bar = 5 μm.

**Fig. 2. Permeabilization of Vero cells.** A: Intact cells stained with 1 μg of FM-143. Note that only the plasma membrane is labelled; B: Cells were permeabilized using SLO (0.25 μg/ml) for 5 min at 37°C and then stained with FM-143 as above. Note extensive labeling of internal membranes; C: Cells were permeabilized with SLO and pre-equilibrated with 1 mM Lucifer Yellow. Cell-associated fluorescence was quantified by digital imaging upon removal of extracellular dye, as described under Experimental Procedures. Data are representative of 3 similar experiments.

**Fig. 3. Estimation of [K+] using the null-point method.** A: FITC-VT1B labeled cells were bathed in 140 mM K⁺ permeabilization medium and pH₆ was measured by ratio imaging. Where indicated, the plasma membrane was permeabilized by addition of SLO. After 3 min, the medium was replaced by permeabilization buffer with the indicated concentration of K⁺. Finally, 5 μg/ml nigericin were added where noted. The figure is a composite of three experiments, each performed at a different [K⁺]. Data are means ± SE of four cells from a typical experiment out of three. B: Estimation of the Golgi luminal [K⁺] by the null-point procedure. The initial rate of pH change induced by nigericin (in pH/min; ordinate) is plotted vs. the K⁺ concentration of the bathing medium and cytosol (in mM; abscissa). Data are means ± SE of 3 experiments. The null-point is equivalent to the intercept on the abscissa.
**Fig. 4. Estimation of the rate of $H^+$ pumping as a function of $pH_G$.** Cells were labelled with FITC-VT1B and $pH_G$ was measured by ratio imaging as in Fig. 3. Where indicated, 30 mM NH$_4$Cl was added to the bathing medium. Upon removal of NH$_4$Cl (wash) $pH_G$ undershoots transiently. Recovery from this undershoot was then recorded in the absence (circles) or presence of 100 nM concanamycin (squares). Data are means ± SE of 4 cells from a typical experiment out of 3. *Inset:* the rates of recovery after the undershoot ($\Delta pH$/min; ordinate) were quantified as a function of $pH_G$ (abscissa).

**Fig. 5. Effects of concanamycin and CCCP on $pH_G$.** Cells were labelled with FITC-VT1B and $pH_G$ was measured by ratio imaging as in Fig. 3. **A:** Where indicated, 100 nM concanamycin (circles) or 2 µM CCCP (squares) were added to the bathing medium. Data are means ± SE of 3 cells from a typical experiment out of 3. **B:** Cell were permeabilized with SLO in a solution containing 140 mM K$^+$. Where indicated, 2 µM CCCP was added and, finally, the bathing medium was changed to a low K$^+$ solution containing 140 mM NMG$^+$. Data are representative of at least three separate experiments of each type.

**Fig. 6. Voltage sensitivity of the $H^+$ leak pathway of the Golgi complex.** FITC-VT1B labeled cells were bathed in K$^+$-glutamate medium and, where indicated, permeabilized with SLO as in Fig. 2. Next, the cells were treated with 1 µM valinomycin in permeabilization medium containing either 140 mM K$^+$ (squares) or 140 NMG$^+$ (K$^+$-free; circles). Lastly, the V-ATPase was inhibited by addition of 100 nM concanamycin. $pH_G$ was monitored by ratio imaging and calibrated as described under Experimental Procedures. Data are means ± SE of 4 cells from a typical experiment out of 3.

**Fig. 7. Effect of ZnCl$_2$ on the $H^+$ leak.** **A:** FITC-VT1B labeled cells were bathed in K$^+$-glutamate medium and, where indicated, permeabilized with SLO as in Fig. 2. The cells were then incubated for an additional 3 min in the absence (squares) or presence of 200 µM ZnCl$_2$.
(circles). Lastly, concanamycin was added to both samples where specified. Data are means ± SE of 4 cells from a typical experiment out of 3. B: Concentration dependence of the inhibitory effect of Zn$^{2+}$ on H$^+$ (equivalent) leakage. Data are means ± SE of 3 experiments like that in A, using varying concentrations of Zn$^{2+}$. Note that 5 mM Mg$^{2+}$ was used in these experiments to minimize chelation of Zn$^{2+}$ by ATP.
A

- Time (min)
- 140 mM K^+ 
- 70 mM K^+ 
- 35 mM K^+ 

SLO Nigericin

B

- ΔpH/min
- K^+ (mM)

0.05 0.15 0.25

20 40 60 80 100 120 140
The graph shows the changes in pH over time with and without the presence of Concanamycin. The x-axis represents time in minutes, ranging from 0 to 25. The y-axis represents pH, ranging from 6.0 to 7.2.

- **NH₄Cl Wash**: The pH changes are indicated by a triangle symbol, showing the initial drop and subsequent recovery.
- **Concanamycin**: The changes are indicated by a square symbol, showing a more pronounced drop and slower recovery compared to the control.

The inset graph on the right shows the rate of pH change (ΔpH/min) over pHG. The y-axis represents ΔpH/min ranging from 0.02 to 0.10, and the x-axis represents pHG ranging from 6.2 to 6.6. The control and Concanamycin treatments are clearly distinguished, with the control showing a slower rate of pH change compared to Concanamycin.
A

\[ \text{pH}_G \]

\[ \begin{align*}
2 & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12 & \quad 14 & \quad 16 & \quad 18
\end{align*} \]

\[ \text{Time (min)} \]

\[ \begin{align*}
6.4 & \quad 6.6 & \quad 6.8 & \quad 7.0
\end{align*} \]

\[ \begin{align*}
\text{CCCP} & \quad \text{Concanamycin}
\end{align*} \]

B

\[ \begin{align*}
\text{140 mM K}^+ & \quad \text{140 mM NMG}
\end{align*} \]

\[ \begin{align*}
\text{SLO} & \quad \text{CCCP}
\end{align*} \]

\[ \begin{align*}
\text{pH}_G
\end{align*} \]

\[ \begin{align*}
5 & \quad 10 & \quad 15 & \quad 20 & \quad 25 & \quad 30 & \quad 35
\end{align*} \]

\[ \text{Time (min)} \]
Table 1. Determinants of Golgi pH

Cells were labeled with FITC-VT1B and pH$_G$ was measured by ratio imaging. The resting pH$_G$ and the initial rate of alkalinization upon addition of 100 nM concanamycin were monitored under the conditions specified in the second column. SLO indicates permeabilization µg/ml of streptolysin O. The concentrations of the agents used were valinomycin, 1 µM; α, α' dipyridyl, 100 µM; TPEN, 20 µM; GTPγS, 25 µM; amiloride, 100 µM; NEM, 100 µM; DCCD, 150 µM; ZnCl$_2$, 200 µM. Data are means of 3 separate experiments.

| Treatment                        | Basal pH$_G$ (± SE) | Concanamycin-induced alkalinization (pH$_i$) |
|----------------------------------|---------------------|---------------------------------------------|
| **Counterion conductance**       |                     |                                             |
| Control                          | 6.48 ± 0.07         | 0.152                                       |
| SCN$^-$                          | 6.53 ± 0.03         | 0.159                                       |
| SCN$^-$ + SLO                     | 6.46 ± 0.04         | 0.153                                       |
| Valinomycin                      | 6.51 ± 0.01         | 0.156                                       |
| **H$^+$/heavy metal co-transporters** |                 |                                             |
| Control                          | 6.54 ± 0.06         | 0.162                                       |
| α, α’dipyridyl                   | 6.49 ± 0.08         | 0.155                                       |
| TPEN                             | 6.51 ± 0.04         | 0.158                                       |
| **Vesicle trafficking**          |                     |                                             |
| Control                          | 6.54 ± 0.02         | 0.164                                       |
| SLO + GTPγS                      | 6.49 ± 0.03         | 0.155                                       |
| **Na$^+$/H$^+$ exchanger**       |                     |                                             |
| Control                          | 6.54 ± 0.02         | 0.162                                       |
| Amiloride                        | 6.46 ± 0.05         | 0.152                                       |
| SLO/Na$^+_e$13 mM                | 6.49 ± 0.03         | 0.155                                       |
| SLO/Na$^+_e$103 mM               | 6.46 ± 0.08         | 0.151                                       |
| **H$^+$ conductance**            |                     |                                             |
| Control                          | 6.50 ± 0.09         | 0.160                                       |
| NEM                              | NA                  | 0.163                                       |
| DCCD                             | NA                  | 0.161                                       |
| ZnCl$_2$                         | NA                  | 0.040                                       |

H$^+$ conductance
Determinants of the pH of the Golgi complex
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