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Abstract

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pH Homeostasis of Cellular Organelles

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An optimal pH is maintained in each secretory and endocytic organelle by the balance between active H^+ pumping and passive H^+ efflux. The progressive acidification of secretory organelles does not reflect an increased proton pumping activity but rather the decreased conduction of a "proton leak" pathway resembling the recently cloned voltage-gated proton channels.

The maintenance of an appropriate pH within membrane-enclosed compartments is a constant challenge for all living beings, from the simplest prokaryotes to complex multicellular organisms. Accordingly, cells have evolved a variety of specialized proton-translocating devices. In mitochondria, cytochromes exploit the flux of electrons to extrude protons, thus producing a proton gradient that is used to generate useful chemical energy in the form of ATP. In contrast, other organelles consume ATP to pump protons into the lumen to generate the acidic pH required for the maturation and processing of secretory proteins and for the dissociation and recycling of endocytosed materials.

Because most enzymes are exquisitely pH sensitive, the pH of each organelle critically determines the coordinated biochemical reactions occurring along the endocytic and secretory pathways. Aberrations of the normal organellar pH homeostasis, either through disease, infection, or by pharmacological means, can lead to significant functional changes. A luminal acidification is essential for the distribution and degradation of internalized ligands in the endocytic pathway, and it regulates posttranslational modification and sorting of proteins along the secretory pathway.

As shown in Fig. 1, The pH varies in different subcompartments of the endocytic pathway, with acidification increasing progressively from the endocytic vesicles and early endosomes to late endosomes and, ultimately, lysosomes. Conversely, the pH of the secretory pathway becomes more acidic as the cargo travels toward the cell surface. Whereas the pH of the endoplasmic reticulum (ER) is thought to be near neutral (6, 8), acidification develops along the Golgi complex and is maximal at the *trans*-Golgi network (TGN) (2, 9, 14). In this review, I will present the new methodology used to measure and manipulate the pH of the cellular compartment and discuss the mechanisms involved in the pH regulation of endocytic and secretory organelles. The regulation of mitochondrial pH, and its potential role in the apoptosis process, has been discussed in detail in recent reviews and will not be covered here.

Measurements of organellar pH

Unlike its secretory counterpart, the endocytic pathway is readily accessible to fluorescent pH indicators. The pH of endocytic compartments can be conveniently measured using internalized FITC antibodies as pH-sensitive probes. FITC is the most popular pH dye because of its optimal pK_a (~6.4), resistance to organellar enzymes, hydrophilicity, stability, and nontoxicity. Using FITC-conjugated ligands binding to well-defined receptors, the pH of specific endocytic compartments can be measured. For instance, internalized FITC-transferrin remains confined to the endosome and thus provides accurate measurements of the endosomal pH (4). This technique, coupled to ratio fluorescence imaging, can be used to follow the kinetic of acidification of endocytic compartments in living cells with good spatial and temporal resolution. Until recently, the determination of the pH of organelles of the secretory pathway, such as the ER, Golgi, TGN, and secretory granules, was limited to the use of static, immunoelectron microscopy-based methods. Over the last few years, however, a variety of ingenious techniques have been developed that allow noninvasive pH measurements of individual organelles in living cells. These include the following:

Trapped dyes. Membrane-permeant esters of the popular pH-sensitive fluorescent dyes BCECF and SNARF accumulate in intracellular compartments under certain loading conditions. This approach allows us to measure the pH of easily identifiable organelles such as the yeast vacuole. However, its lack of targeting specificity precludes its use for small and more dynamic compartments of the secretory pathway.

Size-fractionated liposomes. Liposomes of ~70 nm diameter preferentially fuse with the *trans*-Golgi membrane and can be used to measure the pH of this organelle (13, 14). The procedure, however, is restricted to the *trans*-cisternae of the Golgi, requires microinjection of size-fractionated liposomes, and does not allow long measurements because the fluid phase indicator rapidly leaves the Golgi at 37°C (13).

Bacterial toxins. Toxins produced by *Shigella* and by enteropathogenic strains of *Escherichia coli* bind to surface glycolipids via their nontoxic B subunits and are subsequently transported along an endogenous retrograde pathway, accumulating in the Golgi complex before eventually reaching the ER. This retrograde pathway can be harnessed to deliver probes to the Golgi and the ER, allowing noninvasive measurements

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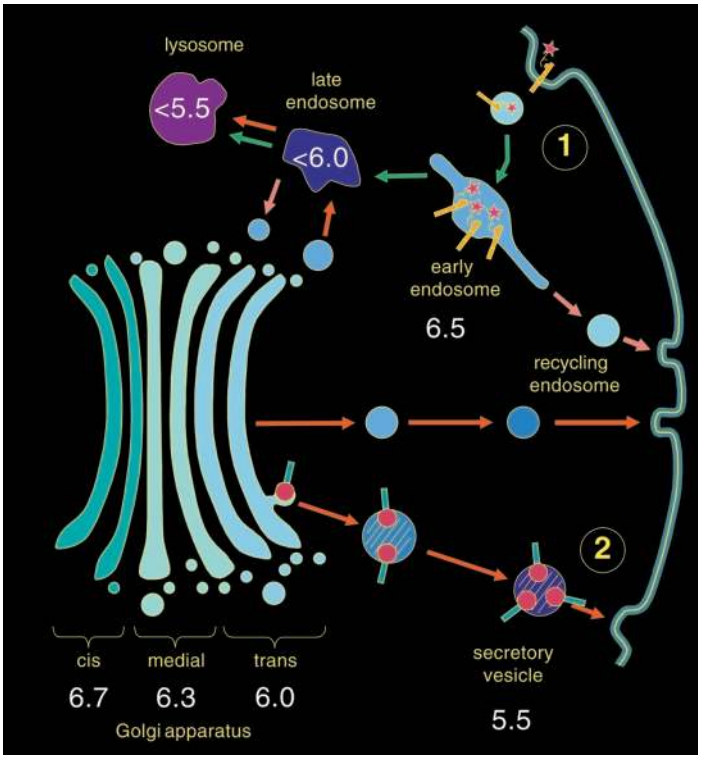

FIGURE 1. pH of endocytic and secretory organelles. Organelles rapidly acidify as they progress along the endocytic or secretory pathway. The pH of endocytic compartments (*1*) can be measured using internalized FITC antibodies as pH-sensitive probes (red stars). A variety of new approaches now allow us to measure the pH of secretory compartments (*2*), using appropriate targeting sequences coupled to pH-sensitive fluorescent probes (red circles).

of the pH within the lumen of these compartments (6, 7). The toxin is labeled with FITC, which is allowed to bind to its plasma membrane receptors, and the fluorophore is chased to the Golgi apparatus, where it remains for several hours (7, 11, 12). Enhanced delivery of the toxins in the ER can be achieved by adding the COOH terminal ER retrieval signal Lys-Asp-Glu-Leu to the B subunit (6).

Targeted probes. The intrinsic retrograde traffic of membranes can also be exploited to target fluorescent probes to the secretory system, because a small fraction of endocytosed molecules reaches the Golgi complex through the retrograde pathway. Certain resident proteins of the TGN, such as furin and TGN38, circulate continuously to and from the plasma membrane. Both the TGN localization and the retrieval from the plasma membrane of these proteins are mediated by their COOH terminus (cytoplasmic) tail. Accordingly, chimeric proteins containing the cytoplasmic sequence of TGN38 or furin localize to the TGN. As the luminal domain is exposed transiently to the outside medium during cycling, intact cells transfected with fusion proteins containing an immunogenic luminal epitope accumulate specific antibodies to the TGN. These fusion proteins can be used as vehicles to deliver pH-sensitive fluorescent probes to the TGN, allowing dynamic measurements of Golgi pH in live cells (2).

Genetically encoded indicators. The green fluorescent protein (GFP) can be used as a noninvasive intracellular pH indicator, allowing us to selectively measure the pH of secretory organelles by transfection of GFP mutants bearing organelle-specific targeting signals. Although the pH sensitivity of the original GFP is poor, several GFP mutants were recently generated that displayed large (>10-fold) and reversible changes in fluorescence as a function of pH (8, 9). The pK_a values of the different mutants ranged from 4.8 to 7.1, making them suitable for pH measurements in both the cytosol and organelles. The pH-sensitive GFPs can be selectively expressed in a defined intracellular compartment by addition of appropriate targeting sequences. For instance, pH-sensitive GFP mutants have been targeted to the *medial*- and/or *trans*-Golgi by fusion with galactosyltransferase and to the mitochondrial matrix by using the targeting signal from subunit IV of cytochrome *c* oxidase (8, 9). Another family of pH-sensitive GFP mutants, generated by structure-directed combinatorial mutagenesis and termed "pHluorins," provide the additional benefit of a pH-dependent shift in the excitation spectra, allowing us to perform quantitative, ratiometric measurements (10).

The targeting specificity conferred by genetically encoded sequences can be combined with the bright and specific properties of chemical fluorophores. The strategy is to express a high-affinity "receptor" at a specific intracellular location to trap a conjugate of a fluorophore linked to a receptor "ligand." Using a single-chain antibody (sFv) as the receptor and cell-permeable conjugates of a hapten [4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx)] and a fluorescent probe (BODIPY FL, tetramethylrhodamine, fluorescein) as the ligand, high-affinity (~5 nM) and specific labeling of the ER, Golgi, and plasma membrane was achieved (3). Using the pH-sensitive phOx-fluorescein conjugate, pH can be measured by ratio imaging microscopy. In an analogous approach, a pH-sensitive, membrane-permeable fluorescein-biotin conjugate was targeted to the ER and to the Golgi complex by achieving localized expression of avidin chimera proteins in these compartments (15). Like the expression of the GFP, the receptor-mediated fluorophore targeting approach provides a specific fluorescence labeling but with the additional advantage of offering the ability to target chemical probes with custom-designed spectral and indicator properties.

In summary, a variety of new techniques now allow in situ pH measurements of individual organelles in living cells by fluorescence imaging. These include the microinjection of pH-responsive probes trapped in size-fractionated liposomes (13, 14), the retrograde transport of Golgi-resident proteins (2) or bacterial proteins (6, 7), the transfection of pH-sensitive variants of GFP linked to organelle-specific targeting sequences (8, 9), and the expression of targeted chimeric constructs that can be used to trap soluble probes in specific organelles (3, 15).

Determinants of organellar pH

The acidic pH of organelles is generated primarily by a vacuolar type (V) H^+-ATPase, which transports protons in the presence of Mg^{2+}-ATP. The extent of acidification varies widely along both the endocytic and secretory pathways (Fig. 1), implying that the steady-state pH of organelles is differentially regulated. This is thought to reflect the differential regulation of organellar pumps and conductances, according to the generally accepted "pump and leak" model. The pump and leak

model postulates that, at steady state, the rate of proton pumping by the V-ATPase is balanced by an equivalent efflux of protons through the organelle endogenous H^+ permeability, or H^+ "leak." Because both proton pumping and H^+ leak are electrogenic, they require the movement of a compensating charge and are thus limited by the permeability of the organelle to counterions.

This model stems from the observation that, under physiological conditions (cytosolic pH = 7.2), the V-ATPase is far from thermodynamic equilibrium. Assuming a stoichiometry of three protons per ATP hydrolyzed, a pH of 3 would be reached in the lumen of organelles if the ATPase attained chemical equilibrium. However, in situ pH measurements consistently reported organellar pH values that, along the secretory pathway, ranged from 6.7 to 5.5 (Fig. 1). This discrepancy could reflect the development of an electrical potential (inside positive) across the vesicular membrane. An electrical potential would develop if the rate of proton pumping exceeded the rate of permeation of counterions. Alternatively, leak pathways could facilitate the escape of protons from within the organelle, thereby partially dissipating the gradient generated by the ATPase. The relative contribution of these pathways to the establishment of the organelle pH will be discussed in detail.

Proton pumping. Proton pumps constitute the "primary active" transport systems that translocate H^+ ions across organelle membranes. They are found in every eukaryotic cell and energize a large variety of organelles and membranes. In contrast to ATP synthase, whose primary function in eukaryotes is to generate ATP by exploiting the proton-motive force, V-ATPases hydrolyze ATP to pump protons into organelles. The proton gradient thus generated is then used to drive a variety of "secondary active" transport processes. Given the highly conserved nature of this enzyme, the variety of acidification levels and energization states achieved across species in organelles is remarkable: some brown and red algae have a vacuolar pH as low as 2, and values close to 0 have been reported in the vacuoles of ascidian cells.

The proton pumping activity of organelles is generally inferred from the pH effects of the macrolide antibiotics bafilomycin A_1 and concanamycin, which are highly specific inhibitors of the V-ATPase. These inhibitors cause a rapid alkalinization of acidic organelles as the organelle pH equilibrates with the cytosolic pH. Using this approach, proton pumping activity was detected in all secretory and endocytic organelles except the ER (6, 15). More direct determination of the rates of proton pumping across organelle membranes requires that the cells are first permeabilized to allow access of ions across the plasma membrane (Fig. 2). This can be achieved using digitonin or streptolysin O. The cells are permeabilized in a cytosol-like medium devoid of ATP to dissipate the pH gradient across the organellar membrane. Addition of ATP to the medium then produces a rapid acidification that directly reflects the rate of proton pumping into the organelle (2). In this configuration, the ionic and pH dependence of the rates of proton pumping can be assessed directly.

Some pH-regulating transporters such as the Na^+/H^+ antiporters are allosterically regulated by their substrate ion (H^+), displaying minimal activity around a pH "set point" close to the steady-state pH levels. The reduced activity has been attributed to the inhibitory effects of the protonation of an allosteric site on the exchangers. This mechanism is unlikely to apply to the V-ATPase, because recent results revealed a near-linear relationship between vacuolar pH and the rate of H^+ pumping within the physiological pH range (3, 12). Using permeant weak bases to impose a transient acidification, the rates of proton pumping were measured to confirm that the V-ATPase was functional even below the resting pH level (12). This contrasts with the sharp decline in Na^+/H^+ exchanger activity at near-neutral cytosolic pH and suggests that an allosteric regulation of proton pumps does not determine the pH set point of organelles.

Rather than reflecting the regulated activity of the pump, the distinct pH of organelles could reflect a different density of pumps in the organelle membrane. Using FITC-labeled transferrin and in situ pH imaging, recycling endosomes containing the transferrin receptor were found to be less acidic than early endosomes in Madin-Darby canine kidney cells (4). The reduced acidification of recycling endosomes correlated with a lack of functional V-ATPases, suggesting that the delivery of pumps to endosomes was followed by their subsequent retrieval as the organelle progressed along the recycling pathway. This suggests that the regulated delivery or assembly of pumps underlies, at least in part, the differential pH of organelles at each step of the endocytic pathway.

A differential targeting of membrane transport proteins was also observed during the maturation of phagosomes, the dedicated compartment of specialized cells that internalize and digest invading microorganisms by phagocytosis. The acidification of phagosomes was directly measured in macrophages

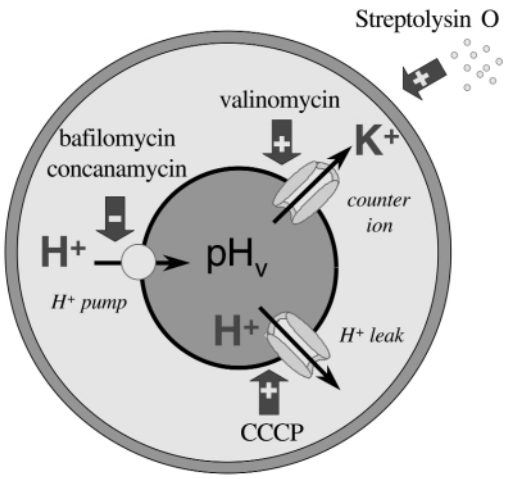

FIGURE 2. Regulation of organellar pH: the pump and leak model. The steady-state pH of organelles is determined by the balance between active proton pumping and passive H^+ efflux, or "leak." Because both the pump and the leak are electrogenic, they might be limited by the permeability of the organelle to counterions (in this case, K^+). Proton pumping can be inhibited by the macrolide antibiotics bafilomycin or concanamycin. An artificial H^+ leak or counterion conductance can be generated with the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) or the K^+ ionophore valinomycin, respectively. The plasma membrane can also be permeabilized with streptolysin O to gain access to the cytosol and impose defined ionic conditions and ATP concentrations.

by ratio fluorescence imaging and shown to involve V-ATPases (5). Na^+/H^+ exchange did not contribute to phagosomal acidification, although the NHE-1 isoform was present and functional in the phagosomal membrane. This reflected the absence of an appropriate lumen-to-cytosol Na^+ gradient due to the lack of Na^+/K^+ pumps in the phagosomal membrane (5). Thus, although both transporters are originally present at the plasma membrane, only NHEs remain in phagosomes, whereas Na^+/K^+ pumps are rapidly retrieved. The retrieval of Na^+-K^+-ATPases and the parallel insertion of V-ATPase ensures that phagosomes rapidly acidify, a mechanism that is critical for the host defense against infections.

Counterions and membrane potential. Due to its electrogenic nature, proton pumping by the V-ATPase can be limited by the generation of a transmembrane voltage across the organelle membrane. Such a mechanism has long been postulated to be the prime determinant of the pH of organelles. The existence of a high Cl^- permeability in endosomes is well established, and Cl^- influx is thought to sustain the rapid acidification of endosomes. By analogy, a Cl^- conductance has been postulated to control the extent of acidification of secretory organelles, and intracellular Cl^- depletion has been reported to dissipate the acidic pH of the Golgi (9). Indirect evidence was also obtained using ionophores to generate an artificial counterion conductance. This procedure is expected to favor the activity of the V-ATPase, to increase the organelle acidification (Fig. 2), and to enhance the luminal acidification in isolated Golgi fractions.

"...a Cl- conductance has been postulated to control the extent of acidification of secretory organelles...."

The notion that the pH of secretory organelles is determined by a limiting counterion conductance was challenged recently by measurements in intact cells. In several studies of Golgi pH, generating an artificial counterion conductance with the K^+ ionophore valinomycin had no effect on the steady-state pH of the organelle (2, 12). The lack of effect of valinomycin indicates that the rate of proton pumping is not limited by the permeability of the organelle to counterions. It also implies that the electrical potential across the Golgi membrane is not significant, because disruption of the transmembrane voltage by valinomycin is expected to alter the rate of electrogenic H^+ pumping by the V-ATPase and thus the steady-state pH of the organelle.

In one study, the free K^+ concentration within the Golgi complex was determined directly by using a null point approach (12). Based on the stoichiometric exchange of K^+ for H^+ catalyzed by nigericin, the Golgi K^+ concentration was estimated to be ~107 mM, i.e., slightly lower than the concentration of the cytosol. These high values might reflect the ability of some Golgi enzymes to perform optimally at high K^+ concentrations. The high K^+ concentrations on both sides of the organelle, together with the lack of effect of valinomycin on Golgi pH, indicate that the endogenous K^+ permeability is high. This implies that the transmembrane potential is almost negligible and that passive efflux of K^+, rather than entry of Cl^-, normally neutralizes the inward pumping of H^+ by the V-ATPase. Thus the rate of H^+ pumping is not limited by the counterion conductance of the Golgi, suggesting that the membrane potential is not an important determinant of the pH of secretory organelles.

H^+ permeability. The existence of an endogenous H^+ permeability, or leak, can be inferred from the effects of V-ATPase inhibitors on the steady-state pH of organelles (2, 3, 7, 9). The resulting alkalinization indicates that passive H^+ efflux contributes significantly to the resting pH, because at steady state the magnitude of the leak is equal to the rate of proton pumping. In organelles devoid of H^+ pumping activity, such as the ER, the magnitude of the leak has been estimated from the recovery rates of organelle pH following an imposed acid load (15). The leak was highest in the ER, whose pH closely followed alterations in the cytosolic pH and was thus indirectly regulated by plasma membrane acid-base transporters. The high H^+ permeability of the ER membrane thus functionally connects the ER compartment with the cytoplasm. The magnitude of the leak decreased along the secretory pathway as organelles became more acidic. This suggests that the different pH of secretory organelles reflects their distinct proton leak rather than a different density or activity of proton pumps.

The molecular nature of the passive H^+ conduction pathways of organelles is unknown, but several insights were provided recently. The net loss of H^+ does not appear to be a consequence of import of alkaline solution from the ER via vesicular traffic nor of delivery of acidic vesicles toward the TGN (12). Similarly, no evidence was found that either Na^+/H^+ exchange or Nramp-related molecules transport H^+ out of the Golgi (2, 12). HCO_3^- transport via an anion exchanger is also unlikely, because most measurements were carried out in nominally HCO_3^--free medium. Instead, at least part of the flux occurs via a conductive pathway, because it was altered by manipulation of the electrical potential across the Golgi membrane (2, 12). The conductive efflux of H^+ from the Golgi was inhibited by micromolar concentrations of Zn^{2+}, a well-known inhibitor of voltage-gated proton channels. This suggests that proton channels might also contribute to the H^+ permeability of intracellular organelles.

Proton channels appear to be unique among pH-regulating transporters because they do not require ATP or coupling to other ions, thus allowing massive flux of protons at no energy cost to the cell. Proton channels are activated by voltage in a pH-dependent manner and, like ion channels, exhibit complex gating kinetics. Unlike most ion channels, however, they are almost perfectly selective, do not have a measurable unitary conductance, and are highly temperature dependent. The recently cloned NADPH oxidase homologue (NOX) protein family members function as proton channels when expressed in HEK-293 cells (1). They contain a critical proton transport motif consisting of a string of histidine residues aligned along the axis of an α-helix that appear to form a "proton wire." This suggests that the mechanism of conduction does not involve permeation through a water-filled pore but proton "hopping" along a H^+-bonded chain. Such a mechanism is consistent with the large temperature coefficient of the Golgi H^+ leak

pathway (12), suggesting that the Golgi H^+ leak pathway might bear a similarity to plasma membrane H^+ channels. However, direct electrophysiological analysis will be required to characterize the endogenous proton permeability of organelles.

Conclusions

Over the last decade, considerable progress has been made in our understanding of the pH regulatory mechanisms of cells. Several new plasma membrane H^+ transporters have been cloned and sequenced, and the structure and topology of these exchangers, cotransporters, and antiporter proteins has yielded much information about their transport properties and regulatory mechanisms. In parallel to this wealth of molecular knowledge regarding the major plasma membrane H^+ transport systems, a variety of innovative approaches have provided insights into the mechanisms regulating the pH of intracellular organelles. These approaches confirmed that the acidity of the lumen increases rapidly along the endocytic pathway, from a pH of ~6.5 in early and recycling endosomes to <6.0 in late endosomes and <5.5 in lysosomes. Conversely, the pH of secretory compartments becomes more acidic as the secretory products approach the plasma membrane, from a pH of ~7.0 in the ER, ~6.7 in the *cis*-Golgi, ~6.3 in the *medial*- and *trans*-Golgi, ~6.0 in the TGN, and ~5.5 in secretory vesicles. This gradient of acidification is accompanied by a decrease in the passive H^+ permeability of the organelles, whereas the counterion permeability remains high. This suggests that the proton leak, rather than the counterion permeability, sets the steady-state pH of secretory organelles. The molecular identity of the proton leak is unknown, but its mechanism of proton conduction resembles that of a voltage-gated proton channel, a family of H^+-translocating cytochromes located both at the plasma membrane and in organelles. Substantial progress will certainly be made in the near future in the molecular identification and functional characterization of these membrane transport proteins.

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