A View of Acidic Intracellular Compartments

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CONSIDERABLE evidence has accumulated over the past ten years that the interior of both the endocytic and exocytic vacuolar apparatus in cells is maintained at a low pH (30, 47, 60). The interior of endocytic vesicles (4, 17, 29, 57, 59), lysosomes (37, 43), portions of the trans-Golgi apparatus (3, 38, 39), certain secretory vesicles (14, 22, 23, 38, 48), and plant tonoplasts (7) is acidic. The maintenance of the high H⁺ concentration depends upon the properties of the surrounding membrane and the vectorial movement of protons mediated by an ATP dependent H⁺ pump (1, 30, 47).

A variety of different techniques have been used to study the function of low pH compartments. (a) Using purified secretory vesicles (24, 36), yeast vacuoles (49), and lysosomes (42, 53), the influx and efflux of low molecular weight solutes has been shown to depend on both a transmembrane H⁺ gradient and an electrical gradient generated by an ATP-dependent H⁺ pump in the membrane of these vesicles. (b) Several laboratories have isolated mutant cell lines that are defective in H⁺ pumping (28, 31, 45); these cells are resistant to certain toxins that gain entry to cells by endocytosis and they are unable to obtain iron from transferrin during receptor-mediated endocytosis. (c) Reagents that either neutralize low pH compartments or dissipate H⁺ gradients inhibit a variety of endocytic and exocytic activities including receptor recycling during receptor-mediated endocytosis (9), the sorting of both content and membrane proteins during exocytosis (19, 33, 58) and endocytosis (9), and the posttranslational processing of macromolecules that pass through the Golgi apparatus (39).

To better understand the function of acidic compartments, investigators have studied H⁺ gradients in the living cell using vital pH indicators. When taken up by cells, these indicators either selectively accumulate in low pH compartments or detect the pH of the compartment in which they reside. As outlined below, recent advances in immunocytochemistry, flow cytometry, and videoenhanced microscopy offer the interested investigator new opportunities for using these indicators to "capture" the H⁺ gradient as it exists in the living cell and detect the chemical reactions that occur in that environment.

Visualization of Acidic Compartments

The first indication that cells had intracellular low pH compartments occurred when Metchnikoff (32) observed that litmus paper phagocytosed by certain protozoa changed color from blue to red. Since that time, acidic compartments in living cells have been extensively studied using vital techniques. Acridine orange and neutral red (2) have been widely used because these basic dyes accumulate in membrane bound vacuoles in response to low pH. Fluorescein-tagged molecules, on the other hand, have been useful for measuring the pH of endocytic compartments because the fluorescence emission spectrum of fluorescein is responsive to pH (17, 37, 43, 59). The first measurement of lysosomal pH in situ (pH 4.7-4.8) was made in macrophages using fluorescein-labeled dextran (37, 43) and the pH of fibroblast endosomes (pH 5.5) involved in receptor-mediated endocytosis was determined with fluorescein-labeled α₂-macroglobulin (57).

Fluoresceinated macromolecules continue to be important reagents. In an exciting new use, Murphy and co-workers have developed methods for measuring the rapid kinetics of acidification by flow cytometry (34, 35). With this technique, the earliest phases of acidification that accompany endosome formation can be detected. Moreover, measurements can be made of the speed of ligand movement through acidic compartments, the pH of the environment the ligand encounters during transit, and the exposure of ligand to proteolytic enzymes (46).

The use of these low pH detecting molecules is limited by several factors: (a) only cultured cells are amenable to study; (b) light microscopy does not have sufficient resolution to identify morphologically and functionally the acidic compartments; (c) cells can only be studied while they are alive. These limitations have prompted the development of a new class of vital dyes that can be used to detect low pH compartments after the cells have been fixed and processed for light and electron microscopy.

Weak bases such as chloroquine accumulate in acidic intracellular compartments (12). Taking advantage of this property, weak bases have been found that are both retained at sites of accumulation after aldehyde fixation and detectable by electron and light microscopic immunocytochemistry. We introduced the use of DAMP (3-[2,4-dinitroanilino]-3'-amino-N-methyl dipropylamine),1 a specially formulated weak base that contains a dinitrophenol group for easy detection with a suitable monoclonal antibody (3, 4, 38, 40, 41). Schwartz et al. (50), on the other hand, used primaquine as

1. Abbreviations used in this paper: DAMP, 3-[2,4-dinitroanilino]-3'-amino-N-methyl dipropylamine; PEDAC, postembedding detection of acidic compartments.
the weak base together with an antibody they prepare against this molecule. We call this procedure PEDAC (postembedding detection of acidic compartments); this technique offers a number of advantages for studying the distribution and function of acidic compartments.

Based on our experiences with DAMP, there are several considerations in applying PEDAC. DAMP appears to behave as an amine with a single titratable group (apparent pKa ≈ 10.6 [4]); therefore, DAMP accumulation should be proportional to the H⁺ concentration in the compartment because as each molecule becomes protonated it acquires a charge that retards diffusion across the membrane. DAMP has a primary amino group that is available for cross-linking to surrounding macromolecules during aldehyde fixation. The amount retained in a compartment, therefore, is dependent on the concentration of DAMP relative to the availability of cross-linking sites, e.g., protein. If DAMP is retained quantitatively after fixation, then protein A gold-labeling techniques allow one to estimate the pH of a compartment using the formula: pH = 7.0 - log D₁/D₂ where D₁ = density of DAMP-specific gold particles in the compartment and D₂ = density of gold particles in a pH 7.0 compartment such as the nucleus. Finally, an important control in all experiments is to demonstrate that labeling is abolished when the proton gradient is disrupted with monensin or chloroquine.

**Morphological Identification of Acidic Compartments**

PEDAC has been used to study acidic compartments in several different types of cells (3–5, 38, 40, 41, 50). The morphologically identifiable compartments that were found to be acidic included: (a) endosomes (or CURL [compartment of uncoupling receptors and ligands]) in fibroblasts (4) and hepatocytes (50); (b) lysosomes (4, 5, 38, 39, 50); (c) a subpopulation of coated vesicles in fibroblasts (4); (d) trans-Golgi vesicles (38, 39, 50); (e) secretory vesicles in insulin-secreting β cells (39); (f) extracellular lacunae in osteoclasts (5). Undoubtedly, the list of acidic compartments will lengthen as investigators examine cells and tissues from a variety of sources.

Although some of these compartments were already known to be acidic (e.g., endosomes, lysosomes, and certain secretory granules), PEDAC has provided some interesting new insights. First, individual endosomes in the same cell appear to vary in pH, regardless of the size or endosomal content, which suggests that the rate of acidification is heterogeneous. Second, the CURL compartment of hepatocytes does not uniformly accumulate primaqine: the receptor-rich tubular extensions take up less than the vesicular body of the organelle (50). This difference may be due to the difference in volume between the tubular and vesicular portions or to the quantity of available cross-linking sites. However, these results could also mean that these two portions of a continuous membrane compartment have a different pH. We made a similar observation in the trans-cisternae of the Golgi apparatus from insulin-secreting cells DAMP accumulated in regions where vesicles were budding but not in the remainder of the stack (38). Third, DAMP appears to accumulate only in coated vesicles associated with the Golgi region of the cell (4). This may mean that endocytic coated vesicles are not acidic. Even though isolated brain coated vesicles have been found to have a H⁺ ATPase (15, 56), recently Fuchs et al. (16) reported that isolated endocytic coated vesicles were unable to acidify. Finally, a comparison of the Golgi region in several different cells suggests that acidification of the trans-cisternae is variable. Whereas all cells have acidic trans-Golgi vesicles, the trans cisternae appear to be acidic only in fibroblasts (3) and hepatocytes (50). Therefore, acidification of this compartment may be regulated to serve the needs of the cell. These observations also indicate that most likely monensin (8, 26) and chloroquine (33, 38) have their profound effects on sorting (33, 38), processing (19), and movement of proteins through the Golgi region (20, 44) by neutralizing the trans portion of the organelle.

**Mapping the Function of Low pH Compartments**

In vitro experiments have shown that protons can modulate the activity of enzymes (e.g., lysosomal hydrolases [11]), the interaction of macromolecules (e.g., ligand–receptor affinity [10, 13]), the conformation of macromolecules (e.g., diphtheria toxin [6] and influenza virus hemagglutinin [51]), and the generation of chemical gradients (e.g., the concentration of biogenic amines in chromaffin granules [24, 36]). Most likely these same activities are regulated by protons within the living cell. With the resolution of the electron microscope it is now possible to pinpoint where in the cell a reaction of interest occurs and determine the relationship between the reactants, the products, and the H⁺ concentration in the surrounding environment.

Helenius and co-workers (21) were the first to localize an intracellular acid dependent reaction with the electron microscope: the membrane of certain enveloped viruses fuse with cell membranes at an acid pH (<pH 6.0). With the electron microscope, they observed that the fusion reaction occurred in endosomes shortly after the virus was internalized by coated pits. Since the fusion reaction is elicited by the pH-dependent change in conformation of a viral surface protein (SI), membrane–membrane fusion serves as an assay for the pH of the endosome environment and the effect of protons on the shape of the surface protein. More recently, these workers have isolated several different mutant viruses, each having a different pH optimum for membrane fusion (25), which extends the pH range that can be detected within the endocytic pathway.

The major advantage of PEDAC is that the pH can be detected in any intracellular compartment. With a suitable histochemical or immunocytochemical marker that identifies a reaction of interest, both the reaction and the H⁺ concentration in the surrounding environment can be colocalized with the electron microscope. Using double-labeled protein A-gold immunocytochemistry, together with suitable quantification techniques, in many cases the magnitude of the H⁺ concentration relative to the concentration of reactants and/or products can be estimated. This is the method that we used to show that the conversion of proinsulin to insulin occurs in an acidic compartment (38, 41).

To detect the conversion step, which requires the removal of the C peptide that joins the A and B chains of insulin, we used either an antibody that binds only to proinsulin (38) or an antibody that exclusively recognizes insulin (41). The concentration of either the precursor (proinsulin) or the product (insulin) in the maturing secretory vesicles is a measure of the activity of the proteolytic converting enzymes; the concentration of DAMP in the same compartment is a measure
Figure 1. Localization of sites of DAMP accumulation in insulin, glucagon, and somatostatin-secreting cells. Rat islet of Langerhans was incubated with 30 μM DAMP at 37°C for 30 min as previously described (38). The islet was then fixed, embedded in lowicryl K4M, and processed to localize sites of DAMP accumulation using protein A-gold (15 nm gold) as described (38).

There are many other potential applications of PEDAC for dissecting the function of acidic compartments. (a) Acidic compartments appear to play an essential role in regulating the intracellular traffic of membrane-bound and -soluble proteins (9, 30, 46, 47). Yet most of our knowledge about this function is based on the effects of drugs that dissipate H⁺ gradients. With PEDAC, the relationship between pH and sorting of macromolecules can be determined precisely by identifying the site of sorting relative to the pH of the sorting compartment. (b) Histochemistry can be combined with PEDAC to examine the distribution of enzymes or ion gradients (e.g., Ca++) in acidic compartments. For example, there are several Golgi apparatus-associated enzymes that have an optimum activity at low pH (e.g., proteolytic enzymes [27, 55], nicotinamide adenine dinucleotide phosphatase [52], acid phosphatase [18], pyridoxal-5-phosphatase [54]). A careful examination of where these enzymes reside relative to the pH of the compartment would give clues about their site of action in the cell. (c) Monoclonal antibody technology offers the potential for developing antibodies that recognize specific protein modifications. Just as it was possible to prepare monoclonal antibodies that distinguish proinsulin from insulin, there are a variety of protein modifications (proteolytic cleavage, glycosylation, sulfation, etc.) or conformational changes (e.g., transferrin to apotransferrin) that could be detected relative to the pH of the compartment with the proper antibody.

**Regulation of pH in Acidic Compartments**

During receptor-mediated endocytosis, some receptors in coated pits carry their ligand into the cell and return to the cell surface in <9 min (9). While endosomes, lysosomes, and recycling vesicles are forming and disappearing, the lumen of each vesicle is maintained at a characteristic pH. Similarly, during exocytosis secretory proteins progressively move through compartments that have different pH. PEDAC has revealed the complexity of pH regulation in these compartments and should be useful in further investigating how regulation is achieved.

PEDAC has shown that the pH of exocytic compartments varies with the type of cell. Even though the trans-Golgi vesicles have been found to be acidic in secretory cells, the secretory vesicles that mature from these vesicles may be more acidic (e.g., insulin-secreting cells [38]) or less acidic (e.g., pancreatic acinar cells [40]). Among endocrine cells, we have found that the pH of the mature vesicle is quite variable. Fig. 1 shows a portion of a pancreatic islet that was processed by the PEDAC procedure. Three different endocrine cells are present: a glucagon, a somatostatin, and an insulin-secreting cell. Whereas the secretory vesicle in the glucagon and insulin cells had similar amounts of immunogold labeling, indicating a similar pH, the somatostatin cell had little labeling of the mature vesicles. Even within the same cell, in this case the polymorphonuclear leukocyte (Fig. 2), two different types of secretory vesicles, the specific and the azurophilic, have quite different proton concentrations.

The variation in pH among vesicles of common origin implies that the H⁺ concentration is regulated to favor the function of the compartment, although in most cases these functions have yet to be identified. One way that acidity may be controlled is by varying the number of H⁺ pumps in the vesicle membrane. This appears to be the way that proton transport is regulated at the surface of certain epithelial cells.
latter molecular probes that are designed to reveal the ionic compartmentation within cells.

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Figure 2. Distribution of DAMP in azurophilic granules (ag) versus specific granules (sg) of human polymorphonuclear leukocytes. Polymorphonuclear leukocytes obtained from peripheral blood and incubated with 30 μM DAMP for 30 min at 37°C before processing for indirect protein A-gold (15 nm gold) localization of DAMP as described (38).

(1). Therefore, at different stages in the life of an endocytic or exocytic vesicle, the number of proton ATPase units may be increased or decreased to achieve the desired pH. With suitable antibodies to the proton ATPase, the relationship between the number of pumps in the membrane and the relative magnitude of the H⁺ gradient could be determined with PEDAC.

Most likely pH regulation in acidic compartments is a complex process that involves multiple molecular events. We have tried to illustrate one way that PEDAC might be useful for distinguishing among different mechanisms. Although biochemical and molecular techniques will be needed to dissect the regulatory events in isolated organelles, PEDAC offers the opportunity to test viable hypotheses in the living cell.

There is much more to learn about how the pH of these compartments is regulated and why. There may even be certain diseases where the pH of either endocytic or exocytic vesicles is not properly regulated. Abnormal regulation could be a primary cause of a pathology or be the consequence of some other defect in cell function. PEDAC offers the interested investigator an opportunity to compare the distribution of acidic compartments in normal and diseased tissue.

Conclusion

Vital pH indicators provide a unique perspective of how acidic compartments function in living cells. With PEDAC it is now possible to obtain high resolution, quantitative information that should contribute substantially to our knowledge about this interesting intracellular compartmentation. Hopefully DAMP will be joined by other specially formu-
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