A Biophysical Characterization of Phagolysosome Acidification

by

Benjamin Ethan Steinberg

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Abstract

Specialized cells of the innate immune system, such as macrophages, employ lysosomal enzymes, together with cationic peptides and reactive oxygen intermediates, to eliminate invading microorganisms ensnared within phagosomes. The effectiveness of this impressive armamentarium is potentiated by the acid pH generated by the vacuolar-type ATPase (V-ATPase). The determinants of the luminal pH of phagosomes and of the lysosomes they fuse with are not completely understood, but the V-ATPase is known to be electrogenic and net accumulation of protons requires charge compensation. For this reason, counter-ion pathways are thought to serve a central role in the control of acidification. It has generally been assumed that a parallel anion influx accompanies proton pumping to dissipate the voltage that tends to build up. In fact, impaired chloride channel activity in cystic fibrosis has been proposed to underlie the defective phagolysosome acidification and microbial killing reported in lung macrophages. In the first part of this thesis, I devised methods to dialyze the lumen of lysosomes in intact cells, while monitoring lysosomal pH, in order to assess the individual contribution of counter-ions to acidification. Surprisingly, anions were found to be completely dispensable for proton pumping, whereas the presence of permeant cations in the lysosomal lumen was essential. Accordingly, defects in lysosomal anion permeability cannot explain the impaired microbicidal capacity of phagocytes in cystic fibrosis. Even though counter-ion permeation pathways exist,
dissipation of the electrical contribution of the V-ATPase may not be complete. If present, a transmembrane potential would alter the rate and extent of proton accumulation in phagosomes and lysosomes. However, no estimates of the voltage across the phagosomes were available. To overcome this deficiency, in the second part of this thesis, I describe a noninvasive procedure to estimate the voltage across the phagosome using fluorescence resonance energy transfer. This novel approach, in combination with organellar pH measurements, demonstrated that proton pumping is not limited by counter-ion permeability.
Acknowledgments

At the outset of my training, the endpoint felt infinitely distant and largely inaccessible. Had it not been for the help of many, I would not have reached this target.

First and foremost, I thank my supervisor and mentor, Dr Sergio Grinstein, for inviting me into his lab. None of the work in this thesis would have been possible without his oversight, patience, and understanding. I could not have asked for a better educator. Throughout my training, he particularly emphasized the quantitative and physical nature of biology, encouraging me to add the tools of the physical sciences to my developing cell biological toolbox. Not only did this help fuel my excitement for science at large, but showed me that a biophysicist can successfully navigate through the world of cell biology. His skill as a scientist is unquestionable, and his probing, yet pragmatic, approach to cell biology is something to which I will forever aspire. Finally, it would be an injustice (however slight) not to also acknowledge his generous gift of new moccasins to replace the old and unsightly pair that I proudly wore when I first entered the lab.

Through their perceptive questions and suggestions, my committee members, Drs Andras Kapus and David Bazett-Jones, helped guide much of the work presented here. Without their advice, my work could not be as complete. For their great insight, support and encouragement, I will be forever grateful. Similarly, Dr Gregory Downey helped ensure a successful start to my graduate training through his discerning advice and strong example.

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To my family – mom, dad, David, Rebecca, Doug, Michael, and Dindi – your support has been the foundation of every success to which I can lay claim. Thank you for the encouragement and, of course, the phone calls.

Lastly, I leave my deepest thanks to Janice, to whom I owe more than I can possibly say.
Data Attribution

I would like to acknowledge Alexandre Brodovitch for performing some of the lysosomal steady state pH, reacidification, and null-point cation determinations presented in Figures 2.1, 2.2, and 2.4, respectively. In addition, Dr Mariana Vargas-Caballero advised me on and helped carry out the electrophysiological plasma membrane voltage determinations by current clamp presented in Figure 3.5.
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<th>Description</th>
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<tbody>
<tr>
<td>ARNO</td>
<td>ARF nucleotide-binding-site opener</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>Bib</td>
<td>Big brain</td>
</tr>
<tr>
<td>bzATP</td>
<td>2’,(3’)-O-(4-benzoylbenzoyl)adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>CCA</td>
<td>Concanamycin A</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CIC</td>
<td>Chloride channel</td>
</tr>
<tr>
<td>COP</td>
<td>Coatamer protein</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell-penetrating peptide</td>
</tr>
<tr>
<td>DACCA</td>
<td>7-Diethylaminocoumarin carboxylic acid</td>
</tr>
<tr>
<td>DiBAC&lt;sub&gt;4&lt;/sub&gt;(3)</td>
<td>Bis-(1,3-dibutylbarbituric acid)trimethine oxonol</td>
</tr>
<tr>
<td>DiBAC&lt;sub&gt;4&lt;/sub&gt;(5)</td>
<td>Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol</td>
</tr>
<tr>
<td>DiSBAC&lt;sub&gt;2&lt;/sub&gt;(3)</td>
<td>Bis-(1,3-diethylthiobarbituric acid)pentamethine oxonol</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GPN</td>
<td>Glycyl-phenylalanine-β-naphthylamide</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LBPA</td>
<td>Lysobisphosphatidic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucammonium</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>pmf</td>
<td>Proton-motive force</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RAVE</td>
<td>Regulator of acidification of vacuoles and endosomes</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SNARF</td>
<td>Seminaphtharhodafluor</td>
</tr>
<tr>
<td>sRBC</td>
<td>Sheep red blood cell</td>
</tr>
<tr>
<td>TMA</td>
<td>Trimethylamine</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar-type ATPase</td>
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Chapter 1

1 Introduction

1.1 Phagocytosis: an overview

Upon infection, the host immune system engages with the invading microorganism, its two major branches – the innate and adaptive immune systems – working in concert to clear the infection. Innate immunity represents the more primitive branch, responding in a non-specific fashion to a wide variety of pathogens. Its components are constitutively present and thus readily mobilized to sites of invasion.

Specialized cells of the immune system, such as macrophages and neutrophils, are the first cells recruited to the site of infection where they function as innate sentinels. Dendritic cells are also present, serving as important players in antigen presentation for ensuing adaptive responses. These cells engage and actively engulf invading microorganisms into an intracellular vacuole by a process termed phagocytosis (1, 2). Inside this vacuole, also known as the phagosome, the invading organisms are killed and degraded by an array of microbicidal and digestive mechanisms.

Phagocytosis, a crucial component of innate immunity, can be conceptually broken down into two modules: the formation of the nascent vacuole containing the ingested particle (phagosome formation), and its subsequent evolution into a degradative compartment (phagosome maturation) (Figure 1.1). Phagosome formation is initiated by the cross-linking of plasma membrane receptors following engagement with their cognate ligands on the surface of the microbe. In turn, receptor cross-linking initiates a complex signaling sequence that culminates with the actin-dependent extension of pseudopods around the particle. The nascent phagosome is formed upon complete enclosure of the particle by the membrane of the advancing pseudopods.

The ensuing maturation program is required because the nascent phagosome is not effectively microbicidal: its contents, with the exception of the prey itself, represent a sampling of the innocuous extracellular milieu and its membrane is derived from the plasmalemma. Consequently, the phagosomal membrane and luminal contents must undergo considerable
remodeling to transform the initially inert environment into a microbicidal one. This maturation process not only helps quell infection, but also generates and routes antigens for presentation on MHC molecules in order to activate the adaptive branch of the immune system (3).

Phagosome maturation is generally considered to involve primarily a complex sequence of fusion and fission events with sub-compartments of the endocytic pathway (Figure 1.1). In many ways, phagosome maturation recapitulates the progression of cargo along the endocytic pathway, with the added advantage that the size of the vacuole enables detailed and unambiguous tracking of the maturing compartment by light microscopy. Characteristic molecular markers used to identify the stage of the maturing phagosome are described below and listed in Table 1.1. During and/or immediately after phagosome closure, the phagosome fuses with early endosomes, acquiring Rab5 and early endosome antigen 1 (EEA1) (4). Through fission events and subsequent interactions with late endosomes, the phagosome divests itself of the early endosomal markers and acquires a late endosome-like phenotype. Late phagosomes are positive for lysobisphosphatidic acid (LBPA), Rab7, and lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2) (2). Ultimately, the organelle fuses with lysosomes to form phagolysosomes. These phagolysosomes represent the decisive degradative compartment, having amassed a potent antimicrobial armamentarium consisting of hydrolytic enzymes, cationic peptides, the NADPH oxidase enzyme complex, and a markedly acid luminal fluid (2). The ability to acidify its contents is fundamental to phagosome physiology and is the primary focus of this thesis.
Figure 1.1 Stages of phagosome formation and maturation. Six stages of phagosomes are shown: I, particle engagement; II, phagocytic cup formation; III, nascent phagosome (1-2 minutes after sealing); IV, early phagosome (2-10 min after sealing); V, late phagosome (10-30 minutes after sealing); VI, phagolysosome (>30 minutes after sealing). As the phagosome matures, it undergoes multiple sequential interactions with early endosomes (EE), late endosomes (LE), and lysosomes (Ly). Adapted from Figure 1 in (5).

<table>
<thead>
<tr>
<th>Maturation Stage</th>
<th>Marker</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early phagosome</td>
<td>EEA1, Rab5, PI(3)P</td>
<td>(4)</td>
</tr>
<tr>
<td>Late phagosome</td>
<td>Rab7, Rab9, LAMP1, LAMP2, LBPA</td>
<td>(2)</td>
</tr>
<tr>
<td>Phagolysosome</td>
<td>LAMP1, LAMP2, fluid-phase markers chased to lysosomes (e.g. ≥2 hrs)</td>
<td>(2)</td>
</tr>
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</table>

Table 1.1 Molecular markers of early and late phagosomes and phagolysosomes.
1.2 Phagosome acidification

In addition to directly affecting bacterial growth, acidification enhances the effectiveness of the other microbicidal components. For example, various hydrolytic enzymes are exquisitely pH sensitive, requiring acidic conditions for optimal activity (6, 7). Proton translocation also allows for the persistence of NADPH oxidase activity (8-10) and provides a substrate for the generation of reactive oxygen species (ROS) within the phagocytic vacuole (11).

The luminal content, which upon phagosome sealing is composed of trapped extracellular fluid, is initially slightly alkaline. Subsequently, the organelle undergoes a gradual acidification, typically reaching pH values less than 5.5 (12-14). The degree of acidification can vary greatly between neutrophils, macrophages and dendritic cells. Macrophage phagosomes display a truly profound and rapid acidification (14), whereas neutrophil phagosomes initially alkalinize, then undergo a modest acidification (12, 15). It has been hypothesized that varying rates of proton consumption by products of the NADPH oxidase account for the disparity in luminal pH between cell types. This was initially suggested for neutrophils (15) and was more recently implicated in the modulation of phagosomal pH in dendritic cells (16). In the latter cells, a rapid acidification would otherwise lead to excessive antigen degradation, rendering it ineffective for presentation. By recruiting active NADPH oxidase complexes to the phagosome, dendritic cells are able to temper the acidification to effectively process and subsequently present phagosome-derived peptides on their surface (16, 17). This mechanism of phagosomal pH modulation is not, however, entirely straightforward. For example, the activity of the oxidase leads to the generation of cytosolic protons that can be translocated into the phagosome, as described below in Section 4.3.1 of the Discussion. In this way, the oxidase can exert both acidifying and alkalinizing challenges to the luminal pH of the phagosome.

Defects in phagosome and endosome acidification may be impaired in some disease states, which may contribute to disease pathology. A prime example is cystic fibrosis (CF), an autosomal recessive genetic condition caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Individuals with CF suffer from mucosal obstruction of various exocrine glands, but lung disease is the major source of morbidity and mortality (18). The lungs of patients with CF are characterized by a complex and heightened state of infection and inflammation (19). Although the detailed mechanism of disease is not clear, a variety of
studies have implicated aberrant organellar acidification as the source of lung pathology (20-25). In one study, the phagolysosomes of alveolar macrophages deficient in CFTR were found to remain alkaline, thereby decreasing the capacity of these cells to clear a bacterial challenge (21). The lung disease seen in CF was accordingly attributed to the resulting impairment of lysosome function. This mechanistic interpretation of CF pathobiology, however, has since been challenged (26). A thorough analysis of lysosome acidification in CFTR-deficient cells is presented in Chapter 2 of this thesis. More recently, impaired endosome acidification in CF was proposed to generate aberrant ceramide metabolism in CF lung tissue, ultimately leading to constitutive pulmonary inflammation, death of respiratory epithelial cells, and increased susceptibility to severe *Pseudomonas aeruginosa* infections (25). While the underlying mechanism of disease in CF has yet to be fully elucidated, the above reports underscore the fundamental position of luminal acidification in phagosomal and endosomal physiology.

Luminal acidification has been traditionally regarded as a functional consequence of maturation, creating the hostile environment that suppresses pathogen growth. More recently, however, acidification has been appreciated to be an integral step of the maturation sequence. The notion that acidification is not just a simple consequence but also a determinant of phagosome maturation was prompted by the finding that dissipation of the transmembrane pH gradient with weak bases can attenuate phagosome-lysosome fusion while promoting phagosome-endosome interactions (27, 28). These findings are reminiscent of reports that a variety of agents that disrupt pH gradients interfere with membrane traffic at multiple stages of the endocytic and secretory pathways. The delivery of cargo to lysosomes appears to be particularly sensitive to dissipation of pH gradients (29, 30). The wide variety of intracellular effects is not entirely unexpected, since all protocols used to date to manipulate pH indiscriminately affect all acidic compartments in the cell. The promiscuity of these pharmacological agents confounds the interpretation of such experiments and precludes the assignment of a precise organellar target.
1.3 Mechanism of phagosome acidification: the vacuolar-type ATPase

The phagosomal acidification process itself is dependent on the V-ATPase (31), a large multimeric enzyme complex that associates with the limiting membrane of the nascent phagosome. The V-ATPase is an active pump that transforms the energy of ATP hydrolysis into the movement of protons across the phagosome membrane. The enzyme consists of a cytoplasmic V₁ sector that hydrolyses ATP, and the transmembrane V₀ sector through which protons flow as shown in Figure 1.2. The energy released from ATP hydrolysis is transduced into the rotation of the ring structure of the V₀ domain, resulting in the displacement of protons across the phagosome membrane.

The V₁ domain is composed of eight different subunits present with a defined stoichiometry of A₃B₃C₁D₁E₂F₁G₂H₁₋₂ whereas the V₀ domain contains six different subunits a₁c₄c’₁c’’₁d₁e₁ (32). A hexamer made from alternating A and B subunits (A₃B₃) form the ATPase module, with three nucleotide-binding sites positioned at the interface between A and B subunits. The hexamer is connected to the proton permeation pathway by two stalks. The central stalk, formed by the D and F subunits, attaches to a ring of hydrophobic subunits (c, c’, and c’’ of V₀) via subunit d (33). Together, subunits D, F and d along with the proteolipid ring, function as a rotary complex. A peripheral stalk (the N-terminal domain of the a-subunit along with the C, E, G, and H subunits of V₁) attaches to the A₃B₃ hexamer forming a mechanical stator.

The c, c’, and c’’ subunits of V₀ are transmembrane proteins, each with a buried carboxyl group essential for proton translocation located near the middle of the bilayer. Subunit a has a buried arginine residue also essential for proton transport (34) as well as two hemi-channels that span half the bilayer. One of the hemi-channels faces the cytosol while the other the organelle lumen. In this way, they allow protons to both reach and leave the buried carboxyl groups on the proteolipid subunits in a coordinated fashion that forms the basis of proton translocation by the V-ATPase (35).
**Figure 1.2** Structural organization of the V-ATPase. The V-ATPase is composed of a peripheral V₁ domain (shown in orange and yellow) and an integral V₀ domain (shown in shades of blue). The hexamer of alternating A and B subunits make up the ATP hydrolysis catalytic core. The V₀ sector includes a proteolipid ring structure comprising the c, c’, and c’’ subunits alongside subunits a and e. The central stalk, composed of subunits D and F from V₁ and subunit d from V₀, and the peripheral stalk containing subunits C, E, G, H and a, bridge the V₁ and V₀ sectors. Proton translocation occurs through two hemi-channels in subunit a that coordinate with the proteolipid ring, as described in the main text. The schematic is adapted from the model presented in (35), based on homology to the F-ATPase and electron microscopy and cross-linking studies.
The catalytic cycle of the V-ATPase is thought to employ a rotary mechanism (36-38). ATP hydrolysis drives the rotation of the proteolipid ring relative to the a-subunit, which is held stationary by the peripheral stalk (or stator). This allows protons to enter through the cytosolic-facing hemi-channel in the a-subunit and protonate one of the buried carboxyl groups in the proteolipid ring. As the ring rotates, the protonated carboxyl group is then brought to the luminal hemi-channel. Once there, the buried arginine residue of the a-subunit stabilizes the carboxyl group in its charged form, thereby releasing the proton into the luminal hemi-channel from where it reaches the luminal space. The rotation of the protonated carboxyl group towards the arginine residue of the luminal hemi-channel brings the carboxyl group of the next proteolipid subunit in contact with the cytoplasmic hemi-channel thereby perpetuating the catalytic cycle and vectorially driving protons into the organelle lumen (35, 39). Based on the three nucleotide binding sites of V_1 and the six to ten protonatable sites in the V_0 proteolipid ring, the H^+/ATP stoichiometry for the V-ATPase is predicted to be between 2 and 3.3 (35), which is in close agreement with the measured value in isolated yeast vacuoles (40). Of note, the macrolide antibiotics bafilomycin A_1 and concanamycin, specific inhibitors of V-ATPase catalysis and rotation, target the c-subunit at the interface between the a-subunit and the proteolipid ring (41, 42). Binding of the inhibitors prevents rotation of the rotary complex, which obstructs any further proton translocation.
1.4 Role of the V-ATPase and luminal acidification in phagosome maturation

Despite the fact that alterations in luminal pH elicit a markedly impaired vesicular traffic, very little is known at the molecular level about the underlying pH-sensitive mechanisms. One of the events altered by the dissipation of luminal acidification is the assembly of coated vesicles that mediate fission from early endosomes. Association of endosomal coatamer proteins (COPs), β- and ε-COP, is necessary for formation of intermediate transport vesicles believed to deliver cargo to late endosomes (43). An inability to form carrier vesicles because of insufficient COP recruitment may explain the pronounced tubulation of early endosomes (44, 45) and the block in late endosome delivery observed in cells treated with inhibitors of acidification. Membrane association of the aforementioned endosomal COPs is regulated by small GTPases, particularly ADP-ribosylation factor (ARF) isoforms (46). It is noteworthy that association of ARF1 with the membrane is itself sensitive to the luminal pH (46).

The effectors that are modulated by the luminal pH of acidic compartments are themselves located on the cytosolic face of organelles. How the occurrence of an internal pH change is transmitted to proteins associated with the cytosolic leaflet is not clear, but the existence of a transmembrane information transducer is implicit. Recent evidence points to the V-ATPase, the enzyme complex itself responsible for proton translocation into the organelle lumen, as the possible transmembrane pH sensor (47, 48). ARF6 and ARF nucleotide-binding-site opener (ARNO; a nucleotide exchange factor of ARF6) are recruited to early endosomes in a pH-dependent fashion (49) through direct interactions respectively with the c-subunit and a2-subunit of V0, the membrane embedded domain of the V-ATPase (47). Disruption of these interactions interrupts vesicular traffic along the endocytic pathway (47). The functional significance of the tripartite complex between the V-ATPase, ARNO and ARF6 remains unclear, although it has been proposed that the interaction between the small GTPases and proton pump may modulate disassembly and reassembly of different sectors of the proton pump (32) (Figure 1.2). Moreover, it is not certain that ARF and COP are the sole or even the primary targets of acidification. It is reasonable to speculate that additional molecular mechanisms must also be involved because additional steps in the endocytic pathway, not known to require ARF, ARNO, or COP, are also drastically altered by the manipulation of pH. New evidence suggests that the activation state of
Rab5, a small GTPase involved in the regulation of early endosome homotypic fusion, is also regulated by the luminal pH (50). Dissipation of pH gradients in macrophages leads to the formation of multiple enlarged vacuoles positive for Rab5 that presumably results from homotypic fusion of early endosomes (K.K. Huynh and S. Grinstein, unpublished observation). In this case, luminal acidification serves as a timer that signals the termination of Rab5 activity and its removal from early endosomes or phagosomes thereby allowing their transition into the later stages of maturation (50).

Emerging data suggest that the proton translocation machinery may also have a direct physical role in the regulation of vesicular fusion along the endocytic pathway. A recent study characterizing the phagocytosis of apoptotic neurons by microglia in the brain tissue of live zebrafish implicated the a1-subunit of the V-ATPase in the progressive fusion of internal vesicles with the phagosome (51). Knockdown of the a1 isoform did not affect the acidification status of internal vesicles compared to wildtype cells; however, it did result in defective heterotypic fusion between the phagosome and lysosomes leading to the accumulation of phagosomes and undigested material within the cells (51).

This evidence parallels previous work that invoked the V-ATPase as a participant in membrane fusion events across the exocytic pathway. In yeast, the V₀ sector was shown to contribute to membrane fusion reactions required for vacuole biogenesis independently from its function in generating a pH gradient (52-54). While the proposal that membrane fusion is accomplished through trans-complex formation of two apposing V₀ sectors remains controversial (32), further evidence for V-ATPase involvement in vesicle fusion is accumulating in other organisms. For examples, various subunit isoforms of the V₀ domain are thought to participate in synaptic vesicle fusion in *Drosophila melanogaster* (55) and vesicular protein secretion in *Caenorhabditis elegans* (56). Likewise, mice lacking specific isoforms of V₀ subunits that normally target insulin-containing secretory vesicles are unable to secrete insulin in response to glucose even though acute pharmacological inhibition of V-ATPase proton pumping does not affect insulin secretion in pancreatic β-cells (57).
1.5 Regulation of the V-ATPase

As the V-ATPase is a primary player in organellar proton transport, it provides an important point of regulation for the acidification process. Multiple mechanisms of controlling V-ATPase activity have been identified and are summarized below and illustrated schematically in Figure 1.3.

One of the first mechanisms of control to be described was the reversible formation of a disulfide bond between conserved cysteine residues in the A subunit at the catalytic site of \( V_1 \) (58-60) (Figure 1.3A). This reaction covalently locks the ATPase into a closed conformation that prevents both the release of the hydrolysis products and the rebinding of a new ATP molecule. In turn, the cleavage of the disulfide is thought to be mediated through the thio-disulfide exchange with another cysteine residue in the A subunit (39). An estimated 50% of the V-ATPase in clathrin-coated vesicles in brain tissue is found in the disulfide-bonded state (60). Nevertheless, the extent to which this modality is employed by cells to regulate endosomal or phagosomal acidification is unclear.

The proton pump density can equally modulate the rate and magnitude of organellar acidification (Figure 1.3B). This mode of regulation is most commonly seen at the apical membrane of epithelial cells, such as the clear cells of the epididymis, which maintain the low epididymal luminal pH needed to keep sperm in a dormant state (61), and renal alpha-intercalated cells of the distal tubule and collecting duct important in acid secretion and acid-base balance in the kidney (62). In clear cells, as the level of cytoplasmic bicarbonate rises, a soluble adenylyl cyclase is activated. By an unknown mechanism, the resulting increase in cyclic AMP concentration, in turn, expands the plasma membrane pump density by increasing exocytosis and/or decreasing endocytosis of the V-ATPase complex (35, 61).

An initial attempt to determine phagosomal pump density found a likely lower limit of approximately 48 V-ATPase complexes per \( \mu \text{m}^2 \) in fully mature phagosome membranes containing opsonized sheep red blood cells as prey (14). Still, the extent to which V-ATPase density varies across both populations of phagosomes and individual phagosomes over time remains unknown. This is due in part to the difficulty in quantifying the number of pumps contained in individual phagosomes across the maturation process. Because of the relatively
Figure 1.3 Mechanisms of V-ATPase activity and vesicular acidification regulation. (A) The formation of a reversible disulfide bond between a highly conserved cysteine residue (yellow star) in the catalytic site of subunit A and a second conserved cysteine (yellow star) in the C-terminal domain of the same subunit prevents ATP hydrolysis and thus proton translocation. (B) Membrane V-ATPase density. In some epithelial cells, regulating the delivery of proton pumps to the cell membrane modulates acid secretion. By providing a higher pump density, an increased acid secretion can be sustained. (C) Modulation of coupling efficiency between ATP hydrolysis and proton transport. In the extreme case of a completely uncoupled complex, ATP hydrolysis does not result in any proton current. (D) Reversible dissociation of the $V_0$ and $V_1$ sectors. This physical uncoupling is used to restrict lysosomal acidification capacity in immature dendritic cells. The subunits reassemble in the mature cell to allow for complete antigen processing (63). (E) Membrane counter-ion permeability. Electroneutrality can be maintained through the parallel influx of anions alongside protons, or the efflux of cations through distinct channels or transporters. Potassium and chloride movement are illustrated, although other ions may equally serve as the counter-ions. Notably, this last modality represents a potential mechanism of vesicular acidification regulation and not a direct mechanism of V-ATPase activity modulation.
small volume of the phagosome, it is somewhat unlikely that pH regulation is achieved by adjusting the number of resident ATPases.

Changes in the coupling efficiency of proton transport and ATP hydrolysis, which could impact on phagosome acidification, represent a third mechanism of control (Figure 1.3C). As described above, an approximately 2H+/ATP coupling ratio is predicted based on subunit stoichiometry. Evidence in the literature, however, suggests that partial uncoupling of proton translocation from ATP hydrolysis, also termed slippage, can occur (64, 65). Its impact on organellar acidification would undoubtedly depend on the degree of uncoupling. At one extreme, where the V₀ and V₁ domains are completely uncoupled, ATP hydrolysis would not result in any proton transport and thus no acidification. Moreover, certain mutations in the A subunit can in fact increase the coupling efficiency (64), which implies that the wildtype V-ATPase is not optimally coupled. Of particular note, patch-clamp analyses of isolated yeast vacuoles suggest that the coupling ratio is itself dependent on the pH gradient across the vacuolar membrane. The ratio was inferred to be approximately 2 H⁺/ATP at pH gradients near 4, but increased toward 4 H⁺/ATP as the gradient was collapsed to zero (40). It is reasonable to envision the V-ATPase more efficiently pumping protons into the nascent phagosome lumen in order to generate a significant pH gradient, only to have its activity tempered through uncoupling as the lumen becomes increasingly acidic. Other physiological signals that alter V-ATPase coupling have yet to be identified.

Another well-defined mechanism for controlling V-ATPase activity is the reversible dissociation of the V₁ and V₀ sectors (Figure 1.3D). This process has been most fully characterized in yeast and insect cells, where the dissociation is activated by nutrient depletion seemingly to conserve intracellular ATP stores (66). Interestingly, the V-ATPase dissociation response to nutrient depletion does not involve many of the signalling pathways typically activated by glucose depletion (67). To date, the identity of the signal transduction pathway that leads to V-ATPase dissociation remains unknown; although recent work suggest that either the PI-3 kinase and protein kinase A pathways may be involved (68, 69). In yeast, the dissociation occurs rapidly, on the order of a few minutes, reversibly and without the synthesis of new protein (70). This dissociation – but not reassembly – is dependent on an intact microtubule network (71). On the other hand, reassembly – but not dissociation – of the V₁ and V₀ sectors upon nutrient replenishment, as well as during normal biosynthetic complex assembly, requires a
heterotrimeric protein complex termed RAVE for the regulator of acidification of vacuoles and endosomes (72, 73). Together, these data suggest that the two processes of dissociation and reassembly are independently controlled.

Work by the Forgac and Kane laboratories has also investigated the impact of α-subunit isoforms and cellular environment on in vivo dissociation of the two sectors of the V-ATPase. Targeting of V-ATPase to different cellular membranes depends on the isoform of the α-subunit (35). For example, in yeast there are two isoforms of the α-subunit: Vph1p targets the yeast vacuole and Stv1p targets the late Golgi compartment (39, 74). V-ATPases containing Vph1p at the vacuole dissociate upon glucose removal, whereas V-ATPases with Stv1p at the Golgi do not (75). However, if V-ATPase complexes containing Stv1p are made to target the vacuole, then they too are able to dissociate upon nutrient depletion, suggesting a dependence of dissociation on intracellular environment. More recent work has shown that V-ATPases made to target the same intracellular membrane, independent of the α-subunit isoform, behave similarly in terms of glucose-dependent dissociation (76). It follows that the cellular – and perhaps intra-organellar – environment may be the principal determinant of the extent of V1-V0 dissociation, although the specific factors modulating dissociation remain unknown. Importantly, an acidic yeast vacuole has been shown to be permissive for dissociation, in contrast to vacuoles alkalinized by treatment with the weak base chloroquine (77). This data implicate luminal pH as one factor that controls sector dissociation. Notably, a recent report suggests that an increase in V1-V0 association from the early to the late endosome helps establish the progressively acidic endosomal lumen (78). Given its putative dependence on the acidification status of the organelle, it will be interesting to determine the extent by which the reversible dissociation of the complex modulates endosomal and phagosomal pH.

More germane to this discussion is the defined role for this mode of regulation in the endocytic protein degradation pathway of dendritic cells (63). The ability of dendritic cells to present antigens to T cells is tightly controlled by their maturation status, a designation that distinguishes between immature and mature phenotypes. The immature dendritic cell proficiently captures antigen by endocytosis but has a limited ability to stimulate the adaptive immune response, whereas the mature dendritic cell does not avidly internalize antigen but is highly immunogenic (79). The different capacities for antigen processing are attributed to enhanced proteolytic activity within the lysosomes of mature dendritic cells. The differential proteolytic capacities
result from a restricted lysosomal acidification in the immature dendritic cell (63). Upon maturation, lysosomal V-ATPase activity increases through the recruitment of additional $V_1$ sectors to the membrane (63). In this way, the low proteolytic capacity of immature dendritic cells allows a considerable fraction of internalized antigen to escape complete degradation thereby enabling their conversion to peptide-MHC complexes. This phenotypic switch highlights the capacity of the reversible dissociation of the V-ATPase sectors to modulate lysosomal acidification, protease activation and the ensuing protein degradation and antigen presentation in dendritic cells.
1.6 Mechanism of phagosome acidification: other players

While phagosome acidification and its regulation represent essential components of phagosome biology, other than the involvement of V-ATPases, the determinants of the phagosomal pH are not well understood. It is nevertheless clear that factors other than the proton pump must be involved. Because the V-ATPase vectorially moves charges across the membrane, its catalytic cycle leads to the generation of a substantial voltage across the phagosome membrane (positive inside). As the electrical potential difference builds up, it becomes increasingly difficult for the V-ATPase to translocate protons into the lumen, causing the process to slow down and eventually cease if left uncompensated. To prevent this self-limiting behaviour, counter-ions permeate the membrane through conductive pathways, tending to dissipate the electrogenic voltage generated by the V-ATPase. Such shunting can be mediated by the influx of cytosolic anions or, alternatively, the extrusion of luminal cations (Figure 1.3E). In this capacity, counter-ion movement is crucial for the proton pump to generate a measurable pH gradient. As the phagosome acidifies, the developing chemical gradient increasingly opposes further accumulation of protons inside the organelle lumen. The proton-motive force (pmf), a thermodynamic parameter representing the total free energy stored within the electrochemical proton gradient, encompasses the combined contribution of the electrical and concentration gradients. The electrochemical potential energy of the gradient is given by

\[ \mu_H = z F \Delta \Psi - 2.303 R T \Delta pH \]

where \( z \) is the valence of a proton (+1), \( F \) and \( R \) are the Faraday and gas constants, respectively, and \( \Delta \Psi \) and \( \Delta pH \) respectively represent the electrical and pH gradients. In this form, the gradients are written as inside – outside (or equivalently for the phagolysosome, lumen – cytosol). The pmf is then computed in volts as

\[ pmf = \frac{\mu_H}{z F} = \Delta \Psi - 2.303 \frac{R T}{z F} \Delta pH \]

The proton buffering capacity of the organelle lumen and the counter-ion permeability of phagosome membrane largely determine the fractional contribution of the pH gradient and the transmembrane electrical potential, respectively, to the pmf. Regardless of the distribution of
the electrical and chemical gradients, the maximal \( pmf \) that the V-ATPase can generate is equal to the amount of energy furnished by ATP hydrolysis. In the intracellular environment, this corresponds to approximately 58 KJ/mol of ATP (80); however, if we assume a coupling stoichiometry of 2 protons per ATP, then the \( pmf \) cannot exceed 29 KJ/mol. To better illustrate the importance of counter-ion flux in phagosomal acidification, consider the electrical component of the \( pmf \). If we conservatively ignore the generation of a pH gradient, at what voltage will the V-ATPase stop transporting protons? From thermodynamic considerations it follows that the energy of ATP hydrolysis is sufficient to generate a transmembrane potential of approximately +300 mV. Importantly, because of the relatively low capacitance of biological membranes (approximately 1 \( \mu \)F cm\(^{-2} \)), very few protons need to be translocated across the phagosomal membrane to generate this substantial voltage. For a spherical phagosome with a diameter of 4 \( \mu \)m and thus a surface area of approximately 48 \( \mu \)m\(^2\), only 1 x 10\(^6\) monovalent ions must be separated across the membrane to produce a 300 mV potential. Even in this artificial scenario, when considering the substantial luminal proton buffering capacity of the phagolysosome (on the order of mM), this amount of proton transport is negligible and not sufficient to generate the large pH gradients seen in the mature phagolysosome (12-14). Moreover, assuming a reasonable turnover rate of 200 ATP/s implies that if the measured 2300 V-ATPases (14) were functioning on the phagosome in isolation, they would generate a prohibitive voltage in less than 2 s. This calculated time scale is considerably abbreviated compared to the known kinetics of phagosome acidification, which occurs on the order of minutes (13, 14, 31, 81).

Notably, while the above provides a basis to appreciate the importance of a counter-ion conductance to accompany proton translocation by the V-ATPase, it implicitly considers the system in (thermodynamic) equilibrium states. This need not be the case in the phagolysosome where dynamic steady states may exist, as further discussed in Chapter 3. It should be apparent, however, that due to the electrogenic nature of the V-ATPase, variations in membrane counter-ion permeability could result in differences in the rate and magnitude of luminal acidification (Figure 1.3E), thus representing an additional and oftentimes neglected mechanism of acidification regulation.

The nature of the counter-ion permeability that allows for proton translocation by the V-ATPase has been investigated to some extent. Cytosolic anions, mainly Cl\(^-\), and luminal cations
represent the two possible sources of counter-ions (Figure 1.3E). The involvement of a parallel Cl⁻ current alongside proton translocation to maintain electroneutrality has long been suspected. A possible role for Cl⁻ was first established \textit{in vitro} using purified endomembrane compartments, including preparations of Golgi (82), endosomes (83-88), and secretory vesicles (89-92). In all cases, a parallel anion (Cl⁻) pathway was required to allow for the establishment of a transmembrane pH gradient. More recently, fluorescence-based organellar Cl⁻ determinations have directly implicated this abundant cytosolic anion as the principal counter-ion in Golgi and endosomal acidification in live cells (93, 94). As such, Cl⁻ remains the primary candidate for dissipating the transmembrane potential across the phagolysosome membrane. The roles of cytosolic Cl⁻ and luminal cations as dissipating counter-ions in lysosome acidification are analyzed in Chapter 2. The specific channels or transporters, however, which support the counter-ion flux have yet to be identified. Similarly, consideration of the osmotic consequences of Cl⁻ influx on organellar volume is wanting. A more general examination of V-ATPase counter-ion conductance in endosomes and phagosomes is included in the Discussion.
1.7 Models of V-ATPase-driven acidification

The above discussion presents a variety of models by which the phagosome could acidify. To date, the identity of the counter-ions that neutralize charge build-up in phagosomes, along with the specific channels through which they permeate, are largely unknown. Regardless, when counter-ion conductance is limiting, most of the $pmf$ generated by ATP is allocated towards the build-up of a large transmembrane electrical potential. As a result, the V-ATPase would generate only a stunted pH gradient. It follows that the progressive acidification of the phagosome reflects a shift between the electrical and chemical components of the $pmf$. In this model, a higher density of counter-ion permeation pathways could explain why mature phagosomes are more acidic than their nascent counterparts. An alternative hypothesis is that a passive proton leak current limits the ability of early phagosomes to become fully acidic. A gradual increase in proton ‘tightness’ would result in reduced leakage, allowing the V-ATPase to develop a more profound acidification. Similarly, the maintenance of organellar pH may reflect a balance between active proton transport and passive proton leak. These models need not be mutually exclusive, reinforcing the complexity of the acidification process.

As yet, the physiology of phagosome acidification remains incompletely understood. Its biological importance notwithstanding, little information is available regarding the detailed biophysical mechanism of phagosome acidification so as to build an accurate model of the acidification process. The primary difficulty has been the physical inaccessibility of phagosomes to direct measurements. Phagosomal pH determinations represent the lone exception, and have been made available through quantitative fluorescence imaging of pH sensors targeted to the phagosome lumen. Such measurements either exploit fusion of the endocytic pathway with the maturing phagosome to deliver the fluorophore to the phagosome or, more simply, covalently couple the pH probe to the phagocytic prey as described below in Sections 3.3.3 and 3.3.7 of this thesis (see also (12, 13, 81, 95) for examples).

Other important biophysical parameters, however, remain altogether elusive. These include the ionic composition of the organelle lumen and the identity of any channel, transporter or pump activities resident within the membrane. Unlike the case of protons, fluorophores responsive to other specific ion species, and that can be targeted to and remain functional within the harsh phagolysosome environment have yet to be developed. Both the luminal ion content and
membrane permeation pathways can directly impact on phagosome acidification by influencing, among other things, counter-ion fluxes and proton leak pathways. In the process, they can modulate the electrical potential difference across the phagosome membrane, a critical physical determinant of acidification.

The transmembrane voltage remains a particularly attractive target for measurement as its magnitude is integrated across the entire organelle, reflecting contributions from multiple components and influencing a broad spectrum of physiological processes. Electrophysiological approaches have traditionally been used to make membrane potential determinations across the cell plasma membrane. The complexity of accessing phagosomes within cells, however, renders them a difficult target for microelectrodes. In some special cases, plasma membrane patch-clamp analysis has been used as a surrogate for the phagosome membrane in studies of the electrogenic NADPH oxidase (see (9, 96-98) for examples). This enzyme complex is able to assemble and function in the plasma membrane of phagocytes, enabling the direct application of patch-clamp microelectrodes. The extensive membrane and luminal remodeling of the maturing phagosome render the plasma membrane a crude proxy at best for phagosome physiology. Nevertheless, from an electrophysiology standpoint, this approximation is presently the best available option. Potentiometric optical dyes that partition across all cellular membranes in a voltage-sensitive manner are similarly available for plasma membrane voltage measurements. Unfortunately, their use for organellar membrane potential determinations is limited by their prohibitively low spatial signal-to-noise ratio.

In summary, a comprehensive biophysical characterization of phagosome physiology awaits the development of quantitative measures of physical parameters within and across the phagosome. Of specific interest to this discussion, the paucity of information regarding phagosome acidification is profound, particularly with regards to the identity of the counter-ion conductance pathways of the phagolysosome that enable V-ATPase activity and the electrical status of the phagosome membrane in situ. To this end, this thesis presents work that addresses:

1. The identification of the counter-ions required for lysosome acidification; and

2. The measurement of the phagolysosomal membrane potential and its contribution to the proton-motive force.
1.8 Rationale, hypothesis, and approach

1.8.1 The identification of the counter-ions required for lysosome acidification

Based on thermodynamic considerations alone, lysosome (and phagosome) acidification necessitates a counter-ion flux, yet little is known of the identity of the conductance. As described above, chloride remains the most likely counter-ion candidate. The anion current that dissipates the voltage generated by the V-ATPase can be carried by a variety of putative channels or transporters. The chloride channel (ClC) family of proteins represents a possible source of the presumed Cl⁻ current as several of its members have been shown to support acidification through knockout experiments (99-104).

On the other hand, a recent report implicates CFTR as the counter-ion conductance for acidification of lysosomes and phagosomes in alveolar macrophages (21). Cells derived from mice with a genetic knockout of CFTR contained alkaline lysosomes (pH ≈ 6.0) relative to controls (pH ≈ 4.5) (21). These primary macrophages were viable, which is perhaps unexpected given the reported defect in acidification and the anticipated ensuing impairment of the endocytic pathway. A role for CFTR in phagolysosome acidification was found exclusively in alveolar and not peritoneal macrophages, implying that any role for CFTR as the counter-ion conductance cannot be generalized. Moreover, a direct relationship between the acidification defect in alveolar macrophage phagosomes and CF pulmonary disease progression was not carried out. Still, given the severity of lung pathology in CF, and because the therapeutic implications associated with this mechanism of disease can be far-reaching, a comprehensive evaluation is warranted.

The notion that aberrant organellar acidification in CF cells represents the underlying mechanistic basis of disease is long-standing, beginning with the observation of a mild acidification defect in the trans-Golgi, endosome, and prelysosome compartments of CF cells (20). In this study, the trans-Golgi, endosomal and prelysosomal compartments were alkalinized by 0.4, 0.5, and 0.25 pH units, respectively, in CF cells compared with wildtype controls (20). The cellular physiology of this initial “pH hypothesis”, however, has been explicitly contested by multiple subsequent studies (105-109). A thorough analysis of the work linking CFTR to the
acidification counter-ion in the phagolysosome as well as previous work dispelling CFTR involvement in acidification processes is reserved for the Discussion.

*I therefore hypothesize that cytosolic anions, namely \( \text{Cl}^- \), function as the counter-ion for lysosome acidification in macrophages; however, CFTR is not the (only) conductance through which they move to dissipate any accumulated potential.*

The approaches I can employ to address the hypothesis rely on measurements of lysosomal pH in live cells using pH-sensitive fluorescent probes specifically targeted to the compartment of interest. This microscopy-based approach allows for the evaluation of the contribution of CFTR to lysosome acidification by comparing the extent of luminal acidification between cells deficient in CFTR function and their wildtype CFTR counterparts. To eliminate CFTR activity within the lysosomes of macrophages, the channel can be inhibited pharmacologically, for instance using the specific inhibitor CFTR\text{INH-172} (110). Alternatively, alveolar macrophages can be harvested by bronchoalveolar lavage from CFTR knockout mice and compared to their wildtype littermate controls.

Independent of the involvement of the CFTR channel, the counter-ion flux required for lysosome acidification can be provided by cytosolic anions, luminal cations or a combination of the two. To evaluate the general contribution of cytosolic anions (mostly \( \text{Cl}^- \)) as counter-ions I can make use of ion substitution protocols whereby organelle acidification status is monitored following replacement of intracellular \( \text{Cl}^- \) with large inorganic ions that do not readily pass through conductive anion channels. Should cytosolic anions be required for acidification, the anion substitution will hinder or completely prevent acidification. By exchanging luminal cations with large inorganic cation substitutes and monitoring the extent of acidification the role of lysosomal cations as counter-ions can be similarly investigated.

Both scenarios are non-trivial as they require access to the intracellular environment in order to make the substitution while monitoring organellar pH and maintaining a viable cell population. This is particularly challenging for the luminal cation substitution because it demands not only access to the cytosol but also requires entry into an intra-organellar compartment. To carry out the cytosol substitution experiments I devised a protocol that allows us to reversibly permeabilize the plasma membrane and dialyze intracellular \( \text{Cl}^- \) in exchange for large anion substitutes. The cation substitution first required loading the cytosol with a bulky cation
replacement using a derivative of the anion-substitution protocol, exchanging cations instead of anions. Dialysis of the lysosome lumen was then carried out by transiently permeabilizing the lysosome membrane through swelling induced by the localized enzymatic generation of osmolytes. The degree of lysosome re-acidification, in both cases, was monitored following a transient luminal alkalinization.

In summary, using cytosolic and organellar ion substitution protocols in live cells, in conjunction with organellar pH measurements, I evaluated the contribution of cytosolic anions and organellar cations to V-ATPase proton pumping in the lysosome membrane. The specific role of CFTR was investigated using a combination of pharmacological and genetic approaches. The methodological details and results are the subject of Chapter 2 of this thesis.

1.8.2 \textit{In situ} transmembrane electrical potential difference measurements across the phagosome membrane

The permeability of the phagosome to ions that dissipate the electrogenic effect of the V-ATPase has been proposed to influence the acidification of the organelle. Validation of this notion requires an accurate measurement of the phagosomal \textit{pmf}. It follows that the electrical potential difference across the phagosome membrane represents a key biophysical parameter in the acidification process. Yet even in a relatively large organelle such as the phagosome, measurements of the phagosomal membrane potential have been refractory to classical electrophysiological analysis due to difficulty in accessing intracellular compartments with microelectrodes. This is in stark contrast to \textit{in situ} organellar pH measurements, which have been widely available for many years (111).

Indirect evidence suggests that the V-ATPase is a principal contributor to the phagosome membrane potential and that the transmembrane voltage is likely to vary over the course of the maturation pathway. Following phagosome closure, the initial phase of maturation supports high rates of acidification of 0.2-0.25 pH/min (31). In addition, the steep pH dependence of the V-ATPase pump rate indicates that higher proton translocation activity can be sustained at the alkaline pH of the nascent phagosome (13). It follows that at this early stage of phagosome maturation, the counter-ion permeability may be limiting and, as a result, the generated voltage may restrict proton transport by the proton pump. The hindered generation of a pH gradient may
be exaggerated by active Na,K-ATPase activity in the phagosome membrane, which would further generate a positive lumen. In fact, the initial rate of phagosome acidification is enhanced by inhibition of Na,K-ATPase by the glycoside ouabain (31), suggesting that the Na,K-ATPase limits proton transport through its rheogenic catalytic cycle.

In contrast, the transmembrane potential of an acidified phagosome is predicted to be minimal. The proton pumping rate is less than the counter-ion permeation capacity of fully acidified phagosomes (13). Moreover, artificially increasing the phagosome conductance with ionophores has no effect on proton translocation rates (13). The above results imply that voltage does not determine to any significant degree the magnitude of the steady state pH and that the chemical gradient is the major contributor to the pmf. These inferences, however, await validation by accurate measurements of the pmf generated across maturing phagosomes.

*Based on these considerations, I hypothesize that the V-ATPase is the primary contributor to the phagosomal membrane potential in macrophages and that the electrical component contributes more significantly to the pmf early in the course of maturation than in the later stages.*

To circumvent the technical difficulties associated with applying standard electrophysiological approaches to the phagosome, I devised a fluorescence microscopy-based approach to evaluate the phagosome membrane potential in live cells using fluorescence resonance energy transfer (FRET) as described in Chapter 3. In this system, the FRET donor is covalently attached to the phagocytic prey, while a potentiometric optical dye is used as the FRET acceptor and voltage sensor. This noninvasive technique was applied to measure the electrical component of the pmf and assess its contribution to the acidification process in live macrophages. In combination with ionophores, such experiments allowed me to determine the electrical status of the mature phagosome membrane, derive information regarding the ionic permeability of the phagolysosome, and generate a model of the acquisition and maintenance of steady-state phagosomal pH.
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Chapter 2

2  A cation counterflux is required for lysosomal acidification

2.1 Abstract

A profound luminal acidification is essential for the degradative function of lysosomes. Because the vacuolar-type ATPase (V-ATPase) responsible for proton-pumping is electrogenic, counter-ion conductive pathways are required to prevent the buildup of a membrane potential that would oppose net proton accumulation. It has generally been assumed that a parallel anion influx accompanies proton pumping, enabling acidification. Indeed, defective anion channel function in cystic fibrosis (CF) has been suggested as the mechanism underlying attenuated lysosomal acidification and impaired microbial killing (21). To assess the individual contribution of counter-ions to acidification, I devised means of reversibly and separately permeabilizing the plasma and lysosomal membranes to dialyze the cytosol and the lumen of lysosomes in intact cells, while monitoring lysosomal pH by ratio imaging. Replacement of cytosolic Cl\(^-\) with impermeant anions did not significantly alter the rate or extent of proton pumping. In contrast, the presence of permeant cations in the lysosomal lumen was required for acidification. A null-point titration method was used to estimate the luminal cation activity of lysosomes. The aggregate content of monovalent cations (50.1 mM K\(^+\) and 20.6 mM Na\(^+\)) suffices to support proton pumping. Because anions are dispensable for lysosomal proton uptake, defects in the lysosomal pH are not anticipated in CF cells. Accordingly, the lysosomes of alveolar macrophages were found to acidify normally in CFTR-deficient cells. I conclude that cations are the primary counter-ions responsible for lysosomal acidification and that defects in lysosomal anion conductance cannot explain the impaired microbicidal capacity of CF phagocytes.
2.2 Introduction

Lysosomes are the terminal compartment of the endocytic pathway. Their various acid hydrolases bestow the organelle with the degradative capacity necessary to break down proteins, lipids and polysaccharides. In certain cells, such as macrophages, lysosomes assume additional, specialized functions. Macrophages are important components of the innate immune response as they engage, internalize and destroy invading microorganisms. In addition to homeostatic degradative functions, their lysosomes contribute antimicrobial capacities by fusing with the intracellular vacuole that ensnares the internalized pathogen. Most lysosomal hydrolases are optimally active in highly acidic solutions (112), which mandates that the organellar lumen be maintained at pH values between 4 and 5. In macrophages, proton transport into phagolysosomes also favors bacterial clearance by supporting NADPH oxidase activity, promoting the generation of toxic oxygen radicals and enhancing the activity of cationic microbicidal peptides. By regulating protease activity, the pH further controls antigen presentation and subsequent adaptive immune responses.

The pronounced acidification of the lysosomal interior is generated by the vacuolar-type ATPase (V-ATPase), a multimeric enzyme complex that utilizes the energy of ATP hydrolysis to translocate protons across membranes (35). While the catalytic cycle of the V-ATPase drives proton accumulation, other factors contribute to the establishment of lysosomal acidification. The vectorial movement of protons across the membrane generates a transmembrane electrical potential difference (positive inside) that, if left uncompensated, curtails the activity of the V-ATPase and limits the magnitude of the pH gradient. To circumvent this self-limiting behavior, counter-ion conductive pathways must operate alongside the V-ATPase to dissipate the mounting voltage. Either the influx of cytosolic anions into the organelle or the efflux of luminal cations can in principle serve as an electrical shunt to facilitate acidification.

Despite their importance to lysosomal acidification, the identity of the neutralizing counter-ions and their conductive channels in lysosomes remains elusive. Studies employing pharmacological approaches alongside organellar pH measurements have largely focused on endosomes, and have implicated chloride as the principal counter-ion that dissipates the endosomal membrane potential generated by the V-ATPase. The intracellular CIC chloride channel family members CIC-3, CIC-4, and CIC-5 have been proposed as the primary endosomal chloride conductances.
ClC family members are also expressed in lysosomes, particularly ClC-6 and ClC-7 (114, 115). However, neither of these channels seems to be essential for lysosomal acidification (116, 117). More recently, the cystic fibrosis (CF) transmembrane conductance regulator chloride channel (CFTR) was proposed as the specific counter-ion conductance necessary for lysosomal acidification in alveolar macrophages (21). The lysosomes in CFTR-deficient macrophages were found to remain alkaline, thereby decreasing the capacity of these cells to clear a bacterial challenge (21). The inflammatory lung disease and excessive bacterial colonization of the airways seen in CF was implicitly attributed to the resulting impairment in lysosome function. This mechanistic interpretation of CF pathobiology, however, has since been challenged (26).

Because of their central role in lysosome biology and potential involvement in the pathogenesis of CF, I analyzed the counter-ion pathways that support lysosomal acidification in macrophages, using novel approaches to manipulate the ionic composition of the cytosol and of the lysosomal lumen in macrophages.
2.3 Materials and methods

2.3.1 Reagents

CFTR\textsubscript{INH}-172 was purchased from Calbiochem (Gibbstown, NJ). Sulforhodamine 101, LysoTracker Red, Oregon Green 514-dextran, fluorescein-dextran, and rhodamine B-dextran were from Molecular Probes (Eugene, OR). All dextrans were of 10,000 MW. Unless specified otherwise, all other reagents were from Sigma-Aldrich (Oakville, ON).

2.3.2 Cell lines and tissue culture

RAW264.7 (ATCC number TIB-71) and J774 (ATCC number TIB-67) murine macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD) and grown at 37°C under 5% CO\textsubscript{2} in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Wisent, St. Bruno, Quebec).

Bitransgenic $cftr$ null mutant ($cftr^{-/-}$) mice, expressing human CFTR in the gut under the control of the rat fatty acid-binding protein ($FABP$) promoter (strain $Cftr^{tm1Unc-Tg(FABPCFTR)}$), and their littermate controls ($cftr^{+/+}$), were obtained from the Cystic Fibrosis Animal Core at Case Western Reserve University (Cleveland, OH). C57BL/6 mice were used as additional controls. All animal protocols were approved by the animal care committee of the Hospital for Sick Children.

To isolate alveolar macrophages, the mice were sacrificed and bronchoalveolar lavage performed. For each lavage, 1 mL of cold phosphate-buffered saline (PBS) supplemented with 1 mM CaCl\textsubscript{2} and 1 mM MgCl\textsubscript{2} was instilled into the lungs by tracheal cannulation. The lavage was repeated 3 times to collect between 2 and 3 mL of fluid. The collected cells from each mouse were cultured on glass coverslips at 37°C under 5% CO\textsubscript{2} in RPMI 1640 medium supplemented with 10% FBS and a penicillin, streptomycin and amphotericin B cocktail. Alveolar macrophages were used within 3 days of harvesting.
2.3.3 Lysosomal pH measurements

Lysosomal pH measurements were carried out by ratiometric fluorescence imaging of a pH-sensitive fluorophore specifically targeted to the compartment of interest using a standard pulse-chase protocol. Cultured cells were plated on glass coverslips and grown overnight in the presence of 500 µg/mL of Oregon Green 514-conjugated dextran. The cells were then washed and the dextran chased to the lysosome by incubating the cells at 37°C for 1 hr in the presence of serum followed by 1 hr in serum-free medium prior to imaging. Where indicated, cells were incubated with the indicated pharmacological agent during the hour in serum-free medium and then maintained in its presence for the duration of the experiment.

For imaging, coverslips were placed in a Leiden chamber maintained at 37°C and mounted on the stage of a Leica DM IRB microscope. pH was measured by fluorescence ratiometric imaging using a filter wheel (Sutter Instruments, Novato, CA) to rapidly alternate between excitation filters. The cells were excited by light from an EXFO X-Cite 120 lamp (Exfo Life Sciences Group, Mississauga, Ontario) transmitted alternately through 485±10 nm and 438±12 nm excitation filters and directed to the sample using a 505 nm dichroic mirror. The emitted light was filtered with a 535±20 nm filter before being captured by a Cascade II CCD camera (Photometrics, Tucson, AZ). The filter wheel and camera were under the control of MetaFluor software (Molecular Devices, Downingtown, PA).

At the end of each experiment, an in situ calibration was performed. The cells were sequentially bathed in isotonic K⁺ solutions (145 mM KCl, 10 mM glucose, 1 mM MgCl₂ and 20 mM of either HEPES, MES or acetate) buffered to pH ranging from 4.0 to 6.5 and containing 10 µg/mL of nigericin. Images were acquired 5 min after the addition of each solution to ensure equilibration of pH across compartments. The resulting fluorescence intensity ratio (490 nm/440 nm) data as a function of pH was fit to a Boltzmann sigmoid and used to interpolate pH values from the experimental ratio data. By bathing the cells in 145 mM KCl, the K⁺ concentration gradient between the medium and the cytosol is virtually eliminated and the ionophore drives the cytosolic pH to that of the bathing medium. Nigericin also inserts into and functions within the lysosomal membrane. To fully equilibrate the lysosomal pH with that of the cytosol (and thus the bathing medium), it is assumed that there is a negligible K⁺ concentration gradient across the lysosome membrane. Based on our null-point titration K⁺ determinations (
Figure 2.4) at steady state, there is approximately a three-fold concentration difference. Were there no buffering power in the lysosome, this gradient would affect the pH measurement by approximately 0.5 (log 3) pH units. However, this error is mitigated by the lysosomal buffering power measured at 61.5 mM, which decreases the discrepancy to approximately 0.13 pH units and by the existence of K$^+$ permeation pathways within the lysosomal membrane that promote equilibration between the lysosomal and cytosolic K$^+$. 

To determine the lysosomal buffering power at steady state pH, 0.5 mM or 1 mM NH$_4$Cl in PBS was added to the medium, the change in pH monitored and the buffering power calculated as described by Roos and Boron (118).

2.3.4 Ion substitution and re-acidification protocols

Lysosomes were loaded with Oregon Green 514-dextran and baseline pH recorded. The cytosol was subsequently dialyzed by incubating the cells in a dialysis medium containing 50 mM KCl, 90 mM K gluconate, 20 mM HEPES, 10 mM glucose, 1 mM EDTA, 1 mg/mL dextran (MW 1500), 2.5 mM Na$_2$ATP and 100 μM bzATP to activate plasmalemmal P2X$_7$ receptors. Where specified Cl$^-$ was isoosmotically replaced with either gluconate or MeSO$_3$-. In all cases, the dialysis medium also contained 500 nM FCCP to alkalinize the lysosomes. Following 15 min of cytosol dialysis and lysosome alkalinization, re-acidification was initiated by washing the cells 3-fold with dialysis buffer supplemented with 3 mM MgCl$_2$ and 1% albumin but without P2X$_7$ agonists and protonophore. An in situ calibration concluded each experiment. A single field was monitored throughout the protocol.

A derivative of the above protocol was used to examine the role of lysosomal cations in lysosomal re-acidification. Following baseline pH measurements, the cytosol was dialyzed by incubation with a P2X$_7$ activation solution containing 90 mM K gluconate, 50 mM KCl, 20 mM HEPES, 10 mM glucose, 1 mM EDTA, 1 mg/mL dextran (MW 1500), 2.5 mM Na$_2$ATP, and 100 μM bzATP for 15 min. Where specified K$^+$ was replaced with NMDG, choline or TRIS. For the remainder of the experiment, the cells were maintained in a deactivation solution similar to the dialysis medium but without the P2X$_7$ agonists and supplemented with 1 mM CaCl$_2$ and 1 mM MgCl$_2$. The cells were allowed 5 min to recover before initiating lysosomal dialysis and
alkalinization through the addition of 200 μM Gly-Phe-β-naphthylamide (GPN) to the bathing medium. This membrane-permeant reagent diffuses into the lysosomes where it is cleaved by cathepsin C. The products osmotically draw water into the lumen leading to organelle swelling and transient permeabilization. After 10 min the cells were washed to remove GPN and given a further 10 min to recover. The protocol concluded with an in situ calibration. At the end of each step of the protocol, images from 5 to 15 fields were acquired.

2.3.5 Null-point titration determinations of lysosomal K⁺ and Na⁺

The ionophores used for the null-point titration experiments catalyze the stoichiometric and electroneutral exchange of protons for alkali cations. In the case of nigericin, which is highly selective for K⁺, no net movement of protons across the membrane—and thus no pH change—will be induced by addition of the ionophore when

\[
\frac{H_l}{H_c} = \frac{K_l}{K_c}
\]

where \(H_l\) is the lysosomal proton activity, \(H_c\) is the cytosolic proton activity, \(K_l\) is the lysosomal potassium activity, and \(K_c\) is the cytosolic potassium activity. From this “null point” the lysosomal potassium activity can be calculated as

\[
K_l = K_c \times 10^{(pH_c - pH_l)}.
\]

Monensin prefers Na⁺, but significant transport of K⁺ also occurs. In this case the null-point is defined by:

\[
\frac{H_l}{H_c} = \frac{Na_l + \left( \frac{P_K k_{Na}}{P_{Na} k_K} \right) K_l}{Na_c + \left( \frac{P_K k_{Na}}{P_{Na} k_K} \right) K_c}
\]
where $H_l$, $H_c$, $K_l$, and $K_c$ are as defined above, $N_a_l$ is the lysosomal sodium activity, $N_a_c$ is the cytosolic sodium activity, $P_K$ and $P_{Na}$ are the potassium- and sodium-ionophore complex translocation rates, respectively; and $\kappa_K$ and $\kappa_{Na}$ are, respectively, the potassium and sodium dissociation rates from the ionophore.

It follows from the above relationship that

$$N_a_l = \left[ N_a_l + \frac{1}{P} K_c \right] \times 10^{(pH_c - pH_l)} - \frac{1}{P} K_l$$

where

$$\frac{1}{P} = \frac{P_K \kappa_{Na}}{P_{Na} \kappa_K}.$$

Our calculations use a selectivity coefficient $P$ of 25, in accordance with published estimates (119-121).

To carry out determinations of lysosomal $K^+$ by null-point titration, RAW264.7 cells were plated on glass coverslips and their lysosomes loaded with fluorescein-labeled dextran using the pulse-chase protocol described above. The cytosolic ion composition was manipulated by incubating the cells for 15 min in titration buffer (10 mM NaCl, 20 mM KCl, 20 mM NMDG Cl, 90 mM NMDG gluconate, 10 mM glucose, 20 mM MES, and 1 mg/mL of 1500 MW dextran, set to pH 6.5 with NaOH) containing 100 $\mu$M bzATP and 2 mM Na$_2$ATP to stimulate P2X$_7$ receptors. The solution was replaced with titration buffer without the P2X$_7$ agonists and containing various concentrations of trimethylamine (TMA) to alkalinize the lysosome plus 1 $\mu$M CCA to inhibit the V-ATPase. After 2-4 min, nigericin (10 $\mu$g/mL) was added to the medium. An in situ calibration was performed at the end of each experiment as described in Section 2.3.3. A single field was monitored by ratiometric imaging at every step of the protocol. Na$^+$ determinations were similar, except the titration buffer consisted of 10 mM NaCl, 40 mM KCl, 10 mM K gluconate, 80 NMDG gluconate, 10 mM glucose, 20 mM MES, and 1 mg/mL of 1500 MW dextran, set to pH 6.5 with TRIS, and monensin (20 $\mu$g/mL) was used instead of nigericin.
2.3.6 Iodide efflux assay

CFTR function was assayed by measuring the cAMP-dependent halide conductance of the plasma membrane in BHK cells stably transfected with CFTR tagged with an HA-epitope at its C-terminal tail (see Figure 2.8) as previously described (122). Briefly, cells were incubated for 1 hr at room temperature in loading buffer (136 mM NaI, 4 mM KNO₃, 2 mM Ca(NO₃)₂, 2 mM Mg(NO₃)₂, 11 mM glucose, and 20 mM HEPES) to replace cytosolic Cl⁻ with I⁻. The cells were then washed 6-8 times with efflux buffer (loading buffer with the 136 mM NaI replaced with 136 mM NaNO₃) to remove any residual extracellular I⁻. The extracellular medium was then replaced every minute with 1 mL of fresh efflux buffer for 6 min, at which point CFTR was stimulated using 500 μM IBMX, 500 μM pCPT-cAMP and 20 μM forskolin. Once added, the agonists were maintained in the efflux buffer for the remainder of the experiment. Efflux buffer was collected every minute for the ensuing 10 min. The I⁻ concentration in each of the collected samples was determined with an iodide-selective electrode. In experiments characterizing the pH sensitivity of CFTR channel activity, following 1 min of agonist treatment the bathing solution was acidified by replacement with efflux solution containing the CFTR agonists but buffered to pH 4.5 with 20 mM acetate. The pH was lowered 1-2 min after channel stimulation to ensure that the acidic pH did not hinder CFTR activation.

In Figure 2.8C a calibration curve obtained at pH 4.5 is compared with a standard calibration at pH 7.4 to demonstrate that low pH did not interfere with the sensitivity of the I⁻ electrode.

2.3.7 Colorimetric determination of intracellular Cl⁻

The colorimetric chloride determination was adapted from Zall et al. (123). Cells were either left untreated or had their cytosol dialyzed by incubation in a nitrate-based P2X₇ receptor activation solution (140 KNO₃, 20 mM HEPES, 10 mM glucose, 1 mM EDTA, 2.5 mM Na₂ATP, 100 μM bzATP, and 1 mg/mL dextran) for 15 min. They were subsequently washed thoroughly with cold NaNO₃ medium (130 mM NaNO₃, 3 mM KNO₃, 1 Mg(NO₃)₂, 1 Ca(NO₃)₂, 10 mM glucose and 20 mM HEPES pH 7.4), then lysed in 1 mM nitric acid. After centrifugation, the supernatant was collected and mixed with an equal volume of the photometric determination solution (1 part 0.417% mercuric thiocyanate in methanol, 1 part 20.2% ferric nitrate solution
and 13 parts water). The ferric nitrate was made by dissolving 20.2 g of ferric nitrate in 31 mL of 4 N HNO₃, which was brought to a final volume of 100 mL with distilled water. The absorbance at 480 nm of the experimental samples and Cl⁻ standards was determined using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). In parallel experiments, cells were incubated in the nitrate-based, chloride-free medium for 1.5 hrs, with frequent washes, and similarly prepared. The signal from these samples consists of non-exchangeable chloride and nonspecific background of the colorimetric reaction, which were subtracted from all experimental samples. Cell number and volume were determined in parallel using the Coulter- Channelyzer in order to calculate intracellular Cl⁻ concentrations.

2.3.8 Data analysis and statistics

Data for lysosome re-acidification following protonophore-induced alkalinization were fit with a single exponential of the form $y = A \times \exp(-Kx) + B$ to calculate the half-life of the pH decay ($\tau = 0.69/K$) reported in Table 2.1. Null-point titration data were fit using least-squares linear regression to determine the null-point equilibrium pH ($x$-intercept).

Data are reported as representative traces or mean±SE for the indicated number of experiments. The statistical significance of differences between multiple groups was determined using analysis of variance (ANOVA) with Dunnett’s multiple comparison test for pairwise comparisons with control data. $P$ values <0.05 were considered statistically significant. Statistical analyses were carried out using either SAS or Prism 4 by GraphPad software.
2.4 Results

2.4.1 The role of CFTR in lysosomal acidification.

To investigate the counter-ions that support acidification, the lysosomes of murine macrophages were loaded with pH-sensitive dextrans and subjected to ratiometric imaging (Figure 2.1A-B). In the RAW264.7 and J774 macrophage lines, the steady state lysosomal pH was 4.71±0.04 and 4.52±0.06, respectively (all values listed as mean ± SE; Figure 2.1C). As expected, lysosomal acidification was dissipated by concanamycin A (CCA), an inhibitor of the V-ATPase (pH≥6.5, the upper limit of pH-sensitivity of our sensor; Figure 2.1B-C). To evaluate its role in maintaining baseline pH, CFTR was pharmacologically inhibited by treating the cells for 1 h with the specific inhibitor CFTR\textsubscript{INH-172}, which has been validated in multiple systems (110, 124, 125). No perturbation in steady state lysosomal pH was observed in either cell line following treatment with the inhibitor (pH = 4.77±0.06 and 4.51±0.05 for RAW264.7 and J774 cells, respectively, at 5 μM of the inhibitor and 4.82±0.08 and 4.75±0.11 at 10 μM; Figure 2.1C). Of note, the concentrations of CFTRINH-172 used are much greater than those required for full inhibition of CFTR (IC\textsubscript{50} ≈ 300 nM) (110).

In the study by Di \textit{et al.} (21), CFTR was implicated in modulating lysosomal pH specifically in alveolar, but not other types of macrophages. To confirm this result, primary murine alveolar macrophages harvested by bronchoalveolar lavage were used to measure lysosomal pH. The steady-state pH (4.64±0.02) was not significantly altered by incubation with 10 μM CFTR\textsubscript{INH-172} (4.75±0.03; Figure 2.1D). That CFTR does not contribute importantly to lysosomal acidification was corroborated comparing alveolar macrophages from wild-type and CFTR-deficient mice. The lysosomal pH was virtually identical in wild-type and \textit{cftr}\textsuperscript{-/-} macrophages (4.64±0.02 and 4.58±0.06, respectively) and CCA fully dissipated acidification in both cases (Figure 2.1D). These observations rule out the possibility that residual CFTR activity, due to incomplete inhibition by CFTR\textsubscript{INH-172}, provided the counter-ion conductance for lysosomal acidification.

Pharmacological inhibition of the V-ATPase with CCA unmasks a proton leak that gradually dissipates lysosomal acidification. Because net proton leakage requires translocation of a counter-ion, the rate of pH change caused by CCA can also be used to assess CFTR-mediated
Figure 2.1 Steady-state lysosomal pH in macrophages. (A) The lysosomes of murine macrophages were loaded with pH-sensitive dextrans and their pH measured by ratiometric imaging. A representative field of RAW264.7 macrophages is shown in (A) with the corresponding differential interference contrast (DIC) image in the inset. At the end of each experiment, an in situ calibration was performed as described in Section 2.3.3 to generate a curve relating the 490 nm/440 nm excitation ratio to pH (B). (C) The macrophage cell lines RAW264.7 (black bars) and J774 (grey bars) were either left untreated (5 experiments with a total of 669 lysosomes, and 5 experiments, 1103 lysosomes, respectively) or treated with 5 μM (3 experiments, 538 lysosomes, and 4 experiments, 810 lysosomes, respectively) or 10 μM (3 experiments, 304 lysosomes, and 4 experiments, 861 lysosomes, respectively) of the CFTR inhibitor CFTR\textsubscript{INH}-172 for 1 hr, and the lysosomal pH recorded. In both cell lines, treatment with 500 nM CCA for 1 hr dissipated the lysosomal pH to values greater than 6.5, the upper limit of sensitivity of the pH-probe. (D) Lysosomal pH in alveolar macrophages harvested from wildtype or cftr\textsuperscript{-/-} mice (5 experiments, 509 lysosomes, and 5 experiments, 395 lysosomes, respectively). Wildtype cells were also incubated for 1 hr in the presence of 10 μM of CFTR\textsubscript{INH}-172 (4 experiments, 450 lysosomes). (E) To determine whether CFTR functions as the counter-ion conductance for the proton leak in lysosomes, wildtype (3 experiments 24 lysosomes) and cftr\textsuperscript{-/-} (4 experiments, 34 lysosomes) alveolar macrophage were treated with 1 μM CCA and the rate of alkalinization measured over the first 5 min.
conductance in lysosomes. As shown in Figure 2.1D, the initial rates of lysosomal alkalization in alveolar macrophages harvested from wild-type and cftr\(^{-/-}\) mice were not significantly different (0.20±0.01 min\(^{-1}\) and 0.16±0.02 min\(^{-1}\), respectively; \(p = 0.28\)), confirming that CFTR is not the predominant conductive pathway of the lysosomal membrane.

### 2.4.2 Role of cytosolic anions in lysosomal acidification

While I failed to implicate CFTR as the primary counter-ion conductance, cytosolic anions, mainly Cl\(^{-}\), remained as leading candidates to provide the counter-charge for proton uptake by lysosomes. To validate this notion I used a cytosolic ion-substitution protocol in combination with lysosomal pH measurements, as depicted schematically in Figure 2.2A. Briefly, the plasma membrane was selectively permeabilized by stimulating resident P2X\(_7\) receptors with the agonist 2\,\(^{\prime}\)-(3\,\(^{\prime}\))-\(O\)-(4-benzoylbenzoyl)adenosine-5\,\(^{-}\)-triphosphate (bzATP). Upon activation, P2X\(_7\) receptors initiate the opening of large pores through which molecules \(M_r \leq 900\) Da are able to pass (126). The well-defined, limited size of the P2X\(_7\) mediated-pores and the fact that they close rapidly upon addition of divalent cations makes this an elegant and conservative means of manipulating the cytosolic ion content. Thus, by bathing the cells in media where Cl\(^{-}\) is replaced by larger inorganic anions – such as gluconate or methanesulfonate (MeSO\(_3\)\(^{-}\)) – during receptor stimulation, the cytosol can be dialyzed of its Cl\(^{-}\) in exchange for the larger anion. Colorimetric Cl\(^{-}\) determinations demonstrated that the intracellular Cl\(^{-}\) content decreased from 70.5±4.2 mM to 8.5±1.7 mM following 15 min of bzATP treatment in media devoid of Cl\(^{-}\) (Figure 2.3A-B). Notably, as the colorimetric assay measures total intracellular Cl\(^{-}\) content, the remaining Cl\(^{-}\) may reflect pools of non-exchangeable Cl\(^{-}\) or organellar Cl\(^{-}\), suggesting that cytosolic Cl\(^{-}\) is virtually depleted. Thorough depletion of cytosolic Cl\(^{-}\) is necessary because the volume of the cytosol is large relative to that of the lysosome, and thus residual Cl\(^{-}\) may represent a non-trivial pool of available Cl\(^{-}\).

To optimize detection of V-ATPase-induced acidification, the lysosomes were transiently alkalinized using the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) concurrently with the cytosolic dialysis step. Re-acidification was then initiated by removal of the protonophore, which was effected concomitantly with the closure of P2X\(_7\)-
A

Untreated → Dialyze the cytosol (PM permeabilization by P2X, activation) and Dissipate lysosomal pH (add FCCP) → Reseal the PM (deactivate P2X, receptors) and Initiate reacidification (remove FCCP) → Record lysosomal reacidification

B

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490/440 Ratio

Control vs. +CCA

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pH

Control vs. MeSO₃

Time (min)
**Figure 2.2** Role of cytosolic anions in lysosome re-acidification following protonophore-induced alkalinization. (A) Schematic of the experimental protocol used to dialyze cytosolic anions and transiently alkalinize lysosomes. Following baseline pH measurements (step I), the cytosol was dialyzed by selectively permeabilizing the plasma membrane through the activation of P2X<sub>7</sub> receptors in the presence of anion substitution buffers (step II). Concurrently, the lysosomal pH was dissipated by treatment with the protonophore FCCP (step II). The plasma membrane was then resealed by removing the P2X<sub>7</sub> agonists and adding divalent cations, while lysosomal re-acidification is initiated by protonophore washout (step III). Lysosomal pH was monitored throughout the protocol. (B) Cells were subjected to the experimental protocol described in (A) while measuring pH. Representative recordings of the fluorescence ratio (proportional to pH) of untreated (filled squares) and CCA-treated cells (open circles) are illustrated (means±SD for 6 and 9 lysosome replicates, respectively). The stages identified by the Roman numerals correspond to those in (A). Note that inhibition of the V-ATPase with CCA immediately prior to plasma membrane resealing and removal of FCCP fully abrogated lysosomal re-acidification. (C) Cytosolic anion substitution with MeSO<sub>3</sub> did not change the rate or extent of lysosome re-acidification. Representative traces of control (filled squares) and anion substituted (open circles) cells are shown (means±SD of 6 lysosomes). Summary statistics are presented in Table 2.1.
Figure 2.3 Effectiveness of the dialysis accomplished by P2X7 activation. (A) RAW264.7 cells were either left untreated or dialyzed of their intracellular Cl- by incubation in a Cl- free medium during stimulation of P2X7 receptors for 15 min. The intracellular Cl- content of cells before and after dialysis was assayed colorimetrically as described in Section 2.3.7. Data are means±SE of 3 and 6 experiments for the control and dialysis treatments, respectively. A sample calibration curve for the assay is shown in (B). (C) Intracellular K+ was measured using the ratiometric K+-sensitive fluorescent dye PBFI. Briefly, RAW264.7 cells were incubated for 30 min with the acetoxyethyl ester of PBFI prior to imaging. Following 5 min of baseline measurements, the plasmalemma was selectively permeabilized using bzATP in the presence of either a cytosol-like (K+-rich) or NMDG-rich solution. Data are representative of ≥3 similar experiments (means ± SD of 6 cells).
Table 2.1 The rate and extent of lysosome re-acidification following cytosolic anion dialysis and protonophore-induced alkalinization. The schematic of the anion substitution experiment refers to the protocol described in Figure 2.2. The differences between the initial pH (pH₀), pH after alkalinization, (pHₐlk) and final pH following re-acidification (pHᵢ) were calculated as indicated. To determine the half-life of the pH decay (τ) the data for lysosome re-acidification following protonophore washout (step III) were fit with a single exponential of the form \( y = A \times \exp(-K \times x) + B \) and τ calculated as \( =0.69/K \). All values are listed as mean ± SE. The \( p \) values, given in parenthesis, were determined by ANOVA using Dunnett’s multiple comparison test to the control (NS, not significant).
induced pores. When the dialysis medium contained 50 mM Cl\(^-\) to mimic the normal Cl\(^-\) content of the cytosol, lysosomes rapidly re-acidified to baseline values following plasma membrane resealing and protonophore washout (Figure 2.2B). Notably, the re-acidification was inhibited by addition of CCA immediately prior to protonophore washout (Figure 2.2B), verifying that the drop in pH is due to the activity of the V-ATPase. Consistent with our previous results, treatment with 10 \(\mu\)M CFTR\(_{\text{inh}}\)-172 did not alter the extent of re-acidification, though the rate was slightly reduced (Table 2.1). More importantly, replacement of cytosolic Cl\(^-\) with either MeSO\(_3\)\(^-\) (Figure 2.2C) or gluconate (data not shown) had no discernible effect on either the rate or extent of re-acidification (Table 2.1). It follows that Cl\(^-\) and other small cytosolic anions are not absolutely necessary for lysosome acidification. As both MeSO\(_3\)\(^-\) and gluconate are poorly permeable through CFTR (127), these data lend further evidence against CFTR as the counter-ion conductance.

### 2.4.3 Lysosomal K\(^+\) and Na\(^+\) determinations by null-point titration

Because I found no evidence that cytosolic anions function as counter-ions I considered whether luminal cations can support lysosomal acidification. To date, the presence and in particular the concentration of free cations inside lysosomes has not been established, likely because the spectroscopic probes of cation activity are incompatible with the extreme acidity of the lysosomal lumen. Instead, I implemented a method that uses ionophores that exchange monovalent cations for protons with defined stoichiometry to indirectly assess cation activity. Based on the theoretical considerations summarized in Section 2.3.5, the steady-state free concentration of Na\(^+\) and K\(^+\) can be estimated by means of a “null-point” titration of lysosomal pH. This required manipulation of the cytosolic monovalent cation concentration, which was accomplished by reversible activation of plasmalemmal P2X\(_7\) receptors, as described above, followed by graded alkalinization of the lysosomal lumen using the weak base tetramethylamine (TMA). Three representative traces using different amounts of TMA for K\(^+\) determination using nigericin are shown in Figure 2.4A. From multiple such determinations I estimated the null-point, i.e. the lysosomal pH at which \(\Delta\text{pH}/\Delta t = 0\) (Figure 2.4B), which was in turn used to calculate K\(^+\) activity as detailed in Section 2.3.5. From 15 independent determinations, each
Figure 2.4 Null-point titration determinations of lysosomal $K^+$ and $Na^+$. Estimates of lysosomal $K^+$ and $Na^+$ were obtained using the null-point method, as described in Section 2.3.5. Lysosomes were loaded with a pH-sensitive dextran, and the cytosolic and extracellular compartments equilibrated using bzATP-activated P2X$_7$ receptors. The lysosomes were alkalinized with the specified amount of TMA (20 mM or 15 mM TMA added where indicated by the filled arrow, and 5 mM TMA where indicated by the open arrow). Changes in lysosome pH upon the addition of a cation/proton exchanging ionophore were measured next to find the null-point, i.e. the condition when addition of the ionophore elicits no change in pH. In the experiment shown in (A), nigericin was added to all samples at 0 min (filled arrowhead). The traces are means±SD of 3 representative experiments. For each null-point titration experiment, the rate of pH change ($\Delta$pH/$\Delta$t) following addition of the ionophore was measured and plotted as a function of the lysosome pH at the time of addition of the ionophore. The data obtained using nigericin and monensin, used to estimate $K^+$ and $Na^+$ are shown in (B) and (C) along with the calculated least-squares linear regressions.
measuring between 6-8 lysosomes, I determined the free [K⁺] in the lumen of lysosomes to be 50.1±2.0 mM.

While nigericin is selective for K⁺/H⁺ exchange, monensin, the ionophore employed in the Na⁺ null-point titration, is only moderately selective. It preferentially exchanges Na⁺ for H⁺ but is also capable of mediating K⁺/H⁺ exchange with reduced efficiency. To calculate intra-lysosomal Na⁺ activity the lysosomal [K⁺], which was determined independently, and the selectivity coefficient of the ionophore were also taken into account (see Section 2.3.5). From monensin null-point titrations shown in Figure 2.4C, and using the lysosomal [K⁺] determined above, I estimate intra-lysosomal free [Na⁺] to be 20.6±2.1 mM. Considering the rate of proton pumping and the lysosomal buffering capacity (61.5±2.3 mM/pH; n=10 experiments), it is clear that the aggregate content of monovalent cations is sufficient to support lysosomal acidification.

### 2.4.4 Role of lysosome luminal cations in lysosomal acidification

To investigate whether luminal cations are required to generate and maintain lysosomal acidification, I employed an ion-substitution strategy. Unlike the approach used to study anions, however, these experiments required manipulation of the internal cation content of the lysosomes. This was accomplished by transient permeabilization of the lysosomal membrane using the dipeptide Gly-Phe-β-naphthylamide (GPN). This membrane-permeant reagent diffuses into lysosomes where it is cleaved by cathepsin C, an exopeptidase found exclusively in these organelles (128). The osmotically active products accumulate, drawing water into the lumen and leading to organelle swelling. When its ability to distend is exceeded, the lysosomal membrane undergoes a transient permeabilization. Under the conditions chosen for our experiments, the pore size generated by the permeabilization procedure is limited. Large molecules such as dextrans are retained by the lysosomes (Figure 2.5B), whereas smaller compounds like sulforhodamine (Mr = 607), and by extension small inorganic cations, are released (Figure 2.5A). As a result the pH of the lysosomal lumen equilibrates with that of the surrounding cytosol, and the lysosomes fail to accumulate LysoTracker (Figure 2.5C). Remarkably, following short (10 min) exposure periods to GPN, the permeabilization process can be reversed by removal of the dipeptide. Within minutes of GPN washout the lysosomes recover the ability to accumulate the
acidotropic dye and the recovery is precluded by CCA (Figure 2.5C), implying that it reflects proton pumping by the V-ATPase.

This transient permeabilization protocol enabled us to manipulate the ionic content of the lysosomal lumen to assess the role of monovalent cations in acidification. A schematic of the lysosome dialysis and alkalinization protocol is shown in Figure 2.6A. The baseline lysosomal pH was initially monitored in cells loaded with indicator probes (step I). The plasma membrane was then selectively permeabilized by P2X7 activation and the cytosol dialyzed while bathing the cells in either a cytosol-like (K+-rich control) or cation-substituted medium (step II). To gain insight into the kinetics of cytosolic cation dialysis, K+ was monitored using the fluorescent K+ probe PBFI. When P2X7 receptors were stimulated in a K+-rich solution, no change in K+ content was observed. In contrast, when the cells were bathed in an N-methyl-D-glucammonium (NMDG)-rich solution during P2X7 activation, intracellular K+ was rapidly depleted within 10 min of dialysis (e.g. Figure 2.3C). Accordingly, the cytosol dialysis (step II) was carried out for 15 min. Following cytosolic dialysis the plasma membrane was resealed by deactivating the P2X7 receptors and the cells allowed 5 min to recover (step III). For the remainder of the experiment, the cells were bathed in the same medium used for cytosolic dialysis, except that the P2X7 agonist was removed and divalent cations were re-added. The lysosomes were next dialyzed (and inevitably alkalinized) by treatment with GPN (step IV). Finally, GPN was removed and the ability of lysosomes to re-acidify was monitored after 10 min (step V). The results of a representative control experiment, where a cytosol-like (K+-rich) solution was used during the dialysis, are shown in Figure 2.6B (the Roman numerals refer to the corresponding steps in the protocol schematic Figure 2.6A). Comparable experiments using either an NMDG-based dialysis solution or NMDG-based solution without the cytosolic dialysis step are illustrated in Figure 2.7.

To evaluate the extent of re-acidification observed after lysosomal cation dialysis followed by removal of GPN, I constructed histograms of pooled lysosome pH determinations from multiple experiments (Figure 2.6C). When K+ was resealed into the lysosomes, mimicking the conditions prevailing physiologically, the lysosomes acidified back to pH=5.44 (median of 1349 determinations), approaching the normal resting value. In stark contrast, lysosomes dialyzed with Na+ and K+-free solutions, containing either TRIS or NMDG, were unable to re-acidify (median re-acidification pH >6.5 and 6.26 for TRIS and NMDG, respectively). The inability to
acidify was not the result of untoward deleterious effects of the organic cations, because when
the ion-substitution protocol was carried out in the absence of bzATP, and thus without actually
dialyzing the cytosol and lysosome lumen, normal lysosomal re-acidification was observed
(median pH=5.27; Figure 2.6C). When choline was used for dialysis, an intermediate level of re-
acidification was observed (data not shown). Together, these observations indicate that, despite
the presence of cytosolic Cl⁻, small inorganic cations are required for proper acidification,
suggesting that in lysosomes a cation counterflux neutralizes the electrogenicity of the V-
ATPase.
Figure 2.5 GPN-induced transient lysosome membrane permeabilization. Lysosomes of RAW264.7 macrophages were loaded with either (A) sulforhodamine or (B) fluorescently-labeled dextrans (MW 10,000), and imaged prior to and following 10 min of GPN treatment. Corresponding DIC images are shown in the insets. (C) The pH of the lysosome was monitored qualitatively using LysoTracker, which accumulates in acidic intracellular compartments. The dye was added to the cells either prior to GPN treatment, following 10 min of GPN treatment, or following 10 min of recovery subsequent to 10 min of GPN treatment. Dye accumulation was abrogated when CCA was added to the cells immediately after GPN washout. Representative fields are shown along with their corresponding DIC images (insets). The fields shown before and after the addition of GPN are not the same.
A

I Untreated

II Dialyze the cytosol (PM permeabilization by P2X$_7$ activation)

III Reseal the PM (deactivate P2X$_7$ receptors)

IV Dialyze and alkalinize the lysosome (GPN-induced osmotic swelling)

V Record lysosomal re-acidification
B

\[
\begin{array}{ccccccc}
& I & II & III & IV & V & \text{pH calibration} \\
\hline \\
490/440 \text{ Ratio} & & & & & & \\
0 & 0.0 & 0.1 & 0.2 & 0.3 & 0.4 & 0.5 \\
\end{array}
\]

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

\[
\begin{array}{ccccccc}
& 0 & 10 & 20 & 30 & 40 & 50 \\
\hline \\
\end{array}
\]

C

\begin{itemize}
\item Cytosol-like
  \[\bar{x} = 5.44 (1349)\]

\item TRIS
  \[\bar{x} = >6.5 (260)\]

\item NMDG
  \[\bar{x} = 6.26 (794)\]

\item NMDG without P2X, activation
  \[\bar{x} = 5.27 (329)\]
\end{itemize}
**Figure 2.6** Role of lysosomal cations in lysosome re-acidification following GPN-induced alkalinization. (A) Schematic of the experimental protocol used to dialyze and transiently alkalinize lysosomes. Following baseline pH measurements (step I), the cytosol was dialyzed by selectively permeabilizing the plasma membrane through the activation of resident P2X7 receptors in the presence of cation substitution buffers (step II). Removal of the P2X7 agonists and the addition of divalent cations resealed the plasma membrane (step III). The lysosomes were then dialyzed and alkalinized by GPN-induced osmotic swelling (step IV). The lysosomes were next allowed to re-acidify following removal of GPN (step V). An *in situ* calibration was performed at the end of each experiment. (B) Determinations of fluorescence ratio (proportional to pH) of a representative control experiment where cells were dialyzed with a cytosol-like solution. Each data point represents the ratio data from an individual region of interest. The steps indicated by Roman numerals refer to the protocol schematic of panel (A). (C) Histograms of pooled lysosome pH determinations after recovery from the GPN treatment (step V of the illustrated protocol). The median pH of the lysosomes after the re-acidification period is given in the figure along with the total number of lysosomes used to compile the histograms. Cells dialyzed in cytosol-like (top-left), TRIS-substituted (top-right), or NMDG-substituted (bottom-left) medium. The bottom-right histogram shows cells that were incubated with NMDG-substituted medium but were not permeabilized. The histograms show collated data from 9, 3, 6, and 2 separate experiments, respectively.
A  Potassium-rich (cytosol-like)

B  NMDG-rich

C  NMDG-rich without cytosol dialysis
Figure 2.7 Lysosome re-acidification following luminal cation dialysis. Raw data of representative lysosome cation-substitution experiments as described for Figure 2.6 in the main text. Lysosomes were either dialyzed with a cytosol-like (A) or NMDG-rich (B) solution. As a control, the same protocol as that used in (B) was carried out but without P2X7 receptor activation, and thus without a cytosol dialysis (C). The different stages of the experimental protocol are indicated by Roman numerals and dashed lines as described for Figure 2.6.
2.5 Discussion

By developing new protocols to reversibly permeabilize the plasmalemma and the lysosomal membrane I was able to separately dialyze the cytosol and the lysosomal lumen to analyze the ionic dependence of acidification in situ. The primary finding of this study is that lysosomal luminal cations – and not cytosolic anions – function as the principal counter-ions that enable lysosomal acidification by dissipating the membrane potential generated by the V-ATPase.

I found that, in otherwise untreated cells, the lysosomes were able to re-acidify fully following a transient protonophore-induced alkalinization. Strikingly, the re-acidification was equally rapid and complete in cells where the cytosolic Cl\(^-\) had been replaced with large organic anions such as gluconate and MeSO\(_3\)\(^-\). Because these are negligibly permeable through most Cl\(^-\) conductive pathways, including ClC channels and CFTR (127, 129), I inferred that cytosolic anions are not essential for lysosome acidification. This conclusion differs from those reached by others, but differences in the experimental design may account for the apparent discrepancies. In some instances, the counter-ion dependence was studied in vitro using lysosomes and/or late endosomes purified by density gradient centrifugation using sucrose solutions that likely depleted the cations from the lumen during the course of isolation (84, 87, 130). This treatment likely made lysosome acidification entirely and artificially dependent on external anions. In another important study, acidification was studied in situ in macrophages and found to require anion transport via CFTR (21). More recently, defective lysosomal acidification was also invoked as the mechanism underlying ceramide accumulation in CF lysosomes (25). These observations were unexpected, since they imply that CFTR can function as a chloride channel while exposed to the extremely acidic luminal fluid of lysosomes. I tested whether CFTR is indeed capable of conducting anions when its extracellular domain, which presumably faces the lysosomal lumen, is bathed in acidic medium. To this end I measured cAMP-stimulated iodide efflux from cells expressing CFTR on their surface membrane. As illustrated in Figure 2.8, I initially verified that under control conditions (extracellular pH=7.4) the flux was attributable to CFTR (Figure 2.8A) and then abruptly acidified the medium to pH=4.5, similar to the value determined in the lysosomal lumen. Acidification produced an acute cessation of iodide efflux (Figure 2.8B), which was not attributable to interference with the ion-selective electrodes used to measure the flux (Figure 2.8C). The reduced ability of CFTR to conduct anions at acidic pH
Figure 2.8 Acidic exofacial pH inhibits CFTR channel function. I investigated whether CFTR is active in an environment that mimics the lysosome using an iodide efflux assay (122). BHK cells stably transfected with an HA-tagged CFTR construct were loaded with iodide. Upon stimulation using a cocktail of 3-isobutyl-1-methylxanthine (IBMX; 500 μM), forskolin (20 μM) and the membrane-permeant cAMP analog 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic monophosphate (pCPT-cAMP; 500 μM), iodide is released from the cells through the activated CFTR channel. The medium was collected at defined intervals and iodide content measured using an iodide-selective electrode. (A) Iodide efflux was readily detectable in CFTR-expressing BHK cells (red squares), but not in their untransfected counterparts (green diamonds). Representative traces of the I efflux from wildtype and CFTR-transfected BHK cells are shown. The inset shows an immunoblot verifying expression of CFTR in the transfected BHK cells, but not in the untransfected control cells. GAPDH is shown as a loading control. (B) Effect of extracellular acidification on CFTR function. After stimulation, the pH of the extracellular medium was lowered to 4.5 while in the continued presence of CFTR agonists. Note that exposure of the extracellular surface (topologically equivalent to the lysosome luminal side) to acid induced a marked decrease in iodide efflux. Representative experiments are shown for the control (red squares) and acid-treated (green diamonds) cases. (C) Calibration curves generated using I standards made in buffer either at pH 7.4 or pH 4.5 demonstrate that the iodide electrode is pH-insensitive in the range studied.
makes its contribution to lysosomal acidification less likely. Indeed the conclusions reached by Di et al. (21) regarding the role of CFTR in lysosomes are not universally accepted. The observations of Verkman and colleagues (26) failed to confirm the involvement of CFTR in acidification, as did ours. I found that pharmacological inhibition of CFTR channel activity did not perturb lysosome pH and that primary alveolar macrophages lacking functional CFTR had no lysosomal acidification defect. Taken together, the available data do not convincingly support a role for CFTR in lysosomal counter-ion conductance and defects in lysosomal acidification cannot fully explain the impaired microbicidal capacity in CF.

Another Cl⁻ transporter, the Cl⁻/H⁺ antiporter ClC-7 has also been invoked in lysosomal acidification (99). If, as proposed for other family members, the stoichiometry of exchange is 2:1, uptake of Cl⁻ via ClC-7 could indeed serve as a neutralizing counter-ion to H⁺ pumping, though the process would be inefficient (three H⁺ pumped for every two accumulated). However, lysosomal acidification appears to be normal in mice lacking ClC-7 (115, 116), implying that alternative counter-ion pathways exist.

I found that not only CFTR, but cytosolic anions in general were dispensable for the re-acidification of lysosomes following a transient alkalinization, which further questions the role of ClC-7. This result prompted us to consider whether lysosomal cations were capable of sustaining proton translocation by the V-ATPase. Because, to the best of our knowledge, the free concentration of inorganic cations in lysosomes had not been reported, the presence of potential luminal counter-ions had to be validated first. Our null-point determinations of Na⁺ and K⁺ activity indicated that inorganic cations are sufficiently abundant to sustain the acidification of lysosomes. More importantly, ion replacement experiments demonstrated that monovalent inorganic cations are sufficient to support for the acidification process. This conclusion is not unprecedented; some in vitro studies had suggested that K⁺ plays a more prominent role in facilitating lysosome acidification than does Cl⁻ (87). It is noteworthy also that lysosomes store significant amounts of Ca²⁺ (131, 132) and efflux of this divalent cation may also contribute to neutralize H⁺ entry, but this possibility has not been directly examined.

It is interesting to speculate why lysosomes may preferentially utilize cations and not anions as counter-ions for acidification. While anion influx and cation efflux would equally neutralize the charge accrued by proton translocation, the osmotic consequences of these processes are
diametrically opposed. The uptake of an anion in parallel with each H\(^+\) results in the net gain of two osmolytes. This is a maximum estimate, as it fails to consider the H\(^+\) buffering power of the lysosomal lumen. In contrast, exchange of a cation for each H\(^+\) is osmotically inconsequential and may result in a net osmotic loss when buffering is taken into account. A mechanism for continuous extrusion of osmolytes may be advantageous for lysosomes, which are required to maintain their volume despite the continued delivery of endocytic solutes. The osmotic implications of the cation exchange mechanism are best appreciated when considering that the buffering power of lysosomes is comparatively high (61.5±2.3 mM/pH in macrophages), likely due to their high protein content. Assuming that the buffering power is constant over the pH 7.4-4.5 range, full acidification of the lysosome would require the parallel transport of ∼180 mmol of counter-ions per liter of lysosomal volume. This gain in anionic osmolytes would cause considerable swelling and possibly lysis of the lysosomes, which are water-permeable and susceptible to osmotically-induced rupture, as revealed by the experiments using GPN. The ensuing collapse of the pH gradient and the release of degradative enzymes would obviously have a detrimental effect on the cell. Use of cations as counter-ions during acidification would guard against volume expansion, obviating the osmotic stress.

To our knowledge no specific cation channels or electrogenic transporters have been shown conclusively to function in the lysosomal membrane. Proteomic approaches are beginning to probe the protein landscape of the lysosomal membrane (133) and, as more extensive lists are developed and consolidated, candidate conductive pathways will be identified. Their ability to function in the unusual environment of the lysosome and their role in lysosome acidification must then be tested.
Chapter 3

3  *In situ* measurement of the electrical potential across the phagosomal membrane using FRET. Contribution to the proton-motive force.


3.1 Abstract

Phagosomes employ lytic enzymes, cationic peptides and reactive oxygen intermediates to eliminate invading microorganisms. The effectiveness of these microbicidal mechanisms is potentiated by the acidic pH created by H\(^+\)-pumping V-ATPases on the phagosomal membrane. The degree of phagosomal acidification varies greatly between neutrophils, macrophages and dendritic cells, and can be affected by diseases like cystic fibrosis. The determinants of phagosomal pH are not completely understood, but the permeability to ions that neutralize the electrogenic effect of the V-ATPase has been proposed to play a central role. When counter-ion conductance is limiting, generation of a large membrane potential will dominate the proton-motive force (*pmf*), with a proportionally diminished pH gradient. Validation of this notion requires direct measurement of the electrical potential that develops across the phagosomal membrane (*Ψ*\(_Φ\)). I describe a non-invasive procedure to estimate *Ψ*\(_Φ\) in intact cells, based on fluorescence resonance energy transfer. This approach, in combination with measurements of phagosomal pH, enabled me to calculate the *pmf* across phagosomes of murine macrophages and to analyze the factors that limit acidification. At steady state *Ψ*\(_Φ\) averaged 27 mV (lumen positive) and was only partially dissipated by inhibition of the V-ATPase with concanamycin A. The comparatively small contribution of the potential to the *pmf* suggests that proton pumping is not limited by the counter-ion permeability, a notion that was validated independently using
ionophores. Instead, phagosomal pH stabilizes when the rate of proton pumping, which decreases gradually as the lumen acidifies, is matched by the passive leak of proton equivalents.
3.2 Introduction

Internalization of pathogens by macrophages, dendritic cells and neutrophils is an essential component of the innate immune response. Invading microorganisms are trapped in a membrane-bound vacuole, the phagosome, where they are subsequently eliminated. Phagosomes acquire microbicidal properties gradually, through a series of fusion reactions collectively known as maturation. A progressive and ultimately profound acidification of the lumen is a distinctive and important feature of phagosomal maturation. The luminal pH attains values \(<5.5\) (12, 13) by a process that depends on vacuolar-type ATPases (V-ATPases) (31). In addition to directly affecting bacterial growth, phagosomal acidification is important for the optimal activity of multiple microbicidal components, including degradative enzymes and the generation of several reactive oxygen intermediates.

While V-ATPases are known to be involved in acidification, the determinants of the phagosomal pH are not well understood. V-ATPases are present in both neutrophils and macrophages, yet only the latter display a rapid and profound phagosomal acidification, while neutrophil phagosomes initially alkalinize and generate only a modest subsequent acidification (12, 15). It has been suggested that \(H^+\) consumption by products of the NADPH oxidase accounts for this differential behavior (15), but other factors may be involved. Specifically, because the V-ATPase is electrogenic (134), differences in the rate and/or extent of acidification could be caused by variations in the counter-ion permeability of the phagosomal membrane. Indeed, a recent report noted that in macrophages from CFTR-null mice phagosomes are less acidic, and attributed this difference to reduced chloride (counter-ion) conductance (21).

If the counter-ion conductance is limiting, V-ATPases would be unable to generate a sizable proton concentration gradient and a larger fraction of the proton-motive force generated by ATP hydrolysis would instead be devoted to the generation of a transmembrane electrical potential (positive inside). Accordingly, a gradual shift in the balance between the electrical and chemical components of the proton-motive force (\(pmf\)) has been suggested as (one of) the mechanisms to account for the progressive acidification of the endocytic and secretory pathways. An increasing abundance of counter-ion channels (or other conductive entities) could explain why lysosomes are more acidic than early endosomes, despite the fact that both organelles are endowed with V-ATPases. Alternatively, differential expression of \(H^+\) “leaks” may be responsible.
Differentiation between these models, and a more complete understanding of the determinants of organellar acidification require a precise quantitation of the components of the \( \text{pmf} \). Reliable methods for \textit{in situ} determination of the pH of phagosomes and other endocytic organelles have been available for over a decade (29, 111). By contrast, it has been virtually impossible to measure the electrical potential across the membrane of endocytic organelles. Here I present a method based on FRET to measure the potential generated across the phagosome membrane in live macrophages. Data from such experiments enabled us to derive information regarding the ionic permeability of phagosomes and to generate a more quantitative description of the proton-motive profile of this organelle.
3.3 Materials and methods

3.3.1 Reagents
sRBC and rabbit IgG against sRBC were purchased from MP Biomedicals (Solon, OH). DACCA succinimidyl ester, DiBAC₄(5), fluorescein isothiocyanate (FITC) and nigericin were from Molecular Probes (Eugene, OR). All other reagents were from Sigma-Aldrich, unless specified otherwise.

3.3.2 Cell lines and tissue culture
RAW264.7 cells (ATCC number TIB-71) were obtained from the American Type Culture Collection (Rockville, MD) and grown at 37°C under 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (Wisent, St. Bruno, Quebec).

3.3.3 Particle labeling and characterization
sRBC labeling was carried out in PBS (pH 8.7) by incubating for 90 min while rotating at room temperature. To couple with DACCA, a 0.15% sRBC suspension was incubated with 25-50 µg/mL of DACCA-SE; FITC labeling was performed using a 6% sRBC suspension with 0.5 mg/mL FITC. Excitation and emission spectra of the DACCA-labeled sRBC (DACCA:sRBC) and DiBAC₄(5) were acquired using a F-2500 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan).

3.3.4 Live-cell imaging and FRET
Coverslips were mounted in a Leiden chamber maintained at 37°C on the stage of a Leica DM IRB microscope equipped with filter wheels (Sutter Instruments, Novato, CA) to independently alternate between different excitation and emission filters. Light from an EXFO X-Cite 120 lamp (Exfo Life Sciences Group, Mississauga, Ontario) was directed to the sample using a dichroic mirror. Emitted light was captured by a Cascade II CCD camera (Photometrics, Tucson, AZ).
The filter wheel and camera were under the control of Metamorph/Metafluor software (Molecular Devices, Downingtown, PA).

In FRET experiments, 3 fluorescent channels were serially acquired. The filter set configurations (listed as excitation / dichroic / emission filters, all in nm ± bandwidth) used in the FRET experiments were: donor (DACCA) 417 ± 60 / 425 / 470 ± 40; acceptor (DiBAC$_4$(5)) 543 ± 22/ 565 / 640 ± 25; and FRET 417 ± 60 / 565 / 640 ± 25. Corrected FRET (cFRET) was calculated using the method of Youvan et al. (135). The donor and acceptor bleed-through coefficients were determined by acquiring images with donor or acceptor alone, respectively, in independent experiments. All microscope parameters, including exposure times and camera gains, were kept constant across all experiments. Fluorescence intensity measurements were performed using Volocity 3 software (Improvison Inc., Lexington, MA). Images were background-subtracted prior to analysis.

3.3.5 Phagocytosis assays

Labeled or unlabeled sRBC were opsonized with rabbit anti-sRBC (20 µg/ml) for 20 min at room temperature. To synchronize phagocytosis, opsonized sRBC were deposited onto RAW cells by centrifugation at 1200 rpm for 1 min. The cells were then incubated for 5 min at 37°C to allow for particle internalization. Remaining uninternalized sRBC were hypotonically lysed by incubation in water for 20 sec, followed by extensive washing. Phagosome maturation was allowed to proceed for 15 min prior to imaging, maintaining the cells at 37°C.

3.3.6 Phagosome membrane potential measurements

Phagocytosis of DACCA:sRBC was initiated as described. Either 125 or 250 nM DiBAC$_4$(5) was added to the bathing media and allowed to equilibrate for 7.5 min prior to imaging. Baseline images of the donor, acceptor and FRET channels were acquired for 10 min. The cells were then treated with concanamycin A (1 µM), NH$_4$Cl (10 mM), or both. Following 3 min of incubation, images in the 3 channels were acquired for 7 min.
To determine the amount of DiBAC\(_4\)(5) within the phagosome, free DACCA:sRBC were placed on a coverslip and imaged. Increasing amounts of DiBAC\(_4\)(5) were added to the particles, and the amount of FRET imaged. In this way, a calibration curve relating cFRET to [DiBAC\(_4\)(5)] was constructed, and the intra-phagosomal [DiBAC\(_4\)(5)] determined. Calibration data was fit using a logarithmic function (\(R^2 = 0.991\)). \(\Psi_\phi\) was calculated as described in the main text.

### 3.3.7 Phagosome pH measurements

Phagosomal pH was measured by ratiometric imaging as described (12). Briefly, RAW cells with FITC:sRBC-containing phagosomes that had been allowed to mature for 15 min, were imaged by rapidly alternating between 485 ± 20 nm and 438 ± 24 nm excitation filters, while using a 505 nm dichroic and a 535 ± 40 nm emission filter. Following acquisition of experimental data, an \textit{in situ} calibration was performed by sequentially bathing the cells in isotonic K\(^+\) solutions (145 mM KCl, 10 mM glucose, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 20 mM of either HEPES or MES) buffered to pH ranging from 4.5 to 7.0 and containing 1 µM nigericin. Calibration curves were generated by plotting the fluorescence intensity ratio (490 nm/440 nm) vs. pH.

To determine the buffering power of sRBC-containing phagosomes, 1 mM or 2 mM NH\(_4\)Cl in PBS was added to the medium, the change in pH monitored and the buffering power calculated as described by Roos and Boron (118).

### 3.3.8 Plasma membrane potential determination

Plasma membrane potential was measured using standard patch-clamp and fluorimetric protocols. To quantify plasmalemmal potential fluorimetrically, RAW cells were equilibrated with the potentiometric dye, DiBAC\(_4\)(5) for 7.5 min, imaged and the intracellular fluorescence computed. An \textit{in situ} calibration was carried out following the acquisition of baseline fluorescence (Figure 3.1). The calibration makes use of gramicidin, a pore-forming ionophore that allows the selective passage of small monovalent cations but not of larger molecules such as N-methyl-D-glucammonium (NMDG\(^+\)). By increasing the membrane permeability to both Na\(^+\)
and $K^+$, the membrane potential approaches the Goldman-Hodgkin-Katz (GHK) reversal potential for monovalent cations, given by the equation:

$$\Psi_{PM} = \frac{RT}{F} \ln \left( \frac{[Na^+]_o + \frac{P_K}{P_{Na}} [K^+]_o}{[Na^+]_i + \frac{P_K}{P_{Na}} [K^+]_i} \right),$$

where $P_K/P_{Na}$ is the permeability ratio of $K^+$ to $Na^+$ through the gramicidin pore. Even though for the gramicidin pore $P_K$ has been reported to be $\approx 3.6$-fold larger than $P_{Na}$, I nevertheless used a $P_K/P_{Na} = 1$ when applying the GHK formulation, because intracellular $Na^+$ exchanges continuously for $K^+$ during the course of the calibration procedure. Since under the conditions used $K^+$ is the predominant permeable species on both sides of the membrane this assumption causes, at most, a very modest and negligible error in the calculations. The calibration method requires independent determination of the intracellular monovalent cation concentrations. $[K^+]_i$ and $[Na^+]_i$, were measured by flame photometry, using Li$^+$ as an internal standard. Briefly, adherent RAW cells were washed three times with ice-cold medium containing 140 mM MgCl$_2$ and 10 mM HEPES, titrated to pH 7.3 with TRIS. The cells were then scraped with a rubber policeman into 1.5 mL of a 15 mM Li$^+$ standard solution. Samples were analyzed in a model 443 flame photometer (Instrumentation Laboratories, Lexington, MA) and compared with Na$^+$ and K$^+$ standards. Intracellular $K^+$ and Na$^+$ concentrations of $136 \pm 11$ and $19 \pm 7$ mM ($n = 4$), respectively, were calculated using a cell volume of $866 \pm 36.7$ fL/cell, estimated in parallel following detachment of cells with trypsin. Cells were counted using a hemocytometer.

The voltage calibration uses an extracellular solution containing 5 mM Na$^+$ with serial substitutions of decreasing amounts of NMDG$^+$ for K$^+$. By substituting extracellular K$^+$ with NMDG$^+$, the permeant cation gradient is altered in a predictable manner, thereby imposing defined transmembrane voltages. By recording the corresponding intracellular DiBAC$_4$(5) fluorescence at various voltages, a calibration curve can be constructed. A sample calibration curve is shown in Figure 3.1.

For patch-clamp measurements, RAW cells were grown on polystyrene dishes (Nalge Nunc, Rochester, NY). Micropipettes with a resistance of 5-8 MΩ were pulled from borosilicate glass capillaries. The pipette and bath were filled with PBS. Recordings were made using an Axopatch-1D (Molecular Devices, Sunnyvale, CA). Signals were filtered at 2 kHz and sampled
Voltage measurements were made in the cell-attached configuration in current-clamp mode (136). Patch resistance was monitored throughout the experiments, and data were accepted for analysis only if a resistance $>10 \text{ G}\Omega$ was obtained. Data analysis was carried out using MATLAB V7 (The MathWorks Inc., Natick, MA).

**Figure 3.1** Sample calibration curve used for the determination of the plasma membrane potential.
3.4 Results and discussion

The small size and inaccessibility of endocytic organelles precludes their analysis by conventional electrophysiological means. To circumvent these limitations, I used a potentiometric fluorescent dye. Such probes are membrane-permeant and can access endocytic compartments. However, the high density and variety of endomembrane organelles makes it impossible to isolate the contribution of endosomes, lysosomes or even larger organelles like phagosomes. It is therefore imperative to circumscribe the measurement to the organelle of interest. To this end I devised a microscopy-based approach, using FRET between an organelle-specific donor and a membrane potential-sensitive acceptor dye. As resonance energy transfer requires close (≤10 nm) proximity between donor and acceptor, targeting one of the FRET partners to the phagosome restricts the measurements to this organelle.

The potential-sensitive probe bis-(1,3-dibutylbarbituric acid)pentamethine oxonol, known as DiBAC₄(5), was used to measure phagosomal potential. This lipid-soluble anionic dye partitions across membranes in accordance to their transmembrane potential. As a donor for FRET I covalently labeled phagocytic particles with 7-diethylamino-coumarin-3-carboxylic acid (DACCA). This was accomplished by incubating sheep red blood cells (sRBC) with the succinimidy1 ester derivative of DACCA, thereby forming stable carboxamide bonds (Figure 3.2A). As shown in Figure 3.2C and Figure 3.2D, the abundance of reactive amino groups on sRBC made particle labeling intense and homogeneous.

The excitation (solid lines) and emission spectra (dotted lines) of the labeled particles (DACCA:sRBC) and of DiBAC₄(5) are shown in Figure 3.2B. The bandwidths used to measure FRET are also indicated. Of note are the considerable overlap between the donor emission and acceptor excitation spectra, predicting robust energy transfer, and the large (≈250 nm) Stokes shift between excitation and sensitized-FRET emission, which minimizes bleed-through of donor emission. Importantly, the fluorescence of both DiBAC₄(5) and DACCA:sRBC is nearly pH-insensitive over a range of pH (4.5 to 7.5), which encompasses the conditions encountered by the particles within phagosomes (Figure 3.3A).
A

\[
\begin{align*}
\text{(CH}_3\text{CH}_2\text{)}_2\text{N} & \quad \text{O} \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{N} & \\
\text{(CH}_3\text{CH}_2\text{)}_2\text{N} & \quad \text{O} \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{N} & \\
\end{align*}
\]

+ \text{H}_2\text{N} \quad \xrightarrow{\text{PBS}} \quad \text{pH 8.7}

\[
\begin{align*}
\text{(CH}_3\text{CH}_2\text{)}_2\text{N} & \quad \text{O} \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{N} & \\
\text{(CH}_3\text{CH}_2\text{)}_2\text{N} & \quad \text{O} \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{N} & \\
\end{align*}
\]

B

Fluorescence (a.u.)

![](Wavelength (nm))

C

DACCA

DiBAC\(_4\)(5)

D

DIC

cFRET

DIC

cFRET
Figure 3.2 Characterization of the FRET donor and acceptor. (A) sRBC were labeled by incubation with the succinimidyl ester derivative of DACCA. (B) Excitation (solid) and emission (dotted) spectra of DACCA:sRBC (black) and oxonol (grey). The grey bars indicate the excitation and emission bandwidths used to visualize FRET. (C and D) Labeled sRBC on a coverslip were incubated with DiBAC₄(5) (C) or without (D). Images acquired in the DACCA, DiBAC₄(5) or cFRET channels, and the corresponding DIC images are illustrated. Scale bars = 10 µm.
Figure 3.3 (A) pH sensitivity of DACCA:sRBC and DiBAC₄(5) fluorescence. Emission spectra of DACCA:sRBC and DiBAC₄(5) were acquired in solutions of varying pH using a fluorescence spectrophotometer. The peak fluorescence intensity of the emission spectra (in arbitrary units) at varying pH is shown. No spectral shifts were observed as pH varied (data not shown). (B) A suspension of DACCA:sRBC was titrated with increasing concentrations of DiBAC₄(5) (in nM) and emission spectra acquired while exciting the donor at 420 nm. A decrease in emission of the donor along with a corresponding increase in emission of the acceptor were observed indicating the development of FRET in an acceptor-concentration dependent fashion.
Figure 3.4 FRET in live macrophages. RAW cells were allowed to internalize either labeled (A-C) or unlabeled (D-F) sRBC, equilibrated with 250 nM DiBAC₄(5), and imaged. DIC (A and D), acceptor (B and E), and cFRET (C and F) fluorescence images of a representative field are shown. DiBAC₄(5) distributes throughout the macrophage (B and E) in contrast to the cFRET, which is restricted to phagosomes (C). The insets in (C) and (F) show donor signal. Scale bars = 10 µm.
When a suspension of DACCA:sRBC was titrated with increasing amounts of DiBAC$_4$(5), a decrease in emission of the donor along with a corresponding increase in emission of the acceptor was observed (Figure 3.3B), indicating that the fluorophores undergo FRET. Similarly, when labeled sRBC were visualized microscopically and incubated with DiBAC$_4$(5), a FRET signal was readily detectable. The emission detected in the FRET channel and corrected for any bleed-through (cFRET) is shown in the inset of Figure 3.2C. As expected, fluorescence in the FRET channel was negligible when either the donor (inset, Figure 3.2D) or acceptor alone (not shown) were present.

Having confirmed the effectiveness of the FRET pair, I proceeded to measure phagosomal membrane potential ($\Psi_{\phi}$). Cultured murine macrophages of the RAW264.7 line (hereafter referred to as RAW cells) were allowed to internalize either DACCA-labeled or unlabeled IgG-opsonized sRBC for 5 min. After removing excess particles, any adherent (not internalized) sRBC were hypotonically lysed and the formed phagosomes then left to mature for 15 min. Prior to imaging, 250 nM DiBAC$_4$(5) was added to the medium and allowed to equilibrate across cellular membranes. The oxonol could be seen throughout the macrophage (Figure 3.4B and Figure 3.4E), but the signal emanating from phagosomes was difficult to discern. However, in macrophages that ingested DACCA:sRBC, a cFRET signal was clearly detectable and restricted to the phagosome (cf. Figure 3.4C and Figure 3.4F). Note that the intensity of the cFRET signal varies between phagosomes even within the same macrophage, indicating an inherent heterogeneity.

Because the amount of DACCA associated with the labeled sRBC is fixed and virtually constant, the cFRET signal will vary primarily as a function of the concentration of DiBAC$_4$(5) in their immediate vicinity. Therefore cFRET fluorescence is, in fact, an indirect measure of $\Psi_{\phi}$. I devised a calibration procedure to deduce the magnitude of $\Psi_{\phi}$ from the cFRET intensity. The rationale applied to the calibrations is illustrated schematically in Figure 3.5A. I envisage the system as consisting of three compartments: (1) the extracellular medium, (2) the cytosol and (3) the intra-phagosomal (luminal) compartment, separated by two membranes. An electrical potential difference ($\Psi_{PM}$) is known to exist across the plasma membrane, which separates compartments (1) and (2), while a distinct potential ($\Psi_{\phi}$) is presumed to exist across the phagosomal membrane. The concentration of the oxonol in each of the compartments will be
dictated by these two potentials, which are in series. Specifically, for a given extracellular concentration of oxonol ($C_1$), the free cytosolic concentration ($C_2$) at equilibrium is strictly a function of $\Psi_{PM}$. In turn, the free intraphagosomal concentration ($C_3$) will be a function of both $C_2$ and $\Psi_\Phi$. Assuming that the free oxonol partitions across the membranes according to the Nernst equation, the relationships between the five variables can be explicitly described by the equations:

$$C_2 = C_1 \exp\left(- \frac{z F \Psi_{PM}}{R T}\right)$$ and

$$\Psi_\Phi = \frac{R T}{z F} \log\left(\frac{C_2}{C_3}\right)$$

where $R$, $T$, and $F$ have their usual meaning, and $z = -1$, the charge of DiBAC$_4$(5). Hence, $\Psi_\Phi$ can be calculated if the values of $C_2$ and $C_3$ are defined. The value of $C_3$ was determined by an external calibration, performed by measuring the cFRET intensity recorded from free DACCA:sRBC while titrating with increasing amounts of DiBAC$_4$(5) in vitro, i.e. in the absence of RAW cells. The resulting calibration curve was then used to calculate the intraphagosomal DiBAC$_4$(5) concentration from the phagosomal cFRET signal recorded in any given phagocytosis experiment. Figure 3.5B presents the calibration curve obtained by averaging 14 individual external titration experiments. The dotted line represents the logarithmic least-squares best-fit ($R^2 = 0.991$) used to interpolate $C_3$ from measured cFRET values.

$C_2$ can be computed knowing $C_1$ and $\Psi_{PM}$. The former is equal to the amount of oxonol added to the bathing medium. $\Psi_{PM}$ was determined empirically by two separate methods (Figure 3.5C). First, I measured $\Psi_{PM}$ electrophysiologically, patching macrophages in the cell-attached configuration and recording in the current-clamp mode (136). These measurements yielded an average potential of $-69.1 \pm 3.7$ mV ($n = 10$ cells; Figure 3.5C). Secondly, I used DiBAC$_4$(5) and a cation ionophore calibration procedure (13) to estimate $\Psi_{PM}$ fluorimetrically (a representative calibration curve is shown in Figure 3.1). The potential estimated by this method averaged $-69.7 \pm 5.7$ mV ($n = 10$ experiments; Figure 3.5C). These measurements are not only
in good agreement with each other, but also with earlier determinations of RAW macrophage membrane potential (137).

Using these parameters, I proceeded to estimate $\Psi_\Phi$. Two different extracellular DiBAC$_4$(5) concentrations ($C_1 = 125$ and 250 nM) were used in order to minimize possible confounding effects caused by dye depletion – due to binding at low concentrations – or by toxic effects or reduced signal-to-noise ratio caused by excess free dye. Using the external calibration curve in Figure 3.5B I calculated a $\Psi_\Phi$ of $+27.2 \pm 5.1$ mV (lumen relative to the cytoplasm) when using 125 nM dye ($n = 8$ experiments) and $+27.8 \pm 2.0$ mV ($n = 24$) when using 250 nM (Figure 3.5D). Similar (10-20 mV) inside-positive potentials were proposed to exist in endosomes, based on measurements of chloride concentration and on the assumption that the conductance to this anion is predominant (93).

As the V-ATPase, the primary contributor to phagosome acidification (31), is an electrogenic pump, I assessed its contribution to $\Psi_\Phi$. Proton pumping was arrested by addition of the specific inhibitor concanamycin A, while monitoring the potential. Inhibition of the V-ATPase decreased the voltage by nearly 50%, to $+10.7 \pm 1.4$ mV ($n = 5$) when measured using 125 nM DiBAC$_4$(5) and to $+17.1 \pm 4.7$ mV ($n = 6$) when using 250 nM (Figure 3.5D), implicating the V-ATPase as a major determinant of $\Psi_\Phi$. Nevertheless, the potential across the phagosomal membrane was not entirely dissipated despite complete inhibition of the V-ATPase, implying the existence of other contributing processes.

A diffusion potential generated by an inwardly directed $K^+$ gradient could in principle account for the residual phagosomal voltage. In the macrophages used in this study the intracellular (largely cytosolic) $[K^+]$ averaged $136 \pm 11$ mM (mean ± SE of 4 experiments), as measured by flame photometry (data not shown). The intraphagosomal $[K^+]$, however, has not been definitively established. Measurements in neutrophils using the electron-probe X-ray microanalysis estimated a $K^+$ content of $\approx 300$ mM (138). While the authors of this report assumed that most of the $K^+$ was free, this assumption is contentious, since it implies that the intraphagosomal osmolarity would greatly exceed that of the cytosol. To obtain an independent estimate of the free phagosomal $[K^+]$ I used a null-point titration method based on the use of an electroneutral $K^+/H^+$ exchanging ionophore (see Figure 3.6 for details). These determinations
Figure 3.5 Phagosomal membrane potential measurement. (A) Variables for the calculation of ΨΦ. The system is comprised of the (1) extracellular, (2) intracellular and (3) intra-phagosomal spaces. The concentration of free DiBAC₄(5) in each compartment is indicated as Cₓ, where x denotes the compartment. Compartments 1 and 2 are separated by the plasma membrane and 2 and 3 by the phagosomal membrane, each with a transmembrane potential of ΨPM and ΨΦ, respectively. (B) External calibration used to calculate C₃. cFRET was recorded from free DACCA:sRBC while titrating increasing amounts of DiBAC₄(5). The amount of cFRET measured within the phagosome was then translated into an intra-phagosomal DiBAC₄(5) concentration (C₃) using the empirically generated calibration curve. Each data point represents the mean ± standard errors of the mean (SEM) of 14 individual calibrations; the logarithmic least-squares best-fit used to interpolate C₃ from a measured cFRET value is shown by the dotted line. (C) ΨPM was measured using both the DiBAC₄(5) (solid black bar; n = 10 experiments) and cell-attached patch clamp recordings in current clamp mode (open bar, n = 10 cells from 3 experiments). A representative calibration curve of DiBAC₄(5) fluorescence vs. ΨPM is shown in Figure 3.1. (D) Two different extracellular DiBAC₄(5) concentrations were added to RAW cells following phagocytosis of DACCA:sRBC and cFRET measured. Data illustrate the ΨΦ measured using 125 nM (solid black bars) and 250 nM DiBAC₄(5) (open bars) before (baseline) and after addition of 1 µM concanamycin A. Data are means ± SEM of 8, 24, 5 and 6 experiments, respectively from left to right. Asterisks indicate a statistically significant (p<0.05) difference from the corresponding baseline, calculated using unpaired t-tests.
Figure 3.6 Intra-phagosomal $[K^+]$ measurement by null-point titration. An indirect estimate of the phagosomal $[K^+]$ can be made using the null-point titration method, which uses pH as a surrogate marker of $K^+$. The general scheme of the protocol is shown in (A). Briefly, RAW cells internalized sRBC conjugated to the pH-sensitive dye FITC. After internalization, the intra- and extracellular compartments were equilibrated using bzATP to activate P2X$_7$ receptors (shown in red on the plasma membrane), which open large-diameter pores within the plasma membrane (step 1). The formation of multiple large pores facilitates rapid equilibration of the cytosolic $[K^+]$ and $[H^+]$ with that of the bathing medium. Concanamycin A and trimethylamine are then added to inhibit the V-ATPase and alkalinize the phagosome, respectively (step 2). Note that trimethylamine was used instead of NH$_4^+$, which may interfere with $H^+/K^+$ exchange by nigericin in the subsequent steps. The extracellular pH is carefully set to equal that of the phagosome, and various solutions with defined $[K^+]_o$ are tested. Next, the $H^+/K^+$-exchanger nigericin is added and the magnitude and direction of any resulting changes in phagosomal pH are monitored (step 3). This step is repeated in parallel experiments using varying concentrations of extracellular (extra-phagosomal) $K^+$. Because the proton concentrations are initially equal, any changes in phagosomal pH are driven by and reflect differences in $[K^+]$ between the two compartments. (B) When the phagosomal potassium concentration ($[K^+]_o$) is greater than the extra-phagosomal value, nigericin will induce a net exchange of phagosomal $K^+$ for extra-phagosomal $H^+$, resulting in a decrease in phagosomal pH (left). The opposite occurs when extra-phagosomal $[K^+]$ is greater than intra-phagosomal $[K^+]$, yielding an increase in luminal pH (right). When no change in pH is recorded, *i.e.* at the null point, the $[K^+]$ in the two compartments must be equal (center). (C) Null-point titrations were performed using $[K^+]_o$ of 1, 10, 40 and 140 mM. For each value of $[K^+]_o$ between 3 and 9 individual experiments were carried out. Data are means ± SEM and are joined by dotted lines.
Table 3.1 Summary of $\Psi_\phi$ measurements. $\Psi_\phi$ was measured in otherwise untreated cells following 15 min of phagosome maturation (baseline) and again following treatment with 1 µM concanamycin A (conc. A), 10 mM NH$_4^+$, or both. Values are positive voltages relative to the cytoplasm. Data are means ± SEM for the indicated experiments (n) carried out using 250 nM DiBAC$_4$(5). The statistical significance of the difference ($p$) between $\Psi_\phi$ measured in treated phagosomes compared to the baseline was calculated using unpaired $t$-tests. There is no statistically significant difference between samples treated with concanamycin A alone vs. concanamycin A plus NH$_4^+$ ($p = 0.82$).

<table>
<thead>
<tr>
<th>Condition</th>
<th>$\Psi_\phi$ (mV)</th>
<th>s.e.m.</th>
<th>n</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>27.8</td>
<td>2.02</td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td>+ CCA</td>
<td>17.1</td>
<td>4.70</td>
<td>6</td>
<td>0.03</td>
</tr>
<tr>
<td>+ NH$_3$</td>
<td>59.1</td>
<td>3.29</td>
<td>12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>+ CCA + NH$_3$</td>
<td>15.7</td>
<td>3.83</td>
<td>6</td>
<td>0.0119</td>
</tr>
</tbody>
</table>
yielded a free [K⁺] of ≈20 mM. The direction and magnitude of the cytosol to lumen K⁺ gradient are therefore sufficient to account for – or contribute to – the electrical potential difference that remains following inhibition of electrogenic proton pumping. Confirmation of the presence of a K⁺-conductive pathway must await direct measurement by electrophysiological means.

While the contribution of the proton pump to the transmembrane voltage is comparatively modest (≈14 mV) in the steady state, it is anticipated to be larger at earlier stages of acidification. This prediction stems from the observation that the rate of acidification recorded immediately after phagosome sealing (Figure 3.7) is much greater than the initial rate of proton leakage unmasked by concanamycin in the steady state (Figure 3.8D), which is in turn equivalent to the pumping rate at that stage. This reduction in the turnover of the V-ATPase is caused, at least in part, by the progressive build-up of a pmf across the phagosomal membrane, which opposes the ATP-driven translocation of protons. Allosteric inhibition by luminal H⁺ may also contribute to the slowdown. Regardless of the mechanism, I was able to demonstrate an increased contribution of the V-ATPase to the membrane potential by clamping the phagosomal pH at a level above the normal steady state. This was accomplished using NH₃, a membrane-permeant weak base. Addition of 10 mM NH₄Cl, which is in equilibrium with 90 µM NH₃ at the pH used, leads to a rapid and sustained elevation of the phagosomal pH (not shown). This alkalinization was accompanied by an increase in the phagosomal membrane potential to a value of +59.1 ± 3.3 mV (n = 12) relative to the cytoplasm (Table 3.1). The hyperpolarization was eliminated by concanamycin A, reflecting increased V-ATPase activity (Table 3.1). Therefore, the phagosomal membrane potential is likely to vary during the course of phagosomal maturation, being maximal at the earliest stages and declining as the pumping rate is reduced by the luminal acidification.

By combining the phagosomal voltage recordings with independent measurements of the transmembrane pH gradient I was able to estimate directly the pmf across the phagosomal membrane. Measurements of the pmf can then be applied to better formulate the energetics of the V-ATPase. Determinations of the luminal pH were made using sRBC covalently labeled with a pH-sensitive fluorescent dye (Figure 3.8A and Figure 3.8B). As reported earlier (12, 13, 31), I found that phagosomal acidification commences shortly after sealing and within minutes reaches a steady value that is sustained for an extended period (Figure 3.7). In 37 determinations...
performed 15-25 min after particle ingestion the phagosomal pH averaged 5.22 ± 0.03. In parallel, I also determined the cytosolic pH using SNARF-5F. These measurements yielded a pH of 7.37 ± 0.03 (n = 7 experiments, each measuring ≥40 cells) for the cytosol of RAW cells and therefore a ΔpH of 2.15 pH units across the phagosomal membrane. From these measurements the pmf across the phagosomal membrane was calculated to reach 15.4 KJ/mol at steady state (15-25 min after particle ingestion), 2.7 KJ/mol of which is contributed by the electrical component.

Our calculations of the pmf enabled us to assess whether the proton pump is at or near electrochemical equilibrium when the phagosomal pH reaches a steady value. The maximum pmf that can be generated by the V-ATPase can be calculated from the free energy of ATP hydrolysis, which under the conditions prevailing in the cytosol approximates 58 KJ/mol (80). At large pH gradients it is assumed that the enzyme complex translocates approximately two protons for every ATP hydrolyzed (40, 139), in which case the pmf cannot exceed 29 KJ/mol. This value is nearly twice as large as the pmf I determined experimentally, implying that the coupling of the overall process is imperfect. This results in part from incomplete efficiency of the pumping reaction (i.e. some of the energy of ATP is dissipated as heat during the pumping cycle) and/or from the presence of a significant H⁺ (equivalent) leak at steady state, in which case the V-ATPase would not attain electrochemical equilibrium. The latter possibility was addressed pharmacologically. As shown in Figure 3.8C, blocking the V-ATPase with concanamycin A while monitoring the luminal pH caused a slow yet reproducible alkalinization, indicative of an ongoing H⁺ leak. Together, these observations indicate that the phagosomal pH is not at thermodynamic equilibrium but in a steady state dictated by endogenous H⁺ (equivalent) leak pathways, as others had concluded earlier (13).

For pH to be invariant, the magnitude of the leak flux must be identical to the rate of pumping. I was therefore able to gain further insight into the properties of the phagosomal V-ATPase by more precisely quantifying the leak in the steady state. The leak flux was calculated as the product of the rate of pH change times the phagosomal buffering power, which was determined to be 113.8 ± 4.6 mM/pH (n = 26) by pulsing with weak electrolytes as described (118). This large value is likely attributable to the high protein (mostly hemoglobin) concentration of the red cells used as phagocytic targets. Assuming an average phagosomal diameter of 4 µm, I calculate the leak flux to be $1.92 \times 10^{-10}$ moles/cm²/min. At steady state, the V-ATPase must therefore be
Figure 3.7 Intra-phagosomal acidification kinetics. Zymosan particles or sRBC were labeled with the pH-sensitive dye FITC and opsonized. The particles were deposited onto RAW cells by centrifugation at 1200 rpm for 1 min in cold media to allow for particle binding but not internalization. The cells were placed on ice and extensively washed to remove any loosely associated particles. Next, the cells were incubated with medium pre-warmed to 37°C to initiate phagocytosis of adherent particles. Phagosomal acidification was monitored by fluorescence ratiometric imaging as described in the text. Representative kinetic profiles of phagosome acidification are shown for both FITC:zymosan (red) and FITC:sRBC (green) phagosomes. Of note, phagosomal acidification commences quickly after sealing and reaches, within minutes, a steady value that is sustained for an extended period. The slight difference in kinetic profiles presumably reflects differences in the buffering power of the respective particles (sRBC > zymosan particles).
Figure 3.8 Energetics of the V-ATPase. FITC-labeled sRBC (A) added to RAW cells as phagocytic prey (inset) were used for phagosomal pH measurements. Phagocytosis was allowed to proceed for 5 min followed by a 15 min maturation period before ratiometric imaging. Following acquisition of experimental data, an *in situ* calibration was performed. A representative calibration curve is shown in (B). An average baseline luminal pH of 5.22 ± 0.03 (n = 37 experiments) was measured. (C) Typical pH profile of an individual phagosome. After acquisition of the baseline pH, concanamycin A was added to inhibit the V-ATPase and the increase in pH monitored. NH₄Cl (2 mM) was added to transiently alkalinize the phagosome in order to compute the buffering power, followed by *in situ* calibration. (D) Phagosomes were allowed to form and reach a steady state pH. Next, where indicated by the arrow, the V-ATPase was inhibited by addition of 1 µM concanamycin A and FCCP was added at the specified concentration, as recording continued. Traces are representative of 3 to 5 experiments for each condition. (E) The rate of alkalinization as a function of FCCP concentration, from experiments like that in D. Data are means ± SEM of ≥25 phagosomes from at least 3 separate experiments. Inset shows the Eadie-Hofsteet linearization of the data.
pumping a $\text{H}^+$ flux of identical magnitude in the opposite direction. Because the pump is electrogenic, this is equivalent to a current of $3.09 \times 10^{-3}$ pA/$\mu$m$^2$. By combining these measurements of pump current with earlier determinations of the turnover rate of the V-ATPase I can estimate the number of active V-ATPases in the phagosome. Phagosomes formed by ingestion of sRBC were calculated to contain 2300 V-ATPases. This calculation assumes a turnover rate of approximately 200 ATP/sec (M. Forgac, personal communication) and a stoichiometry of 2$\text{H}^+$ per ATP (139), and likely provides a lower limit estimate of phagosomal V-ATPase density.

Though measurable, the contribution of $\Psi_{\Phi}$ to the phagosomal $\text{pmf}$ is comparatively small (2.7 KJ/mol of 15.4 KJ/mol). This observation suggests the existence of a sizable counter-ion conductance. To test this notion experimentally, I estimated the counter-ion permeability indirectly by measuring the rate of dissipation of the phagosomal pH gradient. As shown in Figure 3.8C and Figure 3.8D, the spontaneous rate of alkalinization following addition of concanamycin is slow. Addition of the conductive protonophore FCCP markedly accelerated the alkalinization in a concentration-dependent manner (Figure 3.8D and Figure 3.8E), implying that the dissipation of the pH gradient upon inhibition of the V-ATPase is limited by the low intrinsic permeability to $\text{H}^+$. The effect of FCCP is saturable, reaching a maximal rate of alkalinization at $\geq 10$ µM (Figure 3.8E). Because the rheogenic flux induced by FCCP must be matched by an identical counter-ion flux to preserve electroneutrality, measurements of the rate of alkalinization can therefore be used to assign a lower limit to the counter-ion conductance. A maximum rate of alkalinization of 0.11 pH/min was calculated by linearizing the data using the Eadie-Hofstee formulation (Figure 3.8E inset). Considering the buffering power and surface area of sRBC phagosomes, I estimated the counter-ion current to be at least $13 \times 10^{-3}$ pA/$\mu$m$^2$. At the high concentrations of protonophore used, $\Psi_{\Phi}$ can be assumed to approach the $\text{H}^+$ reversal potential, which can be calculated from the determinations of intraphagosomal and cytosolic pH. Using this calculated $\Psi_{\Phi}$, I estimate the counter-ion a conductance to be 0.089-0.11 pS/$\mu$m$^2$. The current carried by this conductance greatly exceeds the current generated by the V-ATPase in the steady state ($3.1 \times 10^{-3}$ pA/$\mu$m$^2$), indicating that pumping is not limited by the counter-ion permeability.
The latter conclusion is consistent with observations made using valinomycin, a conductive K⁺ ionophore. Addition of valinomycin to phagosomes that had reached a steady-state pH did not promote further acidification, even when the lumen contained a high [K⁺] due to the presence of K⁺-rich solution during phagocytosis. Moreover, the rate of dissipation of the pH gradient following inhibition of the V-ATPase was similarly unaffected by valinomycin (not shown), confirming that the endogenous counterion conductance exceeds the H⁺ leak permeability and that the latter limits the rate of pH change.

Based on the preceding observations, I propose the following model to account for the acquisition and maintenance of the steady-state phagosomal pH. Shortly after closure, phagosomes acidify rapidly because the V-ATPase operates unhindered and the back-leak of H⁺ equivalents is minimal. The counter-ion conductance is plentiful so that, while a measurable voltage develops, it does not halt pumping because its contribution to the \( \text{pmf} \) is small compared to the energy of ATP hydrolysis (2.7 KJ/mol vs. 29 KJ/mol of H⁺ pumped). As the phagosomal lumen becomes acidic the \( \text{pmf} \) grows, progressively antagonizing the activity of the V-ATPase. An allosteric inhibition of the pump caused by the acidic luminal pH may also occur. In parallel, the magnitude of the passive H⁺ leakage increases, as the outward [H⁺] gradient grows. Eventually, the rates of pumping and leakage become identical, at which point a steady-state pH is attained. Note that at this stage the membrane potential is small, indicating that counter-ion conductance is not an important determinant of the maximal phagosomal acidification. This conclusion applies to normal macrophages, but not necessarily to cells with abnormally decreased counter-ion permeability, as in the case of CFTR mutants where the reduced anion conductance may limit proton pumping, as described recently by Di et al. (21).

Lastly, I believe that the FRET-based assay introduced here to determine phagosomal membrane potential can be modified and extended to measure the membrane potential of other endomembrane compartments that have remained heretofore inaccessible by other approaches. Such measurements would improve our understanding of ion transport and pH homeostasis in both the secretory and endocytic pathways.
Chapter 4

4 Discussion

4.1 General discussion

The maintenance of an appropriate and differential ionic composition within the cell and its intracellular compartments represents a pervasive challenge for all organisms. To this end, cells have evolved a multiplicity of mechanisms – including pumps, transporters, and channels – that work in concert to establish and preserve this finely tuned organization. Inherently associated with the formation of these gradients is the development of transmembrane electrical potential differences. Together, the chemical and electrical gradients contribute to a variety of cellular processes, including volume regulation and metabolite transport.

It is important to emphasize, however, that ion movement and voltage changes are relevant not only at the level of the plasma membrane, but at the organellar level as well. Stringent modulation of the internal pH in a wide variety of endomembrane compartments underscores the broad importance of ion transport and membrane potential as primary determinants of normal cellular function. This salient feature of cell physiology has been highlighted above in the context of lysosomes and phagosomes, but is certainly not exclusive to these organelles. Along the endocytic and phagocytic pathways the luminal pH drops as vesicles mature from the early endosome to the terminal lysosome. This contrasts with the secretory pathway where the extent of acidification increases as vesicles mature towards the plasma membrane. The lumen of the endoplasmic reticulum is near neutral (140), while the pH decreases through the Golgi stacks (141-143) reaching an acidic maximum in the secretory vesicles (144). The matrix of the mitochondrion, on the other hand, is maintained at an alkaline pH (145, 146).

The functional implications of these varied degrees of acidification are far-reaching. For examples, the slightly alkaline mitochondrial matrix aides in the establishment of the pmf necessary to generate ATP; the redistribution and degradation of internalized plasma membrane receptors and ligands are contingent on the acidification of the lumen of early endosomes (29); and within the secretory pathway, protein post-translational modification and sorting require a progressively acidic lumen (144, 147, 148).
Yet, in stark contrast to the extensive analysis of protein and lipid signaling networks (149-152), organellar ion transport and membrane potential remain neglected aspects of cell physiology and disease pathobiology, in part due to the surprising scarcity of techniques to investigate ion flux and voltage across endomembrane compartments in live cells. This thesis hence described new methodologies to study organellar ion transport and membrane potential in situ in the context of phagolysosome acidification. The work’s two parts will be discussed separately, beginning with the counter-ion conductance involved in lysosomal acidification and followed by the determination of the phagosomal membrane potential. Particular focus will be placed on the biological implications of the findings obtained with the new approaches and on the expandability of the newly developed techniques to other systems. As a case in point, an approach to measure endosomal membrane potential using a FRET-based protocol derived from that designed for the phagosome is discussed.
4.2 Counter-ion conductance for lysosomal acidification

Lysosome acidification by the electrogenic V-ATPase is absolutely necessary for the normal degradative function of lysosomes. The electrogenicity of the V-ATPase mandates that a parallel ion pathway dissipate any membrane potential that would resist a substantial proton accumulation. The general consensus has been that the influx of anions enables acidification by rendering proton transport electroneutral. Indeed, deficient anion channel function in CF has been suggested as the mechanism underlying attenuated lysosomal acidification and impaired microbial killing (21). To assess the individual contribution of counter-ions to acidification, I devised methods to reversibly and separately dialyze the cytosol and the lumen of lysosomes in intact cells, while monitoring lysosomal pH. Replacement of cytosolic Cl⁻ with impermeant anions did not significantly alter the rate or extent of proton pumping. In contrast, the presence of permeant cations in the lysosomal lumen was required for acidification, a notion supported by measurements of the aggregate content of monovalent cations within the lysosome lumen. Because cations are the main counter-ion for lysosomal proton uptake, CF cells are not expected to contain alkaline lysosomes. Accordingly, the lysosomes of alveolar macrophages were found to acidify normally in CFTR-deficient cells. I thus propose that cations are the primary counter-ions responsible for lysosomal acidification and that impaired lysosomal anion conductance cannot explain the diminished microbicidal capacity of CF phagocytes.

4.2.1 Historical perspective

A general historical perspective of counter-ion conductance in endosomes, lysosomes, and phagosomes provides a basis to contextualize these results. A majority of the initial studies used purified endomembrane compartments to evaluate the charge dissipation pathway accompanying the proton pump. Such studies were quick to involve parallel Cl⁻ movement with proton translocation (85, 86, 88, 90, 153, 154). More recently, in live cells, studies employing combinations of pharmacological and ion-substitution approaches alongside organellar pH measurements have largely focused on endosomes, and have also implicated chloride as the principal counter-ion that dissipates the endosomal membrane potential generated by the V-
ATPase. In some cases, the intracellular members of the ClC chloride channel family have been proposed as the primary chloride conductance pathway.

The highly conserved mammalian ClC protein family consists of 9 members with differing tissue and subcellular distributions (reviewed in (155)). The intracellular ClC members are represented by ClC-3 through ClC-7 and are thought to reside primarily in endosomes, lysosomes and the synaptic vesicles of neurons (156). Notably, the ClC-4, ClC-5, and ClC-7 isoforms have been shown to function not as channels but as Cl-/H+ exchangers (99, 129, 157), with a presumed 2 Cl- /H+ stoichiometry (155, 158). It is likely that the vesicular ClC family members all demonstrate Cl-/H+ exchanger activity (155); nevertheless, establishment of the physiological relevance of ClC isoforms is still pending especially given that such a stoichiometry would require the cell to consume more metabolic energy than a simple conductive pathway to generate the same degree of lysosome acidification should lysosomal ClC proteins work as the primary route for counter-ion transport.

Multiple lines of evidence implicating ClC isoforms in endosomal acidification exist. Cells deficient in endosomal ClC-5 exhibit a partial defect in the acidification of early endosomes, whereas late endosomes are unaffected (100, 159, 160). Notably, non-specific inhibition of chloride channel activity decreased early endosomal acidification comparably between wildtype and ClC-5 knockout cells, suggesting chloride conductances other than ClC-5 are present in early endosomes (100). One such candidate is ClC-4, which is thought to have a similar intracellular distribution to ClC-5, and is involved in early endosomal acidification (103). ClC-3 has equally been shown to contribute to the acidification of various vesicular compartments including early and late endosomes (101) and synaptic vesicles in neurons (104). In transfected cells, ClC-3 localizes to both endosomes and lysosomes and mediates a hyper-acidification of the organelar lumen in these over-expression systems (101, 102). On the other hand, ClC-6 and ClC-7 are broadly expressed ClC family members that localize to the lysosomal membrane (114, 115), and yet cells genetically deficient in either do not show impaired organellar acidification (116, 117). These last studies involving ClC-6 and ClC-7 argue against a general role for ClC family members as compensatory electrical shunts for the V-ATPase, especially with regards to the lysosome. The data regarding ClC-7, however, are conflicting as siRNA knockdown of this chloride transporter was reported to dissipate the lysosomal pH gradient in cultured cells (99).
Thermodynamic implications should also be respected when evaluating the role of ClC-7, for example, in lysosome acidification. Consider the somewhat improbable scenario that ClC-7 represents the sole Cl\(^-\) permeation pathway across the lysosomal limiting membrane and that it behaves with the suggested 2 Cl\(^-\) per proton stoichiometry (155, 158). If I reasonably assume a lumen-to-cytosol pH gradient of 2.5 and that the transmembrane potential is fully dissipated (0 mV), then the luminal Cl\(^-\) concentration would be on the order of 200 mM if the system attains thermodynamic equilibrium. Of course, in the case where the V-ATPase (or an alternative electrogenic mechanism) drives the lysosomal voltage to non-zero values (positive inside), even more Cl\(^-\) would enter into the organelle. The osmotic burden would be significant and impart a formidable challenge to the organelle. While the above scenario is built on admittedly simplistic assumptions, it does further indicate the complications associated with attributing acidification counter-ion flux exclusively to the ClC transporters.

Yet, our study surprisingly found cytosolic anions to be expendable for lysosome acidification, instead placing the responsibility on luminal cations. Amid the evidence that generally (but not exclusively) implicates intracellular chloride in organellar acidification, support for cations as the dissipating charge carriers can also be found.

Several \textit{in vitro} studies have investigated ion membrane permeabilities and counter-ion contributions using combinations of ion substitution and ionophore treatment protocols on purified endosomes and/or lysosomes. The organelles of interest were pre-labeled with a pH-sensitive fluorophore and then purified from either cell culture lysates or from rat liver homogenate using density gradient centrifugation (84, 87, 130). Acidification was initiated through the addition of exogenous MgATP to activate membrane-resident V-ATPase enzyme complexes and monitored by changes in the pH indicator present within the purified vesicles.

These cell-free systems uncovered differences in cation and anion permeabilities across the endocytic pathway. Endosomes from rat liver homogenate were found to have an intrinsic K\(^+\) permeability (84). In the presence of Cl\(^-\), however, extra-organellar K\(^+\) had little to no impact on endosome acidification (84, 87). Lysosomes from cultured macrophages were also shown to contain a native K\(^+\) permeability, which was larger than the endogenous proton leak (130). In fact, lysosomes from rat liver were found to be more permeable to K\(^+\) than endosomes, but less
permeable to Na\(^+\) or Cl\(^-\) (87). While Cl\(^-\) was capable of contributing to MgATP-dependent acidification charge compensation in lysosomes, lysosomes were shown to have a limited Cl\(^-\) permeability (87). Decreased lysosome acidification in Cl\(^-\)-free medium when NMDG\(^+\) or Na\(^+\) replaced K\(^+\) highlighted the relative importance of K\(^+\) over other cations in lysosomes acidification (87). These data suggest that in contrast to the case in endosomes, cations – K\(^+\) in particular – play a more prominent role than Cl\(^-\) in facilitating proton pumping in lysosomes.

Supporting evidence that implicates luminal cations as the voltage shunt in acidification is also found within the phagocytosis literature, which functions as a proxy for the endocytic pathway through the phagosome maturation sequence. Using cultured macrophages, Lukacs et al. examined the effect of pharmacological Cl\(^-\) channel inhibition on protonophore-induced phagosome alkalinization following V-ATPase inhibition (13). Cl\(^-\) inhibition resulted in only a partial block in H\(^+\) efflux. Preliminary electrophysiological experiments on excised plasma membrane patches (used as a surrogate of the phagosome membrane) showed little Cl\(^-\) channel activity (13) suggesting either that phagosomes have few Cl\(^-\) channels, the channels are delivered to the phagosome by endosomes, or are simply quiescent in the PM and become activated in the maturing phagosome. These results were loosely suggestive of a parallel cation permeation pathway. This possibility was corroborated by the observation that phagosome acidification occurs when Cl\(^-\) is depleted from cells by electropimulation in K-glucuronate solution. Moreover, K\(^+\) channels blocked by quinine impeded the proton leak by 50\% even though quinine is a weak base, which would be expected to further alkalinize the organelle (13). Together, these \textit{in situ} data signal, albeit indirectly, that K\(^+\) channels may be present within the phagosomal and thus endosomal/lysosomal membranes.

Unlike the multiple anion channels implicated in acidification processes, few specific cations channels have been proposed as contributing a V-ATPase counter current. One candidate was recently identified in \textit{Drosophila}, where the Big Brain (Bib) protein, first recognized as a determinant of cell fate during development (161, 162), was recently shown to be required for endosomal maturation by mediating luminal acidification (163). Cells deficient in Bib exhibited enlarged endosomes arrested in an early stage with alkaline pH (163), a phenotype analogous to that described in alkalinized endosomes of cultured macrophages ((50); K.K. Huynh and S. Grinstein, unpublished observation). Interestingly, Bib belongs to the ion-conducting aquaporin family of channel proteins. Of note, exogenous expression of these channels in \textit{Xenopus} oocytes
imparts a non-selective monovalent cation channel (164). Notably, the \textit{in vivo} coordination of endosome maturation by Bib is completely abolished by a channel-blocking amino acid substitution (163). These data identify Bib as a putative cation counter-ion conductance in the endosomal maturation pathway. Weaker evidence has suggested that the voltage-gated \( \text{Na}^+ \) channel \( \text{NaV}1.5 \) is expressed on the late endosome in macrophages, where it may modulate vesicle pH by mediating compensatory \( \text{Na}^+ \) efflux (165).

It should lastly be noted that from an experimental perspective, the manipulation of cytosolic anions is more readily achieved than that of luminal cations. As a result, studies implicating luminal cations as counter-ions for organelle acidification processes may simply be under-represented for technical reasons.

### 4.2.2 Organellar acidification counter-ion transport and disease

While our work implicates cations as the voltage-dissipating charge carriers, the principal question remains: what channel and/or transporter within the lysosome provides the counter-ion current? This is not purely an academic problem, as impaired counter-ion flux – and thus organelle acidification – can result in disease. Two examples illustrate the importance of the relationship between impaired counter-ion transport in organellar acidification and clinically relevant pathology.

The first and more fully characterized example is Dent’s disease, an X-linked hereditary disorder where patients suffer from tubular proteinuria with a variable degree of nephrolithiasis and nephrocalcinosis (166). Mutations in the gene encoding CLC-5 result in this condition, with more than 40 different mutations having been identified to date (155, 167, 168). The disease pathophysiology and corresponding involvement of organellar acidification have largely been elucidated through knockout mouse models (113, 160, 169). These models demonstrated that there is a broad endocytosis defect encompassing both fluid-phase and receptor-based cargo within the kidney (155). The deficiency was ascribed to impaired acidification in renal endosomes due to the absence of an electrical shunt for proton transport by the V-ATPase (159, 160). As outlined in the Introduction, without an appropriate acidification, normal progression along the endocytic pathway becomes compromised. The low molecular weight proteinuria
specifically manifests through decreased retrieval of filtered proteins by megalin, an apical protein receptor. In particular, the small peptide parathyroid hormone (PTH), normally removed by megalin-dependent endocytosis (170), remains at elevated levels in the proximal tubule lumen. The abnormal PTH metabolism in Dent’s disease can subsequently impact on tubular and intestinal phosphate and calcium reabsorption, leading to kidney stones (156). The pathogenesis of Dent’s disease thus highlights the consequence of impaired organellar acidification on tissue physiology.

CF represents a second, intensely investigated genetic disorder where defective organellar acidification has been directly implicated in disease pathobiology. The impact of CFTR on endosomal/lysosomal acidification is a surprisingly contentious and confusing area of CF research. Using weak base trapping and fluorescence-based measurements to determine organellar pH in nasal epithelium harvested from individuals with CF, Barasch and colleagues found a mild acidification deficit of 0.5 and 0.4 pH units, respectively, in endosomes and the trans-Golgi network (20). This observation quickly led to the formulation of the “pH hypothesis” of disease pathobiology in CF whereby the resulting organellar dysfunction due to high luminal pH culminated in lung pathology. Subsequent work from multiple laboratories, however, failed to show an alkaline lumen in the organelles of CF cells (105-109). These reevaluations of the “pH hypothesis” quelled the initial enthusiasm for an acidification-based mechanism of disease. Contributing further to the confusion, Deretic’s group has since proposed the converse: that deficient CFTR activity results in the hyperacidification of intracellular organelles (22, 24, 171). Of note, the proposed mechanism by which CFTR mutant cells exhibit enhanced acidification is through increased activity of membrane Na⁺ channels that function as the voltage shunts to the V-ATPase (23). In the wildtype case, CFTR acts to limit the Na⁺ efflux thereby establishing an electrical gradient that tempers proton transport (23). A cation counter-current is thus implicit in their model.

4.2.3 Role of CFTR in lysosome acidification in alveolar macrophages

The recent suggestions that defective lysosome acidification in alveolar macrophages is the root of the heightened and persistent state of inflammation in the lungs of patients with CF (21) has further renewed interest in the “pH hypothesis”. The conclusion that CFTR provides a parallel
Cl⁻ pathway to dissipate the voltage generated by V-ATPase proton pumping, however, contrasts starkly with our own and that of others (26). The basis for the discrepant findings are not overtly obvious, still possible reasons for the difference merit further attention and are discussed below.

Methodological differences, some of which have also been noted by Haggie and Verkman (26), are likely to partially underlie the conflicting results. The measurements in (21) were carried out using fluorescein (pKₐ ≈ 6.4) as the pH sensor. The pH indicator must be appropriately chosen so as to give a sensitive measure of organellar acidification. A pH-sensitive fluorophore with a low pKₐ, such as Oregon Green 514 (pKₐ ≈ 4.8), is better suited for the highly acidic lumen of lysosomes and mature phagosomes as maximum sensitivity is achieved when the pKₐ of the sensor matches the pH of the compartment being investigated.

A second crucial procedural difference lies in the type of fluorescence microscopy used to determine lysosomal and/or phagosomal pH. Di et al. (21) use confocal microscopy whereas others use wide-field setups (see (14, 26, 116, 131, 172) for examples). While confocal microscopes provide the user with a higher spatial resolution, they are not necessarily preferable for analytical measurements of organellar pH. The macrophages have a significant height relative to the thin optical sectioning of the confocal imaging. Both lysosomal and phagosomal compartments are motile within the cell (173) and thus difficult to capture by thin sectioning. Moreover, the use of lasers to excite the sample is known to be more damaging both to the fluorophore and to the biological preparation than an attenuated xenon arc lamp, for example. This becomes particularly relevant when two separate fluorophores, such as the rhodamine-fluorescein dextran conjugate used in (21), are employed to construct a ratiometric probe. The two different fluorophores tend to bleach at very different rates, potentially confounding the measurements and calibration.

Methodological differences notwithstanding, several features of the study by Di et al. were particularly striking and unexpected. First, the lysosomal acidification defect in the CFTR deficient macrophages was approximately two pH units. It is reasonable to anticipate that such cells cannot remain viable because the alkaline lysosomes would functionally hinder the endocytic pathway. As described in the Introduction, endosome maturation necessitates the progressive acidification of its lumen. Even in the case of a comparatively small acidification deficit, as seen in Dent’s disease, the cellular repercussions can be substantial. The second
remarkable aspect reflects the generality of the observation. The defect was only observed in alveolar and not peritoneal macrophages (21), which precludes a more universal role for CFTR as a lysosomal counter-ion conductance. It would be interesting to speculate why an alveolar macrophage has opted to employ CFTR in this capacity while other tissue macrophages have not.

The detailed biophysical mechanism of phagolysosome acidification remains incompletely understood. Nevertheless, a general consensus has been established that describes the kinetics of acidification with respect to the maturation pathway. The macrophage phagosome has been shown to acidify rapidly shortly after closure, prior to fusion with lysosomes (see Figure 3.7 and (13, 31, 81, 174-176)). In their study of alveolar macrophages, however, Di et al. observed a delayed phagosomal acidification, where the phagosomes maintained a pH near 6.8 for upwards of 30 min before fully acidifying upon fusion with lysosomes (21). How or why the phagosome pH was held at a mildly acidic value was not addressed. Regardless, this kinetic profile differs remarkably from the quick and profound acidification described in the literature (13, 14, 31, 81, 174-176).

Ultimately the hypothesis that CFTR functions as a counter-ion conductance in lysosomes requires that the channel be present and functional within the organelle membrane. This assumption may not be valid as CFTR has been shown to be degraded upon entry into this compartment (177). Moreover, should it be present in lysosomes, wildtype CFTR channel activity is markedly inhibited by acidic pH (Figure 2.8), although to a lesser extent than the ΔF508 CFTR mutant (178).

4.2.4 Future directions

Our data demonstrate that cytosolic anions are not the necessary counter-ions for lysosome acidification; a role I propose is held by luminal cations. This result, in combination with pharmacological and genetic data, specifically argues against CFTR acting as the primary electrical shunt to proton transport. The identification of the counter-ion conductances therefore remains an open question. An answer is likely to rely on proteomic studies of the lysosomal membrane. Two early reports provide the first proteomic description of the late endosome (78)
and lysosome membranes (133). While their respective objectives were not specific to identifying putative counter-ion conductance candidates, both studies observed several proteins involved in electrogenic ion transport. These include components of Ca\(^{2+}\) ATPases, voltage-dependent Ca\(^{2+}\) channel subunits, and cationic amino acid transporters (78, 133). As more comprehensive and focused analyses are performed, increasingly extensive lists of candidate ion transport pathways will be identified and consolidated.

In parallel, electrophysiological approaches provide a valuable set of tools to directly probe the biophysical properties of lysosomal membranes. Due to their relatively small size, individual organelles are not readily examined by standard patch-clamp techniques; however, enriched preparations of lysosome-derived vesicles can be made to fuse with an artificial planar lipid bilayer thereby directly incorporating resident transmembrane proteins, such as channels, transporters, and pumps. Current-voltage relationships of lysosomal conductance pathways can subsequently be derived by applying electrical potential differences across the membrane and measuring any resultant current. Moreover, with this setup, the solutions on either side of the membrane (representing the luminal and cytosolic environments) can be manipulated in order to perform ion substitution experiments. The current-voltage profile in coordination with ion substitution and pharmacological approaches can help construct a detailed electrical description of the lysosome membrane. This information could be invaluable in identifying candidate lysosomal cation and anion conductances.

Together, proteomic, cell biological and electrophysiological approaches will begin to unravel the biophysical characteristics of the lysosomal membrane. As the description becomes more complete and putative counter-ion pathways recognized, their ability to function within the harsh lysosomal environment and their role in lysosome acidification must then be investigated.
4.3 Phagosome membrane potential measurements

The acidic phagosomal environment enhances the efficacy of luminal antimicrobial agents. Yet, the determinants of phagosomal pH are not completely understood. Similar to lysosomes, the permeability of the phagosome membrane to ions that neutralize the electrogenic effect of the V-ATPase has been proposed to play a central role. When counter-ion conductance is limiting, generation of a large membrane potential will dominate the $pmf$, with a proportionally diminished pH gradient. In order to validate this notion by direct measurement of the electrical potential that develops across the phagosomal membrane, I devised a non-invasive procedure to estimate the voltage across the phagosome in intact cells, based on FRET. This approach employs a fluorescent dye that partitions across intracellular membranes in a Nernstian fashion as the FRET acceptor and voltage sensor. Covalent labeling of the phagocytic prey with a FRET donor allowed for specific targeting of the phagosome. This FRET-based assay, in combination with measurements of phagosomal pH, enabled us to calculate the $pmf$ across phagosomes of murine macrophages and to analyze the factors that limit acidification. At steady state the transmembrane voltage averaged 27 mV (lumen positive) and was only partially dissipated by inhibition of the V-ATPase with concanamycin A. The comparatively small contribution of the potential to the $pmf$ suggests that proton pumping is not limited by the counter-ion permeability, a hypothesis that was validated independently using ionophores and organellar pH measurements. Instead, phagosomal pH stabilizes when the rate of proton pumping, which decreases gradually as the lumen acidifies, is matched by the passive leak of proton equivalents.

From these data, a model of the acquisition and maintenance of the steady-state phagosomal pH was formulated. Following phagosome closure, the lumen acidifies rapidly because the V-ATPase operates unhindered and the back-leak of $H^+$ equivalents is minimal. A plentiful counter-ion conductance keeps the voltage across the membrane small, but measurable. As a result it does not stop proton pumping because its contribution to the $pmf$ is minimal compared to the energy of ATP hydrolysis. As the phagosomal lumen acidifies further, the $pmf$ grows and begins to increasingly impede the activity of the V-ATPase. In parallel, the magnitude of the passive $H^+$ leakage increases, as the outward proton gradient increases. Eventually, the rates of proton leakage and pumping become equal, producing the steady-state pH. At this stage, the
membrane potential is small, indicating that counter-ion conductance is not an important determinant of the maximal phagosomal acidification.

4.3.1 Open questions and future directions

Several questions related to the modulation of phagosome membrane potential remain unanswered. In particular, a temporal profile of voltage changes across the phagosome over the course of maturation is wanting. Our study focused specifically on steady state measurements within the mature phagosome. Through the use of ionophores and weak bases along with phagosomal pH measurements, I was able to formulate the model of phagosome acidification described above (14). The model predicts that the voltage across the phagosomal membrane may vary as it matures into a microbicidal compartment. This proposal awaits direct verification.

Furthermore, our analyses do not consider the impact of the NADPH oxidase on either the membrane potential or pH gradient across the phagosome membrane as the study was carried out in a macrophage cell line that has a very modest NADPH oxidase response. The NADPH oxidase complex catalyzes the vectorial movement of electrons from the cytoplasm into the phagosome lumen, where they reduce molecular oxygen to form superoxide, which is subsequently converted into other highly reactive oxygen species (179). Individuals without this machinery suffer from recurrent and often life-threatening infections (180). Not surprisingly, the activity of the electrogenic oxidase has been found to be highly voltage-dependent (96), therefore mandating the existence of a parallel counter-ion pathway. The charge compensation is largely carried by protons through voltage-gated proton channels (10, 96, 181, 182). The identity of such channels within the phagosome has yet to be elucidated; nevertheless, two groups have independently described a voltage-sensing domain that was sufficient for expression of a voltage-sensitive and proton-selective channel activity (183, 184) in the plasma membrane of heterologously transfected cells. Due to the importance of the phagocytic oxidase in the innate immune response and its sharp voltage dependence, an accurate measure of phagosomal membrane potential in the presence of the active oxidase would contribute significantly to our understanding of phagosome physiology.
Thus far, the susceptibility of the potentiometric oxonol dyes to photobleaching and oxidation precludes the use of our FRET-based approach towards the measurement of voltage changes in individual phagosomes over the course of their maturation or steady state determinations in the presence of a robust NADPH oxidase response. The limited capacity to generate a kinetic map of phagosomal membrane potential could be partially overcome by making measurements in multiple phagosomes using the FRET assay at various points of maturation. The population-based measurements can then be used to reconstruct a statistically averaged temporal voltage profile. This methodology is not ideal as it relies on the accurate synchronization and homogeneity of phagocytosis and maturation. Notably, the assumption of phagosome homogeneity is debatable (185, 186).

A method to measure the electrical status of individual phagosomes over their complete maturation sequence is thus preferable. In order to reach this objective using the FRET system, it is necessary to minimize or completely avoid the direct excitation of the voltage-sensitive dye so as to prevent its photobleaching. Such a scenario can be made possible by exploiting fluorescence lifetime imaging (FLIM) to monitor the FRET interaction between donor and acceptor molecules. Briefly, our study employed a sensitized-emission FRET protocol whereby the donor, acceptor, and FRET channels were serially acquired. The three acquisitions were then used to calculate a corrected FRET image that accounts for donor and acceptor bleed-through into the FRET channel. It is easy to appreciate that the procedure required the FRET acceptor to be exposed to a significant light burden. In contrast, FLIM can be used to measure the fluorescence lifetime – that is, the average time the molecule stays in its excited state before emitting a photon – of the donor molecule. When two fluorophores are undergoing a FRET interaction, the donor molecule is quenched, which translates into a decreased fluorescence lifetime. It thus follows that the FRET interaction, itself a function of phagosomal membrane potential, can be monitored through the changes in donor fluorescence lifetime without having to directly excite the acceptor molecule. This approach would require that illumination of the FRET donor not overly photobleach the oxonol, a reasonable assumption given the substantial Stokes’ shift between the FRET pair.

It is worthwhile noting that these proposals do not address the problem of acceptor oxidation by the NADPH oxidase enzyme complex. On the other hand, electrophysiological analyses of purified phagosomes containing intact NADPH oxidase offer an alternative approach to
investigate phagosomal electrical properties. Similar to what was described above for purified lysosomes, patch-clamping phagosomes is a formidable technical challenge due to their small size. The fusion of purified phagosomes with planar lipid bilayers may thus provide the appropriate setting to characterize the current-voltage relationship of the phagosome membrane. Nevertheless, phagosomes can be on the order of several microns in diameter when they contain a sufficiently large prey, affording the intriguing prospect of direct patch-clamp analysis. Preliminary work in our laboratory, in collaboration with Dr Michael Salter’s group (Hospital for Sick Children, Toronto, Ontario), has demonstrated that purified phagosomes can be captured in the “phagosome-attached” configuration and subjected to electrophysiological analysis (unpublished observation). While technically demanding, these early data suggest that the patch-clamp technique may serve as a viable option in characterizing the electrical properties of the phagosome membrane.

Clearly, patch clamp or planar lipid bilayer protocols only simulate what is seen in the phagocyte; the solutions within and without the purified phagosome only approximate the native intracellular conditions. For example, soluble cytosolic factors, which may modulate channel, transporter or pump activity, are not necessarily present following the purification protocol. Generating a complete in situ biophysical description of phagosomes, however, represents a presently untenable option. Accordingly, only by coupling in situ measurements with in vitro electrophysiological approaches will a more complete biophysical characterization of phagosome physiology, including its acidification process, be possible.

4.3.2 FRET-based organellar membrane potential measurements

The voltage across the phagosome was assessed using a potentiometric optical dye that partitions across all intracellular membranes. Targeting the FRET partner to the compartment of interest and exploiting the requirement for close proximity in the FRET interaction ensured a measurement that was spatially restricted to the phagosome. It follows that this FRET-based assay of membrane potential could be adapted to other organelles simply by targeting the donor molecule to the luminal aspect of a different intracellular compartment.
There are two general requirements for this system to work in other organelles. First, it is necessary to accurately target one of the FRET components to the organelle of interest. To this end, multiple cell biological tools are available to access and probe different intracellular environments. For example, the B subunits of certain bacterial toxins produced by *Vibrio*, *Shigella*, and enteropathogenic *Escherichia coli* are able to bind glycolipids on the cell plasma membrane. Once bound, they traffic in a retrograde manner to the Golgi before eventually arriving in the endoplasmic reticulum (140, 142). By labeling the subunit with one of the FRET components, the voltage across the Golgi membrane can be evaluated upon addition of the corresponding potentiometric FRET partner. Endosomes offer another, particularly intriguing target for transmembrane voltage measurements. Multiple prospective targeting strategies to reach the endosome lumen exist, including fluid-phase and receptor-mediated mechanisms. For example, fluorescently labeled dextrans enter into the endocytic pathway by fluid-phase uptake, while labeled transferrin binds its plasma membrane receptors in order to be subsequently internalized. Another strategy to label endosomes employing a fluorescently labeled lipid is described in more detail below.

The second requirement is to have an appropriate voltage-sensitive FRET partner. Once the intracellular compartment has been accurately targeted with a fluorophore, the potentiometric FRET partner is added to the bathing medium and allowed to partition across intracellular membranes. It is worth noting that there exist numerous voltage-sensitive oxonol dyes, which have a wide variety of spectral properties. As a result, this technique is not limited to the use of the specific coumarin derivative described in Chapter 3 as the FRET donor. For example, red fluorophores can be targeted to a specific compartment and used in conjunction with a green-shifted potentiometric optical dye.

Several open questions relating to endosomal membrane potential make it an excellent example of a biologically relevant target for organelar voltage measurements. For instance, certain fibroblast growth factors (FGF) and cell-penetrating peptides (CPP) may escape from endosomes into the cytosol in a manner driven by the trans-endosomal membrane potential. This notion remains speculative as direct measurement of this biophysical parameter has heretofore not been feasible.
FGF-1 and FGF-2 mediate a variety of biological responses involving cell growth, proliferation, migration and differentiation. It is well established that these growth factors interact with cell-surface receptors to initiate downstream signaling (187, 188). Nevertheless, several reports further suggest that exogenous FGF-1 and FGF-2 are capable of entering into the cell and nucleus, and that this entry is required to establish a mitogenic response in certain cell types (189-192). The mechanism by which these growth factors translocate across the cell membrane is unclear. The polypeptides bind to the plasma membrane and are internalized into endosomes from which they putatively escape. In one model, endosomal escape of both FGF-1 and FGF-2 is driven by vesicular membrane potential, independent of the acidification status of the endosome (189, 190).

CPP are of particular interest in drug discovery because they are able to mediate the intracellular delivery of a wide variety of bioactive molecules, ranging from peptides to plasmid DNA to liposomes (193, 194). They represent a diverse group of peptides, many of which are highly cationic, hydrophilic, and arginine-rich (195). These include the TAT peptide derived from the transactivator of transcription protein encoded in the human immunodeficiency virus 1. It contains six arginine residues and has been used extensively to deliver cargo into cells (196-198). Homopolymeric arginine peptides, ranging from 5 to 11 residues in length, have also been the focus of many studies as they efficiently translocate across the cell membrane into the cytosol (199, 200).

Despite the abundant evidence that CPP can successfully deliver various types of cargo into the cytoplasm or nucleus of cells, their mechanism of membrane translocation remains elusive. CPP are either endocytosed before translocating into the cytosol (201-204) or enter the cytosol directly through the plasma membrane (198). Regardless, both routes require that the peptides traverse a lipid bilayer. The mechanism by which this occurs remains unknown. Recent evidence purports that the arginine residues form extensive hydrogen bond networks with anionic scavengers on the cell surface, such as phospholipids and/or heparan sulfate proteoglycans (202, 204-206), thereby partially neutralizing the strongly basic character of the peptide (206, 207). In this model, the ion pair complexes then partition into the lipid bilayer and migrate through the plasma membrane when in the presence of a permissive membrane potential (205, 207). Interestingly, the movement from the endosomal lumen into the cytosol has been reported to depend on vesicle acidification (208, 209). Recall, however, that there is a strong
association between the acidification process and transmembrane potential through the electrogenic nature of the V-ATPase. Therefore the dependence on acidification is highly confounded by its association with voltage.

4.3.3 An approach to endosomal membrane potential determinations

The above data implicate the endocytic pathway in the internalization of CPPs, with their actual transmembrane movement being variably reported to depend on the electrical and/or acidification status of the organelle. For these reasons it is worth systematically measuring the effect of endosomal voltage on CPP delivery into the cytosol. Clearly this requires that the endosomal voltage be determined. To this end and as a proof of principle, one possible approach to determine endosomal membrane potential using a FRET-based assay is presented.

The FRET donor was made by covalently labeling the headgroup of phosphatidylethanolamine (PE) with the coumarin derivative DACCA. A member of the oxonol family, bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiSBAC$_2$(3)), was used as the FRET acceptor. The structures and spectral characteristics of the two compounds are shown in Figure 4.1A-B.

To demonstrate that the two molecules can undergo an efficient FRET interaction, liposomes containing the fluorescent lipid were titrated with increasing amounts of the DiSBAC$_2$(3) while recording the emission spectrum following excitation of the donor. As the amount of acceptor increases, the donor peak decreases with a corresponding increase in acceptor emission (Figure 4.1C), indicating that the fluorophores effectively undergo FRET.

The endocytic pathway was targeted by loading the fluorescent lipid into the plasma membrane using methyl-β-cyclodextrin as a delivery vehicle, and then chasing the lipid into the endosomal compartment. The oxonol was added to the bathing medium, allowed to equilibrate across intracellular membranes, and the occurrence and magnitude of FRET recorded.
Figure 4.2 shows the distribution of the DACCA-PE and DiSBAC₂(3) in RAW cell macrophages. In the resting state, very little FRET within the endosome was observed suggesting either that there is a negligible or negative-inside transmembrane potential (Figure 4.3A). To demonstrate that this assay is able to record changes in membrane potential, I exploited the electrogenicity of the V-ATPase, as I did in the course of my analysis of phagosomes (see Chapter 3). By alkalinizing the endosomal compartment with a pulse of weak base, the activity of the V-ATPase is dramatically increased. In the presence of ammonia, there was a significant redistribution of the potentiometric optical dye into the endosome compartment and a parallel increase in FRET signal (Figure 4.3B). The increase in FRET signal was abrogated by the application of CCA, indicating that the change was mediated through proton-pumping by the V-ATPase (Figure 4.3C).

This FRET-based system serves as a starting point to carry out endosome voltage measurements and to characterize the factors that contribute to it. For example the role of V-ATPase and Na⁺/K⁺ ATPase activity in the development and maintenance of endosomal membrane potential can be investigated pharmacologically. In addition, this approach may allow us to devise methods to dissociate the manipulation of voltage from that of pH within the endosome. These tools could subsequently be applied to analyze the contributions of pH and electrical gradients to CPP and FGF translocation into the cytosol.
Figure 4.1 Characterization of the FRET donor and acceptor. (A) The structures of DACCA-PE and DiSBAC$_2$(3). The PE was labeled by incubation with the succinimidyl ester derivative of the DACCA directly in either acetonitrile or dimethylformamide. Following the reaction the product was purified by silica-gel column chromatography. (B) Excitation (solid) and emission (dotted) spectra of DACCA-PE (black) and oxonol (grey). The spectra of DACCA-PE were acquire using phosphatidylcholine liposomes containing 1% DACCA-PE. Oxonol spectra were measured in the presence of phosphatidylcholine liposomes. (C) Emission spectra of the DACCA-PE with increasing amounts of DiSBAC$_2$(3). Note the donor quenching and corresponding enhanced acceptor peak with increasing concentrations of the DiSBAC$_2$(3). In the absence of donor molecules there is no detectable fluorescence, even at 1 µM DiSBAC$_2$(3).
Figure 4.2 Distribution of DACCA-PE and DiSBAC$_2$(3) in live RAW macrophages. (A) RAW macrophages were washed and maintained in serum-free medium at 37 deg. In parallel, approximately 36 µg of DACCA-PE and 5 µg of cholesterol were dried under N$_2$. PBS containing 100 mM methyl-β-cyclodextrin was added to the dried lipid and the solution probe sonicated. Following sonication, 40 µL of the lipid-cyclodextrin solution, corresponding to 9 µg of DACCA-PE, were injected directly into the bathing medium. The cells were pulsed with the fluorescent lipid mixture for 5 min before extensive washing with serum-free medium. The lipid was chased into the endocytic pathway for 15 min in serum-free medium followed by 15 min in medium containing 5% FBS prior to imaging. The serum served to back-extract any fluorescent lipid remaining in the outer leaflet of the plasma membrane. The distribution of the DACCA-PE, along with the corresponding DIC image, of a representative field is shown. (B) RAW cells were incubated with 125 nM DiSBAC$_2$(3) and visualized. Images of the acceptor and DIC channels of a representative field are shown.
**Figure 4.3** FRET in live RAW macrophages. Endosomes were loaded with the DACCA-PE as described for Figure 4.2, equilibrated with 125 nM DiSBAC$_2$(3), and imaged (A). The cells were then pulsed with 20 mM NH$_4$Cl for 2 min in the continued presence of the DiSBAC$_2$(3) and visualized (B). The weak base transiently alkalinizes the endosomal compartment leading to increased V-ATPase activity and thus an increased (positive lumen) membrane potential. Increased partitioning of the oxonol into punctate endosome structures and increased corrected FRET signal reflect the increased voltage. The redistribution of the DiSBAC$_2$(3) into the endosomes is abrogated when the V-ATPase is inhibited by 1 µM CCA (C). DIC, donor, acceptor and corrected FRET (cFRET) images of representative fields are shown in all panels.
4.4 Concluding remarks

Organellar ion transport and membrane potential serve important – albeit oftentimes underappreciated – roles in both normal cell physiology and disease pathobiology, as underscored by the exquisite and indispensable regulation of organellar pH. In this thesis, I have developed novel methodologies for performing ion substitutions within and membrane potential measurements across intracellular organelles in intact, live cells. With these approaches I identified the ions that serve as counter-ions to the electrogenic V-ATPase in the lysosome, and further applied the voltage measurements to the macrophage phagosome in order to analyze the biophysics of this organelle’s profound acidification. Importantly, the resulting data call into question the relevance of the pH hypothesis of CF lung disease with respect to alveolar macrophage lysosome acidification.

Although the details of intracellular acidification processes are slowly becoming more apparent, it is worthwhile emphasizing that considerable gaps in our understanding still exist. This work contributes techniques for cell biologists to work towards a better understanding of the complexity of intracellular physiology. For example, by manipulating the counter-ion permeability and organellar membrane potential, one can alter luminal acidification and potentially modify vesicle traffic and the transport of organic and inorganic solutes, such as calcium. In closing, the development of methods for more robust organellar membrane potential measurements, together with increasingly quantitative imaging and electrophysiological modalities, will enhance and facilitate the study of cellular and organellar physiology.
References


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