Determinants of the Phagosomal pH in Neutrophils*

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Andrzej Jankowski‡, Cameron C. Scott‡, and Sergio Grinstein§

From the Cell Biology Programme, Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada and the Department of Biochemistry, University of Toronto, Toronto, Ontario M5G 1X8, Canada

Phagosomes formed by neutrophils are much less acidic than those of other phagocytic cells. The defective acidification seen in neutrophils has been attributed to consumption of protons during the dismutation of superoxide, because a large, sustained acidification is unmasked when the cells are treated with inhibitors of the NADPH oxidase. Consumption of protons transported into the phagosome by dismutation would tightly couple the activities of the NADPH oxidase and the vacuolar type H+-pump (or V-ATPase). We tested the existence of the predicted coupling using microfluorimetry and digital imaging and found that the rate of superoxide generation was independent of the activity of the H+-pump. Moreover, we failed to detect the alkalinization predicted to develop through dismutation when the pump was inhibited. Instead, two other mechanisms were found to contribute to the inability of neutrophil phagosomes to acidify. First, the insertion of V-ATPases into the phagosomal membrane was found to be reduced when the oxidase is active. Second, the passive proton (equivalent) permeability of the phagosomal membrane increased when the oxidase was activated. The increased permeability cannot be entirely attributed to the conductive H+ channels associated with the oxidase, since it is not eliminated by Zn²⁺. We conclude that the NADPH oxidase controls the phagosomal pH by multiple mechanisms that include reduced proton delivery to the lumen, increased luminal proton consumption, and enhanced backflux (leak) into the cytosol.

A complex and highly organized series of events takes place at sites of tissue injury to limit the extent of microbial infection. Neutrophils are crucial contributors to this host response, as they are the first line of defense against invading microorganisms. When activated by bacterial or inflammatory chemoattractants, circulating neutrophils traverse capillary walls by diapedesis and migrate to the sites of infection, where they recognize and eliminate foreign organisms (1, 2). Phagocytosis, the process of internalization of the microorganisms into a membrane-bound vacuole, is central to the microbicidal response (1, 2). The membrane of newly formed phagosomes displays a composition similar to that of the plasma membrane, while the phagosomal contents are initially similar to the extracellular milieu. However, shortly after sealing, the phagosome undergoes drastic remodeling by fusion with endomembrane compartments, while maintaining its approximate size through coordinated fission events. Such remodeling involves delivery into the phagosomal lumen of a variety of microbicidal agents, including lytic enzymes and cationic peptides, as well as insertion into the phagosomal membrane of proteins that also contribute to the killing and disposal of microorganisms (3, 4). The proteins acquired by the phagosomal membrane during maturation include the NADPH oxidase and the H+-pumping vacuolar ATPase (V-ATPase). The NADPH oxidase, an enzymatic complex consisting of both membrane-bound and cytosolic subunits, assembles at the phagocytic membrane, where it facilitates the transfer of one electron from cytosolic NADPH to molecular oxygen. The resulting superoxide anion and the reactive oxygen metabolites generated therefrom, which include hypochlorous acid and hydroxyl radicals, are potent microbicidal agents (5). The V-ATPase catalyzes the vectorial transport of H⁺ into the phagosomal lumen (6).

Like neutrophils, macrophages also utilize phagocytosis to contribute to the innate immune response (2, 7). In these cells, the acidification of the phagosomal lumen, which has been reported to reach pH 5.5 (6, 8), appears to be stringently required for effective bacterial killing (9). Two lines of evidence indicate that the V-ATPase is responsible for the luminal acidification: (a) the accumulation of H⁺ requires cytosolic ATP (8), and (b) acidification is inhibited by specific V-ATPase inhibitors like bafilomycin and concanamycin (6, 8). As V-ATPases are also inserted into the phagosomes of neutrophils, a comparable phagosomal acidification would be expected in these cells. Remarkably, however, phagosomal pH (pHₚ) in neutrophils has been reported to undergo a biphasic change: an alkalinization occurs during the first few minutes, which is then slowly followed by a modest secondary acidification to pH ~6.5. It is unclear whether this apparent discrepancy resulted from differences in the methodology used or whether phagosomal pH is truly regulated differently in these two cell types.

Segal et al. (10) proposed a mechanism to account for the biphasic pHₚ changes reported in neutrophils. The initial alkalinization was attributed to consumption of H⁺ during the process of dismutation of superoxide to H₂O₂, while the secondary acidification was proposed to reflect the ongoing activity of the V-ATPase, which becomes unmasked when the respiratory burst subsides. Consistent with this notion, in neutrophils from chronic granulomatous disease patients, which lack an active NADPH oxidase, pHₚ was found to acidify at rates comparable to those seen in macrophages (6, 10).

Since the original hypothesis of Segal et al. (10), it has

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‡ Recipient of a graduate studentship from the CIHR.

§ A CIHR Senior Scientist, an International Scholar of the Howard Hughes Medical Institute, and the current holder of the Pitblado Chair in Cell Biology Program, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.
become apparent that additional H⁺ transport systems operate in phagocytes, which are activated in parallel with the respiratory burst. In particular, a H⁺ conductive pathway or “channel” was found to exist in both neutrophils and macrophages. While the molecular identity of the channel is still the subject of debate, it is generally agreed that large H⁺-selective currents can be mediated by this entity, which can impact on both the cytosolic and phagosomal pH. Passage of H⁺ through these channels could account for the inability of phagosomes to acidify rapidly and fully in neutrophils, as they do in macrophages.

The objective of the present experiments was to reanalyze the determinants of the phagosomal pH of human neutrophils, with particular attention to the possible contribution of passive H⁺ transport pathways. To this end, we used fluorescence ratio imaging to select and study single phagosomes in adherent neutrophils. This approach overcomes some of the limitations inherent in earlier studies, which may have affected their interpretation (see “Discussion”). We report that the activation of the NADPH oxidase alters pH₅₀ not only by promoting the consumption of intraphagosomal H⁺ (equivalents) but also by altering the permeability and fusogenic properties of the phagosomal membrane.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Nigericin, 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, Oregon Green 514 succinimidyl ester, SNAFL1 succinimidyl ester, SNAFL2 succinimidyl ester, dihydrodorhadename, valinomycin, and zymosan were purchased from Molecular Probes, Inc. (Eugene, OR). Concanamycin A was obtained from Kamiya Biochemical Company (Thousand Oaks, CA). Polyclonal antibody to the 39-kDa subunit of the V-ATPase was prepared as described (11). Monoclonal antibody against CD66b was obtained from Serotec Ltd. (Oxford, England). Fibronectin was obtained from Roche Molecular Biochemicals. Diisopropyl fluorophosphate and diphenylene iodonium (DPI) were from Toronto Research Chemicals Inc. (Toronto, Canada). Human IgG, protease inhibitor mixture for mammalian cell culture, nigericin, 2 μm monensin, 50 mg/ml. Reactive fluorophores (fluorescein isothiocyanate and the sulforhodamine 101 and rhodamine 123. An aliquot of the cell suspension was then applied onto the coverslip, and fluorescence was observed and quantified using the optical setup described above. Superoxide production was assayed spectroscopically using the conventional cytochrome c assay as described previously (13).

**Phagosome Isolation**—To obtain intact phagosomes from human neutrophils, we used a modification of the method of Griffiths and co-workers (14). Neutrophils suspended in Hepes-buffered RPMI 1640 were initially treated with diisopropyl fluorophosphate (2.2 μm), a potent protease inhibitor, for 5 min at room temperature. Following the removal of excess inhibitor by centrifugation, the cells were resuspended at 10⁶/ml in PBS. To induce phagocytosis, latex beads (blue-dyed, 0.8-μm diameter) were sedimented in a microcentrifuge tube, forming a loose pellet onto which 1-ml samples of the bead suspension (10⁶ cells in 0.1 ml) with opsonized zymosan particles (5 mg/ml human IgG followed by a wash and suspension in PBS. Fifty μl of the bead suspension (∼5 × 10⁷ beads) were sedimented in a microcentrifuge tube, forming a loose pellet onto which 1-ml samples of the neutrophil suspension were overlaid, together with an extra 50 μl of the bead suspension. The tubes were again subjected to centrifugation to sediment and promote the interaction between the cells and beads, thereby initiating Fcγ receptor-mediated phagocytosis. The pellet was resuspended and sedimented twice more to increase the contact between particles and neutrophils. The final pellet was then resuspended in cold homogenization buffer (250 mm sucrose, 3 mm imidazole, pH 7.4, at 4 °C) with protease inhibitors (1 mm phenylmethylsulfonyl fluoride and 1:100 dilution of a commercial mixture of protease inhibitors for mammalian cell extracts (Sigma). The cells collected from 10 tubes processed in parallel were broken using a French press (pressure setting: 400 p.s.i. for 5 min). Pressure was released slowly to allow dropwise collection of sample and complete breakage of the neutrophils. A drop of the supernatant was inspected under the light microscope to confirm that cell disruption occurred. The lysate was centrifuged at 1500 rpm (400 × g) for 5 min to remove any unbroken cells. The supernatant containing the homogenate was mixed with an equal volume of 62% sucrose, 3 mm imidazole, pH 7.4, at 4 °C, yielding a final sucrose concentration of 40%. This was then layered on top of a 62% sucrose cushion and overlaid with 35, 25, and 10% sucrose-imidazole layers. The gradient was then subjected to centrifugation at 100,000 × g for 1 h at 4 °C, using a swinging bucket rotor. Upon completion of the centrifugation, the interface between the 10 and 25% Fractionation of the phagosomes was then performed by sequential two or three times while adding protease inhibitor mixture (100 μg/ml human IgG) followed by sonication to disperse the zymosan. This procedure was repeated two or three more times while adding progressively more PBS until the supernatant was completely clear. The final pellet was dissolved in PBS and frozen in aliquots.

The spectral properties of the conjugates were analyzed using a thermostatted Hitachi F-4000 fluorescence spectrofluorometer using 5-nm slits while magnetically stirring the suspensions.
sucrose layers containing the latex bead phagosomes was collected. The sample was diluted into 12 ml of cold PBS and sedimented at 40,000 × g at 4 °C for 15 min. The pellet containing the phagosomes was finally dissolved in boiling Laemmli buffer and used for the electrophoresis and immunoblotting.

**Electrophoresis and Immunoblotting**—Samples were resolved using SDS-PAGE using the Protean II minigel system (Bio-Rad) and transferred onto Immobilon-P transfer membrane (Millipore Corp., Bedford, MA). Membranes were then blocked for 1 h in a buffer containing 2% milk, 0.01% Tween in PBS. The blots were then washed 3-fold (10 min each) with 0.01% Tween in PBS. Blots were next incubated 1:5000 dilutions of peroxidase-coupled anti-rabbit IgG (for V-ATPase) or anti-mouse IgG (for CD66b) for 1 h, followed by three 20-min washes. Finally, immunoreactive bands were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Inc.) and quantified using Image J software (National Institutes of Health).

**RESULTS AND DISCUSSION**

**Measurement of pH**—To examine the pH in situ, we utilized zymosan particles that were labeled with a combination of two pH-sensitive fluorophores: fluorescein isothiocyanate and Oregon Green 514. We took advantage of the different pKa values of these two probes (6.4 and 4.8, respectively) to extend the range of sensitivity of our measurements over 3 pH units, as illustrated in Fig. 1.

**FIG. 1.** Spectral properties of labeled zymosan particles. Rehydrated yeast particles (zymosan) were with both fluorescein isothiocyanate and Oregon Green 514 as described under “Experimental Procedures.” Fluorescence excitation spectra were measured with emission at 510 nm in solutions of the indicated pH.

**FIG. 2.** Fluorescence changes in extracellular and internalized particles. Labeled zymosan particles were added to adherent neutrophils. Internalized particles (solid circles) were distinguished from extracellular particles (open circles) by visualization using differential interference contrast microscopy (e.g. Fig. 4) and by pulsing with ammonia. A, fluorescence intensity using 490-nm excitation. B, ratio of fluorescence at 490/440 nm. Where indicated (Calibration), the pH of the medium bathing the particles was set to the specified values, as described under “Experimental Procedures.” Data are representative of more than five experiments of each type and were normalized to facilitate comparison.

**FIG. 3.** Effect of the myeloperoxidase inhibitor NaN3 on zymosan fluorescence. Adherent neutrophils were treated with 5 mM NaN3 prior to exposure to labeled zymosan. The fluorescence emission of internalized particles was measured with excitation at 440 and 490 nm. The intraphagosomal location of the particles was verified as described under “Experimental Procedures.” A, fluorescence intensity using 490-nm excitation. B, ratio of fluorescence at 490/440 nm. Where indicated (Calibration), the pH of the medium bathing the particles was set to the specified values, as described under “Experimental Procedures.” Data are representative of more than 10 experiments of each type and were normalized to facilitate comparison.
human IgG to promote Fcγ receptor-mediated internalization by neutrophils that had been allowed to adhere to fibronectin-coated coverslips. A high resolution digital imaging system that combines fluorescence ratio imaging with continuous bright field visualization was used to locate cells that interacted with particles, to discriminate between adherent and internalized particles, and to measure the pH of the resulting phagosomes. Observations made in particles that were not internalized (open circles in Fig. 2, A and B) indicate that under the conditions of our experiments both the intensity at 490 nm (the pH-sensitive wavelength) and the 490/440-nm fluorescence ratio remained virtually constant for at least 15 min despite repeated exposures to the excitation light. More importantly, the fluorescence ratio was highly sensitive to the pH of the bathing medium in the pH 5–7.5 range (Fig. 2B), as predicted from the spectral analyses of Fig. 1.

The changes in fluorescence at 490 nm and in the 490/440-nm ratio that follow particle engulfment into phagosomes are illustrated by the solid circles in Fig. 2. A sizable decrease in the 490-nm intensity and in the ratio was observed to develop during the 10–15 min after phagocytosis. In view of the sensitivity of the fluorescence to pH, this would normally be interpreted as a progressive decrease in pH. However, subsequent calibration indicated that the pH was near neutral. In addition, it became obvious that the pH responsiveness of the probes had been drastically reduced after internalization (i.e. the dynamic range of the fluorescence ratio when pH varied between 7.5 and 5.0 was very small, a fraction of that seen in extracellular zymosan) (see Fig. 2B). This was not attributable to incomplete equilibration of the phagosomal pH with the extracellular medium during calibration, since identical results were obtained with ionophores and following disruption of cellular membranes with Triton X-100.

It was reported earlier that fluorescein derivatives similar to the probes used here to monitor pH were susceptible to chlorination by phagocytic cells (15). Klebanoff and co-workers (15) suggested that myeloperoxidase combines H2O2 with chloride, yielding the highly reactive hypochlorous acid, which probably chlorinates the aromatic rings of fluorescein derivatives. Importantly, it was found that the fluorescence of these molecules is severely altered upon chlorination. This raised the possibility that the pH probes covalently attached to zymosan underwent chemical modification inside phagosomes upon generation of reactive oxygen intermediates, perhaps accounting for their altered dynamic range. This notion was tested using NaN3, a potent inhibitor of myeloperoxidase activity (16). As shown in Fig. 3A, the presence of NaN3 prevented the progressive fluorescence decrease noted in untreated samples (internalized particles in Fig. 2A). Moreover, the dynamic range of the ratio as a function of pH was also maintained (Fig. 3B). These observations indicate that chlorination of the probe can occur during phagosomal development in neutrophils and that inhibition of myeloperoxidases is es-

FIG. 4. Effect of concanamycin on pH and on NADPH activity. A, measurements of phagosomal pH. Adherent neutrophils were pretreated with (open circles) or without (solid circles) DPI and allowed to internalize labeled zymosan. Phagosomal pH (ordinate) was measured as detailed under “Experimental Procedures.” Where indicated, 100 nM concanamycin was added to both samples. B–D, measurements of NADPH oxidase activity. Cells were incubated with unlabeled zymosan in the presence of dihydrodihydrorhodamine. Intraphagosomal particles were identified by differential interference contrast microscopy (D), and the generation of rhodamine 123 was quantified by digital imaging (C). The mean ± S.E. of three determinations performed in the presence (open bar) or absence (solid bar) of 100 nM concanamycin is presented in B.
sentential for reliable measurements of pH by fluorescence. Therefore, all subsequent experiments were performed in media supplemented with NaN3. Whereas NaN3 is also an inhibitor of the respiratory chain, neutrophils have very few mitochondria and depend almost entirely on anaerobic sources for their metabolism (17). Accordingly, their ability to ingest particles and to pump H+ was unimpaired by NaN3 (see below).

Under conditions where destruction of the probe was precluded, we found that the phagosomal pH of neutrophils remains near neutral for a period of at least 20 min (e.g. Fig. 3B). Two criteria indicate that the particles were fully internalized: (a) the addition of ammonium/ammonia to the extracellular space induced a sizable alkalinization, attributable to preferential permeation of ammonia across biological membranes, and (b) the particles were inaccessible to antibodies added extracellularly (not illustrated). It is noteworthy that, unlike Segal et al. (10), we were unable to detect an alkalinization phase shortly after particle ingestion. This could conceivably be attributed to the modest sensitivity of the pH probes used in the alkaline range, since the pH of fluorescein isothiocyanate is ~6.4 and that of Oregon green is even lower. To facilitate detection of a possible alkalosis, the starting pH was lowered by using acidic solutions during phagocytosis. However, no initial alkalosis was recorded, whether the extracellular pH at the time of phagocytosis was 7.0 or even 6.5 (not shown). In addition, we tested another set of pH-sensitive fluorescent probes, namely SNAFL-1 and SNAFL-2. The pH values of these dyes are considerably higher (7.8 and 7.7, respectively), making them more suitable to study pH in the alkaline range. Nevertheless, no phagosomal alkalinization could be detected using these probes. Together, our findings indicate that in single adherent neutrophils, the phagosomal pH does not exceed the pH of the extracellular solution that is internalized along with the particle.

Role of the NADPH Oxidase—We next proceeded to evaluate the contribution of the NADPH oxidase to the establishment of pH. This was accomplished using DPI, a powerful inhibitor of the oxidase (18). When neutrophils were allowed to internalize particles in the presence of DPI, a rapid and profound acidification of the phagosome was recorded (Fig. 4). In nine experiments, pHp equilibrated at 5.1 ± 0.5 within 2–8 min and remained at this level for at least 10 min. The acidification was fully dissipated by 100 nM concanamycin (Fig. 4A), confirming that it is generated by V-ATPases.

**Coupling of the NADPH Oxidase and the V-ATPase**—The model proposed earlier by Segal and colleagues (10) suggested that H+ pumped by the V-ATPase provides the counterions for superoxide generation and the substrate for its dismutation and implied that the two processes are intimately coupled. This model predicts that impairment of the V-ATPase would result in the generation of a sizable membrane potential and/or the development of a very alkaline pH. The development of an opposing electrochemical potential would in turn reduce the activity of the oxidase. These predictions were tested experimentally.

The effect of concanamycin on pH was tested in cells where the oxidase was active. Contrary to the model described above, the V-ATPase inhibitor did not elicit an alkalinization (Fig. 4A, solid circles). Second, we measured the rate of generation of reactive oxygen intermediates using dihydrorhodamine. This reduced, nonfluorescent compound is converted to the fluorescent derivative rhodamine 123 upon oxidation. To ensure that phagosomal (as opposed to plasmalemmal) oxidase activity was being detected, we carried out experiments where phagosomes were identified by differential interference contrast microscopy, and only the oxidized products accumulated therein were simultaneously measured by digital fluorescence imaging (Fig. 4, C and D). We then compared the rate of rhodamine-123 generation in cells incubated in the presence and absence of concanamycin. As summarized in Fig. 4B, there was no significant difference in the activity of the oxidase when the pump was active versus inactive. In three experiments, the average fluorescence per phagosome, recorded after 5 min, was 424 ± 27 and 384 ± 89 units in the absence and presence of concanamycin, respectively.

These findings indicate that the V-ATPase is not an important source of counterions for the electron-carrying NADPH oxidase. It is noteworthy that the conversion of dihydrorhodamine to...
The occurrence of a marked phagosomal acidification upon phagocytosis reaction. Phagosomes were isolated from neutrophils that were exposed to opsonized latex beads, as described under “Experimental Procedures.” A, immunoblotting of the 39-kDa subunit of the V-ATPase. Samples are as follows (from left to right): a rat brain homogenate, whole neutrophil extract, phagosomes obtained from control cells, and phagosomes from DPI-treated cells. Identical amounts of protein were loaded in each lane. As a control for nonspecific binding, an equivalent amount of beads was added to a cell lysate after homogenization and used for immunoblotting. B, comparison of the content of the 39-kDa subunit of the V-ATPase and of CD66b in phagosomes isolated from DPI-treated (left lane) and untreated cells (right lane). Identical amounts of protein were loaded. C, quantitation of four experiments like that in B. Data are means ± S.E. of four separate experiments. Data were normalized with respect to the control (untreated with DPI).

rhodamine-123 is catalyzed by H₂O₂, suggesting that similar amounts of the latter oxidant were available in phagosomes with and without functional V-ATPases. This would suggest that the H⁺ pumped by the V-ATPase is not the sole and possibly not the most important source of H⁺ for the dismutation reaction.

Irreversible Effects of the NADPH on Phagosomal Permeability—The occurrence of a marked phagosomal acidification upon inhibition of the NADPH oxidase was initially interpreted to mean that consumption by superoxide or its derivatives prevents proton accumulation in the phagosomal lumen. To test this notion further, we introduced DPI at various times following initiation of phagocytosis. As illustrated in Fig. 5A, the inhibitor blocks the oxidase totally within 5 min, even when added after the enzyme has reached maximal activity. Fig. 5B compares the effect of DPI added before or 5 min after initiation of phagocytosis. As before, a rapid and pronounced acidification was observed when the oxidase was inhibited from the outset, and little change in pHᵢ was recorded in its absence. However, the addition of DPI after 5 min resulted in a much smaller and slower acidification. While in untreated cells pHᵢ remains near neutral for extended periods (see Fig. 3B), the effect of DPI became progressively smaller with time (not shown). These observations were unexpected, since continuous consumption of H⁺ by the oxidase was postulated to account for the failure of the phagosome to acidify. We concluded that other processes must contribute to the inability of the phagosome to acidify, particularly at longer times.

Several potential mechanisms can be envisaged for the gradual loss of ability of the phagosomes to become acidic. First, the activity of the proton pumps may diminish over time. This in turn could be due to inactivation of the phagosomal V-ATPases or to a reduction in their number. Second, the activity of the pumps, which are electrogenic (19), may become limited by a reduction in the permeability of the phagosomal membrane to counterions. Third, the passive permeability to H⁺ equivalents (i.e. the H⁺ “leak”) may have increased. These possibilities were analyzed individually.

Quantitation of Phagosomal V-ATPases—The plasma membrane of quiescent neutrophils, which contributes to form the nascent phagosome, is devoid of V-ATPases. Instead, the V-ATPases are delivered to the maturing phagosome by fusion with endomembranes. There are isolated reports that the activity of the oxidase may alter the mobilization of intracellular granules, including some observations in patients with chronic granulomatous disease (10). It was therefore possible that the generation of oxygen radicals in some way antagonizes the insertion of V-ATPases into the phagosomal membrane. This was tested by quantifying the amount of V-ATPase integrated into the membrane of isolated phagosomes. Following phagocytosis in the presence and absence of DPI, the neutrophils were disrupted, and the phagosomes were purified by density gradient fractionation. The V-ATPase content of the purified phagosomal membranes was studied using SDS-PAGE and immunoblotting with antibodies to the 39-kDa subunit of the ATPase. As expected, V-ATPases were enriched in purified phagosomes, compared with the original whole cell lysate. More importantly, however, the accumulation of ATPases was considerably greater in phagosomes from cells treated with the NADPH oxidase inhibitor DPI (Fig. 6, A and B). Scanning of blots from four individual experiments indicated that the density of V-ATPases was nearly 4-fold greater in phagosomes from DPI-treated cells (Fig. 6C). This difference is unlikely to be due to altered immunoreactivity of the V-ATPase and is more likely to reflect diminished fusion with endomembrane compartments. This view is supported by the finding that DPI treatment also increased the amount of CD66b recovered in purified phagosomes (Fig. 6, B and C). CD66b is a marker of the membrane of neutrophil secretory (secondary) granules. These findings suggest that one or more products of the NADPH oxidase reduce the efficiency of granule fusion with the maturing phagosome. As a result, the density of V-ATPases on the phagosomal membrane is lower, accounting in part for the impaired ability of the phagosome to acidify.

Assessment of Counterion Conductance—Although comparatively low in density, V-ATPases are still present in the membrane of phagosomes, yet these failed to acidify detectably over extended periods (see Fig. 7). This implies that other factors contribute to the inability of these phagosomes to acidify. A complex regulatory mechanism could involve a decrease in the counterion flux required to balance the electrogenic nature of the V-ATPase. Conceivably, pathways that normally allow the passage of counterions may be inactivated by products of the NADPH oxidase. To test the role of counterions, phagocytosis was allowed to occur in Na⁺-free/high K⁺ solution, thereby ensuring that this cation would be the main constituent of the phagosomal lumen. This procedure did not affect the activity of the phagosome, which induced a large and rapid acidification in cells.
Phagosomal pH in Neutrophils

Assessment of Passive H\(^+\) (Equivalent) Conductance—Backflux into the cytosol of H\(^+\) pumped by the V-ATPase can minimize acidification of the phagosome. This backflux or “leak” is an important determinant of the pH of intracellular organelles (21). It is possible that products of the NADPH oxidase may have augmented this leak, contributing to the dissipation of the pH gradient. To define the base-line conductance, we first analyzed the possible presence and nature of the passive H\(^+\) flux into the cytosol of H\(^+\)-sensitive pathways involved are insensitive to Zn\(^{2+}\). We then considered whether an enhancement of this Zn\(^{2+}\)-sensitive pathway is responsible for the inability of phagosomes to acidify, the pathways involved are insensitive to Zn\(^{2+}\). To explore the possibility that other types of leak pathways develop as a result of oxidase activity, we compared the passive H\(^+\) equivalent permeability of phagosomes in cells that were either treated with DPI or left untreated. Neutrophils were allowed to develop mature phagosomes and were plated onto coverslips. To compare H\(^+\) permeability under similar conditions, the V-ATPases were inhibited with concanamycin, and the phagosomal pH was artificially lowered by incubating the cells in Na\(^+\)-free medium of pH 5.8. Next, a pH gradient was

pretreated with DPI (Fig. 7). As in normal medium, K\(^+\)-rich phagosomes formed by cells with an active oxidase failed to acidify. To assess whether this was attributable to limiting counterion conductance, the cells were then treated with valinomycin, a conductive K\(^+\) ionophore. By permeabilizing the phagosomal membrane to K\(^+\), valinomycin should allow this cation to exit the lumen, counteracting the buildup of charge due to inward H\(^+\) pumping. We demonstrated earlier that valinomycin reaches endomembranes in neutrophils, since the ionophore rapidly collapses the mitochondrial potential when added to intact cells (20). As shown in Fig. 7, the addition of valinomycin did not facilitate the acidification of phagosomes, implying that the process was not limited by the counterion permeability.

Assessment of Passive H\(^+\) (Equivalent) Conductance—Backflux into the cytosol of H\(^+\) pumped by the V-ATPase can minimize acidification of the phagosome. This backflux or “leak” is an important determinant of the pH of intracellular organelles (21). It is possible that products of the NADPH oxidase may have augmented this leak, contributing to the dissipation of the pH gradient. To define the base-line conductance, we first analyzed the possible presence and nature of the passive H\(^+\) (equivalent) conductance in phagosomes from DPI-treated cells. As shown in Fig. 8, despite the ability to generate a sizable pH gradient, such phagosomes nevertheless have a significant leak pathway, which can be unmasked when the V-ATPase is inhibited by concanamycin. Because the plasma membrane of phagocytes expresses a Zn\(^{2+}\) - and Cd\(^{2+}\)-sensitive H\(^+\) conductance (22), we considered whether a similar pathway could contribute to the observed leak. To this end, neutrophils were allowed to internalize zymosan in a medium containing 200 \(\mu\)M Zn\(^{2+}\) as well as DPI. As shown in Fig. 7, the presence of Zn\(^{2+}\) reduced significantly the rate of dissipation of pH upon the addition of concanamycin. This suggests that a Zn\(^{2+}\)-sensitive pathway contributes to the backflux of H\(^+\) from the phagosomal lumen to the cytosol.

We then considered whether an enhancement of this Zn\(^{2+}\)-sensitive pathway is responsible for the inability of phagosomes to acidify when the oxidase is functional. Cells were allowed to form phagosomes in the presence of Zn\(^{2+}\) as above, but DPI was omitted from the solution. Despite the inhibition of Zn\(^{2+}\)-sensitive pathways, the phagosomes were unable to acidify significantly when the oxidase was active (Fig. 8). We tentatively concluded that if increased H\(^+\) leakiness contributes to the inability of these phagosomes to acidify, the pathways involved are insensitive to Zn\(^{2+}\). To explore the possibility that other types of leak pathways develop as a result of oxidase activity, we compared the passive H\(^+\) equivalent permeability of phagosomes in cells that were either treated with DPI or left untreated. Neutrophils were allowed to develop mature phagosomes and were plated onto coverslips. To compare H\(^+\) permeability under similar conditions, the V-ATPases were inhibited with concanamycin, and the phagosomal pH was artificially lowered by incubating the cells in Na\(^+\)-free medium of pH 5.8. Next, a pH gradient was

![Fig. 7. Role of the counterion conductance in the establishment of pH\(_{\text{pH}}\). Adherent neutrophils were pretreated with (open circles) or without (solid circles) DPI and allowed to internalize labeled zymosan in a Na\(^+\)-free/high K\(^+\) solution. Phagosomal pH (ordinate) was measured as detailed under “Experimental Procedures.” Where indicated, both samples were treated with 1 \(\mu\)M valinomycin. Data are representative of four experiments.

![Fig. 8. Role of the Zn\(^{2+}\)-sensitive pathways in the establishment of pH\(_{\text{pH}}\). Adherent neutrophils were pretreated with (circles) or without (asterisks) DPI and allowed to internalize labeled zymosan. Where noted (open circles and asterisks), 200 \(\mu\)M Zn\(^{2+}\) was present at the time of phagocytosis. Phagosomal pH (ordinate) was measured as detailed under “Experimental Procedures.” Where indicated, the samples were treated with 100 \(\mu\)M concanamycin. Data are representative of three experiments.](http://www.jbc.org/)

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imposed across the phagosomal membrane by rapidly restoring the cytosolic pH to near physiological values. This was accomplished by abruptly changing the bathing pH to 7.5 and reintroducing Na\(^+\) to activate Na\(^+\)/H\(^+\) exchange across the plasmalemma. By recording the rate of alkalinization of the phagosomal lumen, we could estimate the passive H\(^+\) (equivalent) permeability of cells with active or inactivated oxidase. The results shown in Fig. 9 illustrate that the rate of pH\(_p\) recovery was greater in untreated than in DPI-treated cells. In four experiments, the rate was at least 6-fold faster in control than in DPI-treated cells. This implies that the activity of the oxidase increased the passive permeability of the phagosomal membrane to H\(^+\).

In summary, we used an imaging procedure to analyze the determinants of pH\(_p\) in intact neutrophils. Our experimental system represents a significant improvement over previously used models in several respects. (a) It studies neutrophils while adherent to fibronectin, which resembles the physiological situation more closely than the suspended neutrophils used in the past. (b) We took precautions to minimize the chlorination of the fluorescein derivatives used for pH determination. Chlorination may have occurred in earlier studies, adversely affecting the interpretation of the measurements. (c) By imaging single phagosomes, we ensured that only internalized particles and not those adhering to the neutrophil surface were measured. (d) We used internal calibrations after each individual experiment. This procedure estimates pH\(_p\) more accurately than calibrations made post facto in parallel samples.

Using our approach, we confirmed that the phagosomes of neutrophils differ from those of macrophages in that their pH is more alkaline. As suggested earlier, the difference can be attributed to the activity of the NADPH oxidase, which is much more robust in neutrophils. However, our findings indicate that the neutralization of pH is not due primarily to the consumption of H\(^+\) by the dismutation of superoxide. Instead, we find that two factors combine to minimize the acidification: (a) inhibition of the delivery of V-ATPases to the phagosomal membrane and (b) induction of a passive H\(^+\) leak on the phagosomal membrane. The former effect is associated with a reduced fusion of secretory granules with the phagosomal membrane. The mechanism underlying this process is obscure at present, but it may be related to depressed flux of calcium across the phagosomal membrane, resulting from an oxidase-induced depolarization of the electrical potential. The nature of the oxidase-induced leak pathway also remains elusive. It is not sensitive to Zn\(^{2+}\) and therefore differs from the outward rectifying H\(^+\) conductance reported to exist in leukocytes. Last, we cannot rule out the possibility that reactive oxygen intermediates, in addition, directly inhibit the V-ATPase. Indeed, the V-ATPase is known to contain critical sulfhydryl groups that are very susceptible to oxidation (23) and might be targets of the products the NADPH oxidase.\(^3\)

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