Phagosomal Acidification Is Mediated by a Vacuolar-type H\(^+\)-ATPase in Murine Macrophages*

Gergely L. Lukacs§§, Ori D. Rotstein§, and Sergio Grinstein††

From the §Division of Cell Biology, Research Institute, Hospital for Sick Children, Toronto M5G 1X8, Canada, the ‡Department of Surgery, Toronto General Hospital, Toronto M5G 2C4, Canada, and The Institute of Medical Science, University of Toronto M5S 1A8, Toronto, Canada

The mechanism underlying phagosomal acidification was studied in thioglycolate-elicited murine macrophages. The pH of the phagosomal compartment (pH\(_p\)) was measured fluorimetrically in macrophage suspensions following ingestion of fluorescein isothiocyanate-labeled Staphylococcus aureus. At 37 °C, pH\(_p\) decreased rapidly, reaching a steady state value of 5.8–6.1, while the cytoplasmic pH remained near neutrality, pH 7.1. The phagosome to cytosol pH gradient could be collapsed by addition of nigericin, monensin, or weak bases. The substrate dependence and inhibitor sensitivity profile of phagosomal acidification were investigated in intact and permeabilized cells. Phagosomal acidification was inhibited when ATP was depleted using metabolic inhibitors or permeabilizing the plasma membrane by electroporation. In permeabilized cells, acidification could be initiated by readdiction of both Mg\(^{2+}\) and ATP. Neither adenosine 5'-(β,γ-imido)triphosphate nor adenosine 5'-dithio)triphosphate supported phagosomal acidification. Inhibitors of F,F\(_1\)-type H\(^+\)-ATPases such as oligomycin and azide, and the E,F\(_1\)-type H\(^+\)-ATPase inhibitor vanadate had no effect on phagosomal acidification. In contrast, the rate of phagosomal acidification was reduced by micromolar concentrations of N-ethylmaleimide and N,N',dicyclohexylcarbodiimide. In permeabilized cells, nitrate inhibited the acidification with an apparent K\(_i\) of 25 mM. Phagosomal acidification was also effectively blocked by the macrolide antibiotic bafilomycin A\(_1\), with an apparent K\(_i\) of ~3 nM in both intact and electroporated cells. In this concentration range, bafilomycin A\(_1\) selectively inhibits vacuolar H\(^+\)-ATPases. The substrate requirement and inhibitor susceptibility profile of phagosomal acidification strongly suggest that proton translocation across the phagosomal membrane is mediated by a vacuolar-type H\(^+\)-ATPase.

The ability of phagocytic leukocytes to ingest and kill microorganisms is one of the major mechanisms whereby microbial invasion is thwarted by the host. Following attachment of the opsonized microorganisms to the cell surface, extension of the phagocytic cell membrane around the particle results in total enclosure and formation of a sealed intracellular compartment, the phagosome. Using pH-sensitive dyes, several investigators have demonstrated the occurrence of progressive acidification of the phagosomal compartment to levels as low as pH 5.5–6.5 (1, 6). This ability of the cell to lower phagosomal pH appears to be crucial to its microbicidal functions. The elevated proton concentration is directly lethal for certain microorganisms (7). In addition, low phagosomal pH promotes the spontaneous dismutation of superoxide to hydrogen peroxide (8), provides optimal conditions for the activity of certain hydrolytic enzymes (9), and appears to be prerequisite for the process of phagosome-lysosome fusion (6). Indeed, certain microorganisms such as Legionella pneumophila (10) and Toxoplasma gondii (6) appear capable of preventing phagosomal acidification, thereby evading killing by phagocytic cells of the host.

Despite the well-documented occurrence of phagosomal acidification, the mechanism underlying this process remains unclear (see Ref. 11). Proposed mechanisms that might participate in phagosomal acidification include increased lactic acid production during phagocytosis (12), proton generation during superoxide formation (13), carbonic anhydrase-coupled proton transport (14), Na\(^+\)/H\(^+\) exchange (Ref. 15, but see also, Ref. 16), proton pumping via H\(^+\)-Pump ATPases (17), and phagosome-lysosome fusion (18). Available evidence supporting each of these potential mechanisms is mostly indirect.

In the present study phagosomal pH was measured by monitoring the fluorescence of FITC-labeled, opsonized Staphylococcus aureus that were ingested by thioglycolate-elicited peritoneal macrophages. Using this technique the putative role of H\(^+\)-pumping ATPases in phagosomal acidification was investigated. A direct examination of this mechanism was made possible by two recent developments: 1) the availability of bafilomycin A\(_1\), a macrolide antibiotic isolated from Streptomyces sp., which is a potent and, at low concentrations very selective inhibitor of vacuolar-type H\(^+\)-ATPases (19 and 2) the implementation of electropermeabilization techniques (20), which can render the plasma membrane leaky without affecting the phagosomal membrane. This procedure provides direct access to the cytoplasmic face of the phago-

1 The abbreviations used are: FITC, fluorescein isothiocyanate; pH\(_p\), pH in the phagosomal compartment; pH\(_c\), cytoplasmic pH; [Ca\(^{2+}\)], cytoplasmic-free calcium concentration; BAPTA, 1,2-bis (N-methylpiperazone)-N,N',N',N'-tetraacetic acid, EGTA, [ethylendiylenbis(oxyethylenenitrito)]tetraacetic acid; BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES-RPMI, bicarbonate-free RPMI 1640 medium buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid–NaOH; DCCD, N,N'-dicyclohexylcarbodiimide; NEM, N-ethylmaleimide; AMP-PNP, adenosine 5'-dithio)triphosphate; ATPyS, adenosine 5'-dithio)triphosphate; MES, 4-morpholineethanesulfonic acid.

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†† Recipient of the Merck-Frosst Surgical Infectious Diseases Fellowship.

§§ Recipient of a Medical Research Council Scientist Award. To whom correspondence should be addressed. Tel: 416-598-5727; Fax: 415-598-6897.

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some, without grossly disrupting the cellular architecture. Using this approach we were able to analyze the ATP and Mg2+ dependence of the acidification process and to test the effects of poorly permeant inhibitors. The data provide evidence that vacuolar-type H+-ATPases play a central role in phagosomal acidification.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Medium RPMI 1640 (with l-glutamine, HEPES-K, and 0.5 mM glucose or 2-deoxy-D-glucose (as indicated), 10 mM HEPES, pH 7.35, at 37 °C. The permeabilization medium contained 140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.35, and 20 mM HEPES-K, and 0.5 mM EGTA to give a final free (Ca2+) of <30 nM. The osmolarity of the media was adjusted to 290 ± 5 mosM with NaCl or NaHCO3 (pH,). These alkali cation/proton exchangers collapse the sodium concentration, antifluorescein-IgG quenched the fluorescence of a fluorophore in macrophages containing phagocytosed FITC-labeled S. aureus are illustrated in Fig. 1C. In the experiment shown in Fig. 1C, addition of antifluorescein-IgG decreased fluorescence intensity by 21.00% at pH 7.4, indicating that 37% of the total fluorescence originated from the extracellular space. In 23 similar experiments, the average fluorescence intensity and this fraction did not increase after electroporation (23.6 ± 7.1%). This signal was diminished by the presence of antifluorescein-IgG to an average of 8.5% of the total fluorescence intensity, representing only a minor contaminating component of the overall fluorescence determinations. Lowering the pH value of the bacterial suspension (therefore unquenching pH) from 7.4 to 5.0 reduced the fluorescence intensity, as described for the bacterial suspension (Fig. 1A). There was a slight increase in the apparent pK of the bacterial suspension (determined as described below) of FITC-labeled S. aureus in the phagosomal space (pK, = 6.7 ± 0.1, n = 15) compared to that of the free bacterial suspension (pK, = 6.4 ± 0.1, n = 8). The higher apparent pK might be due to the different environment of the fluorescein moiety in the phagosomal space, or to the failure of the ionophores to fully equilibrate pH with the extracellular pH. The preservation of the pH sensitivity of the fluorophore in macrophages contrasts with the results reported for neutrophils, where fluorescein in the phagosomal space became chlorinated losing its pH sensitivity which resulted in an apparent decrease in the pK (29).

2 The latter possibility is unlikely as the pH calibration curve was not affected by permeabilization of the plasma membrane with nystatin (50 μg/ml). The occurrence of permeabilization in the presence of nystatin was confirmed measuring colloidiosmotic swelling in Na+ medium using a Coulter counter/channelizer combination.
An in situ calibration technique was used to determine pH, during the course of phagosomal acidification. At the end of each experiment, monensin (4 μM) and nigericin (20 μM) were added, pH, was decreased gradually by adding to the medium aliquots of MES (Fig. 2A) and fluorescence was recorded. In a parallel sample, a comparable titration was carried out in the absence of ionophores. In the latter case, a small rapid fluorescence decrease was observed after each downward pH step, followed by a slower decrease. The rapid steps are attributable to contaminating extracellular fluorescence and the pH sensitivity of the ingested bacteria measured as in panel A. D, pH dependence of the fluorescence intensity (in arbitrary units) of FITC-labeled S. aureus in bacterial suspension (triangles) and after phagocytosis (circles), determined as in A and C, respectively. Excitation wavelength was 490 nm and emission was 525 nm.

$$F = \frac{F_{max} + F_{min} \times 10^{(pH-pK)}}{(10^{pH-pK} + 1)}$$

(see Ref. 23), where F is the corrected fluorescence intensity (total - extracellular) at a given pH; $F_{max}$ and $F_{min}$ are the asymptotes of the minimum and maximum fluorescence intensity at extremely acidic and alkaline pH, respectively, and pK is the apparent dissociation constant (pKa) of FITC-labeled S. aureus for protons. The values of pK, $F_{max}$, and $F_{min}$ were determined in each experiment with the regression program and used to calculate pH.

It was important to determine whether phagocytosis of contaminating extracellular bacteria could occur during the course of the pH measurements. For this purpose, macrophages were allowed to adhere, but not phagocytose bacteria by co-incubation at 0 °C. Following bacterial adherence, the macrophages were washed three times in the cold as above and resuspended in Na+ medium at 37 °C in the fluorimeter cuvette. Under these conditions the fluorescence intensity, originating entirely from extracellular bacteria, remained at a constant level for at least 12 min. This indicates that phagocytosis did not occur during this period or that no fluorescence changes are associated with phagocytosis itself and that these newly formed phagosomes did not acidify significantly during the course of the experiment. Thus, phagocytosis of adherent extracellular bacteria during the course of the fluorescence measurement, if occurring at all, does not contribute measurably to the phagosomal pH determinations. Cells allowed to phagocytose unlabeled S. aureus displayed only negligible autofluorescence (less than 5% of that of cells containing FITC-labeled bacteria).

Electroporation of Macrophages—Macrophages containing phagocytosed S. aureus were permeabilized immediately before use for pH, determinations. To achieve permeabilization, 1–2 x 10^6 cells were resuspended in 0.8 ml of ice-cold permeabilization medium and subjected to two or three 2.3 kV/cm discharges from a 25-microfarad capacitor, using the Bio-Rad Gene Pulser. The suspension was gently stirred between pulses. The cells were utilized immediately after permeabilization, or stored on ice up to 7 min. The efficiency of the electroporation procedure was assessed by quantifying the percentage of trypan blue-positive cells in Na+ medium containing 0.2% trypan blue.
blue, determined by phase-contrast microscopy.

Determination and Manipulation of the Cytosolic Calcium Concentration—[Ca\(^{2+}\)], was measured fluorometrically using Indo-1. Cells were loaded by incubation in HEPES-RPMI with 2 \(\mu\)M of the acetoxymethyl ester precursor of Indo-1 for 15 min at 37 °C. After washing the cells with ice-cold Hank's solution, FITC-labeled bacteria were adhered and ingested as described above. After 10 min of phagocytosis, macrophages were washed three times in ice-cold Hank's solution. Fluorescence of the cell suspension (10\(^6\) cell/ml) was determined in nominally Ca\(^{2+}\)-free Na medium with an excitation at 331 nm (4-nm slit) and emission at 410 nm (10-nm slit). Calibration was carried out with ionomycin and Mn\(^{2+}\) (26), using a \(K_0\) of 254 nm and a ratio of \(F_{\text{max}}/F_{\text{min}}\) of 12. To buffer [Ca\(^{2+}\)], cells were loaded with BAPTA-AM (15 \(\mu\)M) in HEPES-RPMI containing 4 mM EGTA (<100 nm [Ca\(^{2+}\)]) for 12 min at 37 °C.

Cytosolic pH Determination—Cytosolic pH (pHc) was measured fluorometrically in cells loaded with the pH-sensitive dye BCECF. Cell suspensions (10\(^7\) cells/ml in HEPES-RPMI) were incubated with 2 \(\mu\)g/ml of the acetoxymethyl ester form of BCECF for 12 min at 37 °C, washed, and then allowed to phagocytose unlabeled S. aureus. Free bacteria were eliminated by washing three times with Hank's solution at 4°C. Fluorescence of a suspension containing 10\(^6\) cells/ml was measured with excitation at 495 nm and emission at 525 nm, using 5- and 10-nm slits, respectively. Calibration was performed using monensin and nigericin in Na\(^+\) medium as previously described (16).

Light and Electron Microscopy—To visualize the lysosomal compartments by fluorescence microscopy, resident and thioglycolate elicited macrophages were incubated for 3 h in RPMI containing rhodamine B isothiocyanate/dextran (3 mg/ml) or Lucifer yellow (0.5–1 mg/ml). The cells were then washed with ice-cold Hank's solution and chased in dye-free RPMI overnight. Staining of acidic compartments with acridine orange (3 \(\mu\)M) was carried out for 5 min at 37 °C in RPMI. Cells were observed live, using a Leitz Dialux 22 microscope and photographed on Kodak (200 or 800 ASA) films. For electron microscopy, samples were fixed with 2% glutaraldehyde, postfixed in osmium tetroxide, and stained with uranyl acetate and lead citrate as described (16).

RESULTS

Phagosomal Acidification—To measure phagosomal acidification, macrophages were allowed to phagocytose FITC-labeled bacteria and were then resuspended in Na\(^+\) medium in the presence of antifluorescein-IgG to quench extracellular dye (Fig. 2A). A spontaneous decrease in fluorescence was consistently recorded. The following observations indicate that this reduction in fluorescence represents a true acidification of the phagosomal compartment rather than leaking, quenching, or degradation of the dye or the result of ongoing phagocytosis of adherent bacteria. First, addition of the alkali cation-proton exchangers nigericin and monensin increased the fluorescence intensity, presumably through dissipation of the pH gradient across the phagosomal membrane, elevating pHc (Fig. 2A). Second, the fluorescence was increased by addition to the medium of weak bases such as ammonium chloride (Fig. 2B), methylamine, or chloroquine (not shown), as their hydrophobic unprotonated form diffused into the phagosomal compartment and associated with protons, thereby increasing pHc (27). Third, the spontaneous fluorescence decline was abolished when monensin and nigericin were present from the onset of the recording (Fig. 2C). It is noteworthy that addition of nigericin plus monensin after spontaneous acidification restored the fluorescence intensity to the level noted in cells that were prevented from acidifying by the continuous presence of the ionophores (cf. Fig. 2, A and C).

After ingestion of bacteria, and prior to the measurement of fluorescence, the macrophages were stored at 4 °C to prevent further acidification and phagosome-lysosome fusion. When cells were allowed to carry out phagocytosis for only 2 min, the initial pHc recorded upon rewarming to 37 °C was 7.4 (similar to the extracellular pH). In contrast, when cells were allowed to phagocytose for 10 or 20 min prior to the measurement, the initial pHc recorded was 6.4 ± 0.15 (n = 28) and 6.47 ± 0.11 (n = 8), respectively, indicating that phagosomal acidification had already taken place and was (at least partially) preserved during the subsequent washes and incubation in the cold. Because the intervening incubation on ice was up to 4 h long, dissipation of the transphagosomal membrane pH gradient during storage appears to be comparably slow. Indeed, to attain complete equilibration of pHc with the cytoplasmic pH, cells had to be incubated overnight on ice after phagocytosis. Unless otherwise specified, a phagocytosis period of 10 min was chosen for the experiments described hereafter. Even though the initial acidification was smaller when using shorter periods, the low absolute level of fluorescence internalized made detection of pHc inaccurate.

Upon rewarming to 37 °C, pHc decreased at an initial rate of 0.20 ± 0.4 pH unit/min (n = 20) and reached a steady state at 5.89 ± 0.18 (n = 28) in 6–8 min. In parallel experiments, changes in the cytosolic pH were measured during this period of phagosomal acidification, using BCECF. To maintain parallel experimental conditions and to avoid interference with the cytosolic pH measurements by bacterial FITC fluorescence, the macrophages were allowed to ingest opsonized, unlabeled S. aureus prior to loading with BCECF. The cytosolic pH slightly increased from 7.0 to 7.1 after resuspension of the cells (not shown). These observations confirm the independence of the two compartments.

ATP-dependence of Phagosomal Acidification—Based on the well-defined role of vacuolar-type H\(^+\)-ATPases in the acidification of intracellular organelles (see Refs. 11 and 28–31 for reviews), it was hypothesized that phagosomal acidification was mediated via a H\(^+\)-ATPase, likely of the vacuolar type. We first examined the ATP dependence of the process. When ATP synthesis was partially inhibited by substituting the medium glucose with the nonmetabolized glucose analog 2-deoxy-D-glucose, the rate of acidification was reduced but not abolished and the pHc at steady state was higher (Fig. 3A). The combination of 2-deoxy-D-glucose plus the mitochondrial respiratory chain inhibitors antimycin A (10 \(\mu\)g/...
The phagosomal pH gradient was dissipated with nigericin (NIG, 20 μM) or 2-deoxy-D-glucose (2DG); B, cells suspended in glucose-free medium with 10 mM 2-deoxy-D-glucose plus either rotenone (ROT, 10 μM) or antimycin A (ANT, 10 μM). The phagosomal pH gradient was dissipated with nigericin (NIG, 20 μM) plus monensin (MON, 4 μM) addition. The traces are representative of four similar experiments.

To attain a more efficient and reversible ATP depletion, the plasma membrane of macrophages was permeabilized by electrical discharges. The electroporation capacitance and voltage were selected empirically, based on their ability to permeabilize the plasma membrane, without disrupting the phagosomal membrane. In preliminary experiments, the appropriate voltage range was selected by electronically measuring the colloidosmotic swelling of macrophages that occurs when the plasma membrane is permeabilized in a Na+-containing medium. With an optimal electric field (2.3 kV/cm), about 80% of the cells lost their ability to exclude trypan blue from the cytoplasm, meanwhile preserving their content of FITC-labeled bacteria. Successful permeabilization of the plasma membrane was confirmed examining the loss of the normally impermeant fluorescent indicator BCECF from the cytoplasm. For these studies the cytoplasmic compartment of intact macrophages was loaded with BCECF, the cells were next allowed to phagocytose unlabeled S. aureus, and then permeabilized under the conditions described above. Ninety s after electroporation, the BCECF content of the cells had already decreased to 29.2 ± 3.5%, n = 5, and to 19.8 ± 2.6% after 11 min. In contrast, intact cells lost only a negligible amount of BCECF after 11 min (data not shown). The incomplete depletion of BCECF in the permeabilized cells might be due to the presence of a small fraction of intact cells and/or to the accumulation of BCECF in intracellular compartment(s) other than the cytosol (see Ref. 32). These results suggest that electroporation must have rendered the plasma membrane permeable to Mg<sup>2+</sup>, ATP, and related nucleotides, since these molecules are smaller than BCECF (M, 520) and trypan blue (M, 960.8), the markers used. Parallel studies were performed to ensure that, under the conditions selected, electroporation did not effect permeabilization of intracellular compartments. Lucifer yellow (M, 457) which had been previously taken up by pinocytosis (for 10 min), did not leak from the endosomal compartment following electroporation (not illustrated). More importantly, the phagosomal compartment seemed to be largely unaffected by the electrical discharges. FITC-labeled bacteria remained within the phagosomes following electroporation and, as shown below, the phagosomes retained their ability to acidify in an ATP-dependent manner.

Following electroporation, macrophages were kept on ice for 5 min in a medium devoid of ATP and Mg<sup>2+</sup>. In contrast to intact cells, the pH<sub>e</sub> of porated cells remained near neutrality (7.01 ± 0.06; n = 8) after resuspension at 37°C, presumably due to depletion of ATP and/or Mg<sup>2+</sup> through leakage (Fig. 4A). Reintroduction of ATP and Mg<sup>2+</sup> initiated a rapid drop in FITC fluorescence. This fluorescence decrease was also attributable to phagosomal acidification, as it was reversed by the addition of nigericin plus monensin or of ammonium chloride (Fig. 4). The considerable acidification rate obtained in the presence of both agents was not observed when either ATP or Mg<sup>2+</sup> alone were added, suggesting that the simultaneous presence of the nucleotide and the divalent cation is required for phagosomal acidification. The rate of Mg<sup>2+</sup>-ATP-stimulated acidification in permeabilized cells averaged 0.16 ± 0.04 pH/min (n = 8) and the final pH<sub>e</sub> reached was 6.16 ± 0.14 (n = 8). These figures resemble the properties of the spontaneous acidification observed in intact cells (see above), suggesting a common mechanism.

The hydrolysis of the high energy γ-phosphate bond of ATP appeared to be required for H<sup>+</sup> (equivalent) translocation, since the nonhydrolysable ATP analog AMP-PNP failed to support the acidification (Fig. 4B). In addition, ATPγS which can be utilized as a substrate by some protein and lipid kinases, did not support phagosomal acidification (Fig. 4B). Moreover, these analogs antagonized the effect of ATP on pH<sub>e</sub>, presumably by interacting competitively with ATP nucleotide-binding site of the transporter (Fig. 4B). Other nucleotides and divalent cations were also examined for their ability to restore phagosomal acidification in depleted cells. GTP, ITP, and UTP also supported acidification, albeit less effectively than ATP. Precise determination of the substrate specificity of the proton pump from such measurements was hampered by the nucleoside diphosphokinase activity of the cells. Substitution of Mg<sup>2+</sup> by Ca<sup>2+</sup> greatly diminished the rate of acidification (data not shown). When considered together, these data are consistent with the notion that a H<sup>+</sup>-pumping ATPase is involved in acidification of the phagosome. To further define the nature of this ATP-dependent mechanism, the inhibitor sensitivity profile of phagosomal acidification was examined.

**Inhibitor Sensitivity of Phagosomal Acidification in Intact and Electroporated Macrophages**—There are three general groups of proton pumping ATPases: those that form phosphorylated intermediate or F<sub>0</sub>,F<sub>1</sub>-ATPases, the mitochondrial-type F<sub>0</sub>,F<sub>1</sub>-ATPases, and the vacuolar-type ATPases (34, 35). The three families of pumps differ in their pharmacological properties (see Refs. 11 and 28–31 for reviews): K<sub>v</sub>-type ATPases are inhibited specifically by vanadate, F<sub>0</sub>,F<sub>1</sub>-ATPases by oligomycin and azide, and vacuolar-type H<sup>+</sup>-ATPases by NEM, nitrate, and by low nanomolar concentrations of bafilomycin A<sub>1</sub> (19). The lipid-soluble carbodiimide DCCD is a potent inhibitor of both vacuolar and F<sub>0</sub>,F<sub>1</sub>-ATPases (36, 37). We have exploited the substantially different inhibitor sen-
Phagosomal Acidification by a \( H^+ \)-ATPase

A. P"no addition

6.3

B- 7.3

6.9

6.1

MON

NIG

\( \text{FIG. 4. Phagosomal acidification of electroporated macrophages induced by Mg-ATP.} \)

Macrophages (2 \( \times \) \( 10^6 \) cell/ml) were electroporated in permeabilization medium as described and incubated on ice for 5 min. Then, 0.4 ml of the cell suspension was transferred to a fluorimeter cuvette containing 0.8 ml of permeabilization medium and 0.8 unit/ml antifluorescein IgG. A, top trace: no further additions. Middle and lower traces, 0.5 mM ATP and 5 mM \( \text{MgCl}_2 \) added where indicated. In the middle trace the medium contained 50 mM NEM; B, the medium contained 5 mM \( \text{MgCl}_2 \). Top trace, AMP-PNP (A-PNP, 0.5 mM) added at the first arrow and 0.5 mM ATP at the second arrow. Middle trace, ATP\( \gamma \)S (ArS, 0.5 mM) at first arrow; 0.5 mM ATP at second arrow. Bottom trace, 0.5 mM ATP at second arrow. Bottom trace, 0.5 mM ATP; C, \( \text{MgCl}_2 \) and ATP added to both traces where indicated, as above. The medium in the top trace contained 200 nM bafilomycin A\( \text{i} \), Nigericin (NIG, 20 \( \mu \)M), monensin (MON, 4 \( \mu \)M), or ammonium chloride (30 mM) added where specified. Traces are representative of 2-8 experiments.

sitivity of the three types of \( H^+ \)-ATPase to confirm that phagosomal acidification is due to a proton pump and to identify its type. The phosphorylated ATPase inhibitor vanadate, at concentrations of 100-200 \( \mu \)M, did not alter the rate of Mg-ATP induced phagosomal acidification (Table I). The effect of vanadate was tested in permeabilized cells to ensure the access of vanadate to its site of action on the cytoplasmic side of the ATPase. This precaution was not required in the case of the membrane permeant inhibitors of the \( F_1 F_0 \)-ATPase, oligomycin, and azide, which were tested in intact cells. As shown in Table I, the rate of phagosomal acidification was also unaffected by these agents. These results indicate that the phagosomal pump is neither of the \( E_1 E_2 \)-type nor of mitochondrial-type, but is possibly a vacuolar \( H^+ \)-ATPase. This notion was further supported by results obtained with several vacuolar-type \( H^+ \)-ATPase inhibitors. DCCD inhibited

### Table I

| Inhibitor    | Condition     | Rate of acidification (% of control) |
|--------------|---------------|-------------------------------------|
| Vanadate (100 \( \mu \)M) | Electroporated cells | 102.5 \( \pm \) 14.7% (3) |
| Oligomycin (5 \( \mu \)g/ml) | Intact cells | 98.2 \( \pm \) 4.7% (4) |
| Azide (1 mM) | Intact cells | 98.7 \( \pm \) 4.9% (3) |
| NEM (1 mM) | Intact cells | 25.8 \( \pm \) 5.4% (4) |

![FIG. 5. Inhibitor sensitivity of phagosomal acidification. A, measurements of phagosomal acidification in intact (triangles) or porated (squares) cells in the presence of increasing concentrations of DCCD. The rate of phagosomal acidification was determined in electroporated cells as illustrated in Fig. 4B; B, rate of phagosomal acidification of porated cells in the presence of increasing concentrations of \( \text{NEM} \). C, rate of phagosomal acidification in electroporated cells in the presence of increasing concentrations of \( \text{NO}_3^- \). To maintain isosmolarity, glutamate was replaced by the indicated concentration of \( \text{NO}_3^- \) (C). Inset: typical \( \text{pH}_6 \) recording in the presence and absence of \( \text{NO}_3^- \) (40 mM). The data are means \( \pm \) S.E. of 3-5 determinations. Where absent, error bars were smaller than the symbol.](http://www.jbc.org/Downloadedfrom)
Phagosomal acidification in both intact and electroporated cells with comparable potency (approximate $K_i \approx 20 \mu M$; Fig. 5A). This is similar to the range of concentrations reported to block vacuolar ATPases (10-50 μM; see references in Refs. 11 and 30), but substantially higher than the concentrations needed to inhibit the F0F1-ATPase (36). NEM has been reported to inhibit vacuolar H+ATPases at micromolar concentrations (11, 32-34). In intact cells, however, substantial inhibition was detected only at millimolar concentrations of this agent (Table I). In contrast, the Mg-ATP induced accumulation of extracellular H+ for K'. The traces are representative of 4 determinations.

Nitrates, at millimolar concentrations, selectively inhibit vacuolar-type H+-ATPases (38-40). When added externally to intact macrophages, nitrate had no immediate effect on phagosomal acidification. However, the Mg-ATP induced acidification of electroporated cells was inhibited in the presence of nitrate. A representative experiment and a detailed concentration dependence of the inhibitory effect of nitrate are illustrated in Fig. 5C. Half-maximal inhibition was obtained at 25 mM.

Bafilomycin A1 is the most potent and specific inhibitor of the vacuolar-type H+-ATPase available. In the nanomolar range, this antibiotic blocks the activity of vacuolar, but not of E1E2 or F0F1-ATPases (19). Preliminary control experiments indicated that bafilomycin had no direct effect on the fluorescence of FITC-labeled bacteria (not shown). When added to intact cells at 5 mM, bafilomycin A1 inhibited the spontaneous phagosomal acidification by 58% and at 200 nM inhibition was complete (Fig. 6A). This implies that continuous proton pumping is required for the maintenance of steady state pH. The trace in Fig. 6 also demonstrates that the blocking effect of bafilomycin A1 was very rapid, in keeping with its hydrophobic nature. Dose-response curves examining the effect of bafilomycin A1 on the rate of phagosomal acidification in intact cells yielded an apparent $K_i$ of 3 mM (Fig. 7), a value that is similar to the $K_i$ reported for inhibition of the pump in isolated chromaffin granules, Neurospora crassa vacuoles, Golgi vesicles, and yeast vacuoles (19, 41, 42).

The acidification initiated by addition of ATP plus Mg2+ to permeabilized cells was also found to be sensitive to bafilomycin A1 (Fig. 4C) and the apparent $K_i$ was 5 nM, very similar to that determined in intact cells (not shown). In addition, pretreatment of macrophages with bafilomycin A1 caused irreversible inhibition of the acidification process (not shown). Taken together, the sensitivity of phagosomal acidification to bafilomycin A1, NEM, nitrate, and DCCD and its resistance to vanadate, oligomycin, and azide, are fully compatible with the notion that phagosomal proton transport is mediated by a vacuolar-type H+-ATPase.

When added to the cells after pH had reached a steady state, maximally inhibitory doses of bafilomycin A1 (500 nM) evoked a slow dissipation of the pH gradient, indicating the existence of a measurable proton "leak" permeability of the phagosomal membrane. This implies that continuous proton pumping is required for the maintenance of steady state pH.

The rate of the bafilomycin-induced alkalization was substantially accelerated by addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (Fig. 6B). This finding indicates that "leakage" is limited by the proton (equivalent) permeability and not by the counterion conductance.

The Origin of the Phagosomal Pumps—Phagosomes are known to fuse with lysosomes and other vesicular organelles (1, 43, 44). It is therefore conceivable that delivery of the acidic contents of organelles such as lysosomes, endosomes, or tertiary granules into the lumen of the phagosome contributes to phagosomal acidification.

A limited number of experiments were performed to determine the origin of the phagosomal H+-ATPases. The phagosomal pumps might be an intrinsic component of the plasma membrane (45, 46) that becomes internalized during phagocytosis (see "Discussion"). Alternatively, the pumps may be incorporated into the phagosome by fusion of an acidic endomembrane compartment. Earlier reports have indicated that substantial phagolysosomal fusion is apparent after ~15 min (1, 2, 47). We therefore examined whether significant fusion of lysosomes with phagosomes occurred under our experimental conditions. Morphological identification of lysosomes of thioglycolate-elicited macrophages was facilitated by the swelling of these organelles after accumulation of the osmotically active, indigestible components of the thioglycolate medium (47). Such swollen lysosomes could be visualized by fluorescence microscopy following pinocytosis of rhodamine B isothiocyanate/dextran or Lucifer yellow and also by

![Fig. 6. Effect of bafilomycin A1 on phagosomal acidification.](image-url)
accumulation of acridine orange, indicating their acid nature (data not shown). The lysosomes, filled with thioglucolate can also be readily identified by electron microscopy, due to their size and content of electron-dense fibrillar material, likely the agar present in the thioglucolate broth (not shown; the swollen structures containing fibrillar material were absent in resident macrophages). After 10 min of phagocytosis, 98% of the internalized bacteria are present in a compartment that is separate from the large lysosomes, as evidenced by the small size of the phagosome and by the absence of dense fibrillar material in their lumen. These observations suggest that no substantial fusion of phagosomes and large acidic lysosomes had occurred during the incubation period utilized to measure the intraphagosomal pH. These experiments, however, do not exclude the possibility that smaller thioglucolate-free lysosomes fuse to the phagosome.

It was recently demonstrated that the elevation of [Ca\(^{2+}\)], that accompanies phagocytosis is required for successful phagolysosome fusion (48). This observation enabled us to assess the role of phagolysosome fusion in phagosomal acidification. The rate of acidification was determined under conditions where the [Ca\(^{2+}\)], increase normally associated with phagocytosis was prevented by omission of extracellular calcium and by loading the cytosol with BAPTA, a calcium chelator. That BAPTA markedly enhanced the cellular calcium buffering capacity was demonstrated by suspending cells in calcium-free medium with EGTA and pulsing with ionomycin, a divalent cation ionophore. Ionomycin induced a modest [Ca\(^{2+}\)], increase (20–30 nM) in BAPTA-buffered cells, whereas an increment of 300–500 nM was observed in unbuffered cells. The combination of BAPTA and calcium omission also effectively precluded [Ca\(^{2+}\)], increases during phagocytosis. In samples taken at various intervals during and after phagocytosis, [Ca\(^{2+}\)], remained 30–40 nM below the normal resting level of 130 nM as determined with indo-1. Despite the obliteration of the [Ca\(^{2+}\)], increase, there was no substantial difference in the initial rate of acidification (0.2 ± 0.01 pH/min versus 0.18 ± 0.003 pH/min; six determinations from three independent experiments) or in the pH\(_{50}\) recorded upon transpermanon after 10 min of phagocytosis (6.55 ± 0.07 versus 6.61 ± 0.04) in BAPTA loaded and control cells, respectively. These results further support the notion that phagosome-lysosome fusion does not play a major role in the initial phase of phagosomal acidification.

**DISCUSSION**

The process of phagosomal acidification following ingestion of an infecting microorganism has been well documented and appears to be a crucial step in the normal degradative process characteristic of phagocytic cells (1–6). Despite its important role, however, the mechanism underlying the acidification has been incompletely studied (e.g. Ref. 11). The present study provides evidence supporting the concept that phagosomal acidification is mediated via a vacuolar-type H\(^+-\)ATPase active in the phagosomal membrane, which translocates protons from the cytoplasm into the intraphagosomal space. This conclusion was based on two major approaches. First, the Mg-ATP dependence of the process was demonstrated directly. ATP depletion of intact cells by incubation with 2-deoxy-D-glucose plus antimony A or rotenone inhibited phagosomal acidification. Similarly, when ATP was depleted by leaching electroporated macrophages, acidification was also obliterated. In the latter case, acidification could be restored by the addition of exogenous Mg-ATP to the permeabilized cell suspension. That acidification required ATP hydrolysis was indicated by the failure of AMP-PNP or ATP\(_{\gamma}\)S to support acidification.

The second line of evidence indicating involvement of proton pumps was mainly pharmacological. In addition, this approach revealed that the phagosomal pumps are of the vacuolar type. Briefly, the acidification was insensitive to oligomycin and azide, ruling out the involvement of mito-chondrial type (F,F\(_{0}\)) H\(^+-\)ATPases. Similarly, phagosomal acidification in electroporated cells was insensitive to vanadate, implying that proton transport is not mediated by E,EType H\(^+-\)ATPases. In contrast, a variety of well-known inhibitors of the vacuolar-type H\(^+-\)ATPase diminished or prevented phagosomal acidification. NEM in permeabilized, and DCCD in both intact and permeabilized cells substantially inhibited phagosomal acidification at concentrations reported to inhibit other vacuolar pumps (11, 28–31). Moreover, the acidification in permeabilized cells was blocked by nitate with an apparent K\(_{i}\) of 25 mM, which is very similar to the K\(_{i}\) calculated for the pump of coated vesicles (40). Perhaps the most convincing evidence was obtained with bafilomycin A\(_1\), a highly selective blocker of vacuolar-type H\(^+-\)ATPases (19), which completely inhibited phagosomal acidification at sub-micromolar concentrations both in intact and permeabilized cells. Bafilomycin A\(_1\) was similarly effective in blocking phagosomal acidification in human neutrophils after internalization of FITC-labeled *S. aureus*.

The origin of the phagosomal proton pump has not been resolved. Vacuolar-type ATPases have been isolated from clathrin-coated vesicles which may have originated from the plasma membrane or other intracellular organelles (4). In addition, the bafilomycin-sensitive recovery of macrophages from a cytoplasmic acid load has been attributed to a plasmaemmal proton pump (45, 46). Therefore, the phagosomal pumps might be an intrinsic component of the plasma membrane. On the other hand, proton pumps might be incorporated into the phagosomal membrane upon fusion of lysosomes or other intracellular organelles (1, 2, 44, 47). However, in our experimental system phagosomal acidification persisted under conditions where increases in [Ca\(^{2+}\)], were prevented, thereby precluding phagosome to lysosome fusion (48). Moreover, little fusion of phagosomes and large lysosomes was observed in electron micrographs 10 min after initiation of phagocytosis. These observations are in accordance with the findings of Knapp and Swanson (47) who demonstrated using macrophages that phagocytosed red blood cells reached the lysosomal compartment 15 min after ingestion. Moreover, bafilomycin A\(_1\)-sensitive acidification was noted in our system as early as 2 min after phagocytosis had been initiated. This time period is considered to be too short for the occurrence of phagosome-lysosome fusion. It would therefore appear that acidification is initiated prior to lysosomal fusion. This conclusion is in keeping with earlier results demonstrating that phagosomal acidification is detectable before phagolysosomal fusion occurs (2) and prior to the appearance of lysosomal enzyme activity in the phagosomes (3).

While lysosomes appear to be an unlikely source of the phagosomal proton pumps, rapid fusion of other acidic organelles with the phagosome cannot be discounted. Such organelles have been described in *Paramecium caudatum* and called "acidosomes" (49) and in *Dictyostelium discoideum*, where they were termed "prelysosomal acidic vacuoles" (50). In *D. discoideum* this prelysosomal vacuolar compartment is

\(^2\) G. Lukacs, O. D. Rotstein, and S. Grinstein, unpublished data.

\(^3\) There is uncertainty as to whether the clathrin-coated vesicles containing proton ATPases originate from the plasma membrane and/or from the Golgi (see Ref. 11).
thought to provide 7-chloro-4-nitrobenz-2-oxa-1,3-diazole-sensitive proton pumps for the acidification of phagosomes and pinosomes (50). The presence of a similar organelle in macrophages, capable of rapid fusion and proton pump delivery to the phagosomal compartment is a definite possibility, but direct evidence is presently lacking.

The ability of intracellular parasites such as L. pneumophila (10) and T. gondii (6) to prevent phagosomal acidification has been implicated as a mechanism whereby they are able to survive intracellularly. The present studies demonstrating the role of vacuolar pumps in phagosomal acidification suggests that these pathogens might diminish acidification by directly interacting with the vacuolar \( \text{H}^+ \)-ATPase. Alternatively, the parasites or their products may decrease the permeability of the counterions required for net \( \text{H}^+ \) translocation, or increase the leakage of acid out of the phagosomal space. Further characterization of the regulation of phagosomal acidification is clearly required, as it might suggest alternative approaches to the treatment of the diseases caused by these microorganisms.

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