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V-ATPases IN PHAGOCYTIC CELLS

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Summary

V-ATPases in phagocytic cells are known to mediate the acidification of most intracellular organelles. Proton-pump-mediated acidification of these organellar compartments is vital to numerous cell processes, including receptor recycling, protein processing and sorting and microbial degradation. Recent studies have suggested a role for V-ATPases in cytoplasmic pH homeostasis. The present discussion will review the current knowledge regarding the presence of V-ATPases in both the plasmalemmal and organellar membranes of phagocytic cells, the regulation of proton pump activity in these locations and the functional significance of pump-mediated proton translocation.

Introduction

The existence of V-ATPases has been well documented in both neutrophils and macrophages. These proton pumps are primarily responsible for the acidification of several of the intracellular organelles, including endosomes, lysosomes, Golgi-derived vesicles and phagosomes. Proton-pump-mediated acidification of organelles appears to be a crucial event in numerous cell processes, such as receptor recycling, protein sorting and intracellular microbial degradation (Mellman et al. 1986). Recent studies have provided evidence for the presence of V-ATPases in the plasmalemma of macrophages and neutrophils (Swallow et al. 1988, 1990a; Nanda and Grinstein, 1991). In this location, these pumps appear to contribute to cytoplasmic pH (pHi) homeostasis and possibly to acidification of the pericellular environment. The purpose of this paper is to review the current knowledge regarding the presence of V-ATPases in both the plasmalemmal and organellar membranes of phagocytic cells, the regulation of proton pump activity in these locations and the functional significance of V-ATPase-mediated proton translocation.

V-ATPase-mediated cytoplasmic pH regulation in macrophages

Definition

Early studies investigating the mechanisms of pHi regulation in cells of monocyte/macrophage lineage clearly documented the contribution of the Na⁺/H⁺ antiporter and the Cl⁻/HCO₃⁻ exchanger to the ability of these cells to recover from an imposed cytoplasmic acid load (Ladoux et al. 1988). Using thioglycolate-elicited murine

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peritoneal macrophages, we observed that significant pH recovery remained when both Na+/H+ antiport activity and anion exchange mechanisms were rendered inactive, suggesting an alternative recovery process (Swallow et al. 1988). Fig. 1 illustrates pH recovery in cells acid-loaded by incubation in 40 mmol l⁻¹ ammonium chloride for 15 min. In nominally bicarbonate-free medium, the addition of amiloride retarded but did not prevent pH recovery. Subsequent studies revealed that this recovery was mediated by an ATP-dependent H⁺ extrusion mechanism, which had the characteristics of a V-ATPase. This conclusion was based on several lines of evidence: (1) cellular ATP depletion prevented recovery; (2) measurement of extracellular pH following imposition of a cytoplasmic acid-load revealed an acidification with similar kinetics to that observed for pH recovery; (3) recovery was retarded by a variety of known H⁺-ATPase inhibitors, including N-ethylmaleimide (NEM) and dicyclohexylcarbodiimide; and (4) specific inhibitors of V-ATPases, e.g. bafilomycin A₁ (Bowman et al. 1988), nitrate and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), impaired pH recovery whereas inhibitors of F-ATPases (e.g. oligomycin and azide) and of E₁E₂-type H⁺-ATPases (e.g. vanadate) were without effect. Proton pumping was shown to be associated with hyperpolarization of the plasma membrane and was inhibited by cytoplasmic chloride depletion (Swallow et al. 1990a). When considered together, these data indicated that an electrogenic V-ATPase was an important contributor to the pH homeostatic mechanisms in thioglycolate-elicited peritoneal macrophages. Our laboratory as well as other investigators have documented similar activity in both resident and endotoxin-elicited murine macrophages as well as in rabbit alveolar macrophages (Bidani et al. 1989; Tapper and Sundler, 1992). Further, proton-pump-mediated H⁺ extrusion has recently been reported in human neutrophils (see below).

This pH regulatory proton pump mechanism has not been precisely localized. These pumps are probably situated in the plasmalemma, where they serve to translocate protons.
Proton pumps in phagocytic cells

Proton pumps in phagocytic cells

from the cytoplasm directly to the pericellular environment. This concept is supported by
the finding that the kinetics of extracellular acidification mirrors that of pH recovery, by
the observation that hyperpolarization of the plasma membrane occurs during proton
extrusion and finally by the fact that intracellular organelles derived from invaginations
of the plasma membrane (e.g. endosomes and phagosomes) are acidified by V-ATPases
in their delimiting membranes. In addition, osteoclasts, which are cells of
monocyte/macrophage lineage, acidify their pericellular milieu and thereby effect bone
resorption by a plasma membrane V-ATPase (Blair et al. 1989). Studies confirming the
plasmalemmal location of these pumps are being made in our laboratory.

Mechanism of activation

Under resting conditions, proton-pump V-ATPase-mediated proton extrusion occurs at
a slow but steady rate, as shown by NEM-sensitive inhibition of extracellular
acidification. As expected, the rate of proton extrusion by this mechanism following
cytoplasmic acid loading is markedly increased, suggesting activation of the pump. In
urinary epithelial cells, V-ATPases are mobilized to the apical membrane in response to
cytoplasmic acidification by exocytic translocation of submembrane vesicles (Cannon et
al. 1985; see Gluck, 1992). This process is signalled by a rise in cytoplasmic calcium
level and is dependent on an intact microtubule system. The fact that macrophages
contain numerous intracellular organelles capable of exocytosis suggested a similar
mechanism of activation. However, the results of several experimental approaches
indicate that exocytotic translocation of intracellular organelles is an unlikely mechanism
underlying the activation of the proton pump following cytoplasmic acidification
(Swallow et al. 1990a). Neither buffering of the acid-induced calcium rise nor disruption
of microtubules with colchicine altered the rate of pH recovery from an acid load.
Further, cytoplasmic acidification did not induce lysosomal degranulation or exocytosis
of early or late endosomal compartments. These data, therefore, argue against exocytic
pump insertion and are instead consistent with the hypothesis that V-ATPases are
constitutive to the plasmalemma of macrophages and are directly activated by
acidification.

Modulation of V-ATPase activity

Factors modulating V-ATPase activity have been most frequently investigated in
isolated endosome preparations. These studies have shown widely divergent effects of
cyclic AMP on H+-ATPase-mediated acidification of endocytic vesicle preparations
(Gurich and Dubose, 1989; Bae and Verkman, 1990). In addition, stimulation of guanine-
nucleotide binding proteins has been reported to augment H+-ATPase activity (Gurich et
al. 1991). Further discussion of regulation of proton pump activity in intracellular
organelles will be presented below. In this section, various mechanisms shown to regulate
the function of the plasmalemmal V-ATPase in whole cells will be reviewed.
Recent studies (Marletta, 1989) investigating macrophage effector functions have
demonstrated that L-arginine metabolism by these cells results in the generation of a
short-lived, highly reactive nitrogen intermediate, nitric oxide (NO). NO has been shown
to contribute to macrophage microbicidal and tumoricidal activity, as well as to modulate macrophage functions, such as phagocytosis and superoxide production (Albina et al. 1989). The effect of L-arginine metabolism on cytoplasmic pH recovery by proton pumps was studied by examining pH recovery from an imposed acid load in cells incubated in L-arginine-containing and arginine-free media (Swallow et al. 1991). Cells maintained in L-arginine-replete medium had a significantly slower rate of pH recovery than cells in arginine-free medium. This effect appeared to be mediated by a product of L-arginine metabolism, since the competitive inhibitor of L-arginine metabolism, N-monomethyl-L-arginine, reversed the effect, as did co-incubation with the enzyme arginase, which degrades L-arginine. Several lines of evidence supported the concept that NO was the responsible metabolite. First, scavengers of NO, such as myoglobin and iron(II) sulfate, reversed the inhibition. Second, the addition of sodium nitroprusside, which spontaneously generates NO intracellularly, mimicked the inhibition. Finally, cells incubated in L-arginine generated higher concentrations of the stable end product of NO decomposition, nitrite, than did cells incubated in L-arginine-deficient medium. We further proposed that NO-induced elevation of cyclic GMP level might be responsible for the effects of NO. In keeping with this hypothesis, sodium nitroprusside was shown to increase cyclic GMP levels and, furthermore, the membrane-permeant 8-bromo cyclic GMP mimicked the inhibitory effect. These data suggest that cyclic GMP might exert an inhibitory effect on V-ATPase activity.

The effect of various factors present in the inflammatory microenvironment on proton pump activity has recently been investigated. Endotoxin derived from gram negative enteric bacteria was shown to inhibit proton pump activity. This effect was dependent upon the presence of L-arginine in the medium, suggesting a role for nitric oxide generation in mediating the inhibition. This concept is consistent with the well-documented ability of endotoxin to augment L-arginine-derived nitric oxide production in macrophages. By contrast, the proinflammatory cytokine interleukin 1 enhanced proton pump activity, as shown by an increase in bafilomycin-sensitive proton extrusion and in the rate of pH recovery from an imposed acid load. Present studies are endeavouring to define the mechanisms responsible for the modulatory effect of these mediator molecules on proton pump activity.

Functional significance of plasmalemmal V-ATPase activity

Maintenance of cytoplasmic pH near physiological levels is crucial to the preservation of normal cell function. In macrophages, pH homeostasis may be particularly stressed by metabolic acid generation during cell activation and also because these cells must function within an acidic microenvironment commonly found at sites of inflammation and neoplasia. The contribution of proton pump activity to the maintenance of cytoplasmic pH and normal cell function was examined in media adjusted to various pH levels (Swallow et al. 1990b). It was reasoned that pump-mediated H⁺ extrusion might be particularly important to pH regulation at low extracellular pH, since activity of the Na⁺/H⁺ antiporter and anion exchange would be rendered less effective under these conditions. In keeping with this deduction, bafilomycin significantly reduced phorbol-
ester-stimulated superoxide production when tested in a medium of low (pH 6.7) but not neutral pH. This effect was directly attributable to the requirement for a functioning proton pump to maintain pHi close to the physiological range when cells were incubated in an acid extracellular environment. By contrast, at neutral extracellular pH, inhibition of proton pump activity was compensated for by activation of the Na⁺/H⁺ exchanger.

Studies from our laboratory have focused on the contribution of the plasmalemmal V-ATPase to the regulation of cytoplasmic pH homeostasis. However, numerous other roles are important. In particular, proton-pump-mediated extracellular acidification probably contributes to the activation of various degradative enzymes, such as the acid hydrolases and may also be important to the activation of selected cytokines, such as transforming growth factor beta. Finally, plasma membrane V-ATPases may serve as a source of proton pumps for organelles that are derived from invaginations of the plasma membrane, such as endosomes and phagosomes.

Role of V-ATPases in cytosolic pH regulation in neutrophils

Unstimulated cells

Because of its potency and selectivity, bafilomycin has also been used to study V-ATPase involvement in pHi regulation of human neutrophils. As described for macrophages, the putative contribution of the pumps is best studied under conditions where regulation of intracellular pH by other systems, such as the Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers, is minimized. For this reason, Na⁺ and HCO₃⁻ were nominally absent from the solutions used for these experiments. Under such conditions, addition of nanomolar doses of bafilomycin induced a moderate (0.05–0.1 pH unit), yet reproducible, acidification of the cells, measured fluorimetrically. Such acidification could be indicative of a contribution of V-ATPases to the steady-state maintenance of the cytosolic pH, possibly by extrusion of protons across the plasma membrane. However, it is also possible that inhibition of existing pumping into acidic endomembrane compartments, such as secretory granules, unmasks a continuous leakage of organellar acid into the cytosol, lowering the pH of the latter.

To clarify the role and location of the bafilomycin-sensitive pumps, cells were acid-loaded by prepulsing with ammonium, as described above. Unlike macrophages, which recover rather rapidly from acid loads in the absence of Na⁺ and HCO₃⁻, the restoration of cytosolic pH in neutrophils occurred much more slowly. Moreover, only a small fraction of the recovery was inhibited by bafilomycin. From these measurements, it is impossible to determine whether protons are traversing the plasma membrane and whether V-ATPases participate effectively in cytoplasmic pH regulation.

Determinations of acid extrusion from cells, carried out in lightly buffered media, were equally inconclusive. Only a minute fraction of the proton equivalents extruded from the cells was suppressed by bafilomycin. Thus, the role of V-ATPases in pHi regulation of unstimulated human neutrophils remains unclear. It is evident, however, that the transporting capacity of the proton pumps is small relative to that displayed by the electroneutral cation and anion exchangers.
Stimulated cells

From a metabolic viewpoint, unstimulated neutrophils are relatively quiescent. When activated during infection, however, they undergo a sudden and large metabolic burst. The increased metabolic rate is largely attributable to two pathways: the NADPH oxidase pathway, which generates microbicidal oxygen radicals and the hexose monophosphate shunt, which regenerates the NADPH required for the function of the oxidase (Sha’afi and Molski, 1988). Upon neutrophil activation, the plasma membrane potential depolarizes, supposedly because of the electrogenic mechanism of action of the NADPH oxidase pathway, which is thought to release superoxide anions to the external medium while liberating protons into the cytoplasmic compartment. Continued operation of the oxidase requires dissipation of the electrical and pH gradients it generates. This is accomplished in part by a proton-conductive pathway that is activated concomitantly with the oxidase (Nanda and Grinsten, 1991). Outward leakage of protons, driven by the prevailing electrochemical gradient, tends to collapse the intracellular acidity and restore the membrane potential to resting levels. This is best illustrated using Zn$^{2+}$, a potent blocker of the conductive pathway. When activated in the presence of this divalent cation, the depolarization and cytosolic acidification of neutrophils are accentuated.

In addition to the protons released during the oxidation of NADPH to NADP$^+$, metabolic acid equivalents are also produced by the hexose monophosphate shunt. Although the electrochemical potential created by the oxidase can drive a fraction of the protons out of the cell, additional mechanisms are required to prevent intracellular acidification by the shunt. One of these was identified earlier as the Na$^+$/H$^+$ antiporter. This electroneutral cation exchanger was detected in the plasma membrane of neutrophils and was found to be stimulated by chemoattractants, phorbol esters and other agents that mimic activation by infection. Conditions known to preclude antiport activity, such as omission of extracellular Na$^+$ or addition of amiloride, were indeed reported to accentuate the internal acidification recorded in stimulated cells. Nevertheless, substantial extrusion of acid from the cells proceeds even under conditions where the antiport is inactivated and the conductive pathway is blocked.

These observations prompted us to consider the possibility that, like macrophages, neutrophils possess a vacuolar-type proton pump capable of removing excess acid from the cytosol. Since the putative pumps were not clearly detectable in quiescent cells, it was conceivable that pumping was activated upon stimulation. The multiplicity of acid-generating and pH-regulating processes activated during neutrophil stimulation, however, made this possibility difficult to assess. To overcome this complexity, we adopted a reductionist approach, eliminating as many pathways as possible by defined pharmacological means. First, we minimized metabolic acid production by addition of diphenylene iodonium (DPI), an inhibitor of the flavoprotein component of the oxidase (Ellis et al. 1988). Because availability of NADP$^+$, one of the products of the oxidase reaction, is the parameter that determines the activity of the hexose monophosphate shunt, the latter pathway is also inhibited by DPI. Second, Na$^+$/H$^+$ exchange was precluded by removal of extracellular Na$^+$. Third, the proton conductive pathway was eliminated by addition of Zn$^{2+}$. To ensure that the activity of an electrogenic proton pump would not be limited by slow counterion permeation, the potassium ionophore
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Valinomycin was added. A typical experiment performed under these conditions is illustrated in Fig. 2. Stimulation of the cells with an active phorbol ester elicited a significant cytosolic alkalosis, with a maximal rate averaging 0.057±0.012 pH units min⁻¹. Initial indications that this alkalization was mediated by a V-ATPase were obtained with bafilomycin A₁. The antibiotic not only prevented the increase in pHᵢ, but unmasked a small, yet significant, acidification triggered by activation of protein kinase C.

A more detailed characterization of bafilomycin-sensitive pH changes was undertaken in acid-loaded cells. By lowering the cytoplasmic pH, the magnitude of the fluxes was enlarged, optimizing the detection of activity of the putative pumps. We have used two different approaches to acidify the cytosol, with very similar results. For some experiments, the classical ammonium prepulse paradigm was used. In other cases, the source of the acid was endogenous, generated metabolically by the cells upon stimulation. To attain controlled metabolic acidification, cells were suspended in Na⁺-free medium containing Zn²⁺ and then stimulated with phorbol ester. The extent of the acidification was dictated by terminating the activity of the NADPH oxidase with DPI at chosen times. The ensuing recovery of pHᵢ was then analyzed to estimate the contribution of V-ATPases.

Valinomycin was used to determine whether the pHᵢ recovery process in stimulated neutrophils was sensitive to the electrical potential across the plasma membrane. The alkalization was found to be considerably faster in cells depolarized by suspension in medium with high (140 mmol l⁻¹) K⁺ compared to cells in low (3 mmol l⁻¹) K⁺. In the

![Fig. 2. Assessment of vacuolar-type H⁺-ATPase activity in stimulated neutrophils. Peripheral blood neutrophils were isolated from human donors, loaded with the pH-sensitive fluorophore BCECF and the cytosolic pH was monitored fluorimetrically as described by Nanda and Grinstein (1991). Neutrophils were suspended to 10⁶ cells ml⁻¹ in a K⁺ medium consisting of 140 mmol l⁻¹ KCl, 1 mmol l⁻¹ CaCl₂, 10 mmol l⁻¹ glucose and 20 mmol l⁻¹ Hepes, pH 7.50. The NADPH oxidase inhibitor diphenylene iodonium (DPI; 2 µmol l⁻¹) and the potassium ionophore valinomycin (VAL; 1 µmol l⁻¹) were included in the medium from the beginning. The cells were treated with ZnCl₂ (Zn²⁺; 50 µmol l⁻¹) and then stimulated with 4β-phorbol 12-tetradecanoate 13-acetate (TPA; 50 nmol l⁻¹) where indicated by arrows. Bafilomycin A₁ (BAF; 100 nmol l⁻¹) was present from the outset in the lower trace. Representative of at least three similar experiments.](image-url)
latter case, osmotic balance was maintained with N-methyl-d-glucammonium and not with Na⁺, to preclude Na⁺/H⁺ exchange. The observed effects of membrane potential were not due to the passive conductive pathway described above, since maximally inhibitory concentrations of Zn²⁺ were present throughout. Further evidence against the involvement of a passive conductance was obtained with neutrophils suspended in acidic (pH 6.5) K⁺-rich medium. In these cells, the cytosolic pH recovered to levels above the extracellular pH. Subsequent addition of an exogenous conductive protonophore led to a rapid intracellular acidification, implying that the direction of the net electrochemical gradient for protons was inward. It follows that acid extrusion from the stimulated cells was an active process or at least indirectly (secondarily) coupled to an active step.

As described briefly above, bafilomycin was a potent inhibitor of the pHl recovery process. Half-maximal inhibition was attained with 3 nmol l⁻¹ of the A₁ form of the antibiotic and similar concentrations of the B₁ form were also inhibitory. Moreover, inhibition was also observed with NEM, dicyclohexylcarbodiimide and NBD-C₁, whereas ouabain and vanadate were without effect. This pharmacological profile is characteristic of V-ATPases and differs from that of other known transporters of protons, including other types of proton pumps.

Several lines of evidence indicate that activation of the V-ATPase pump by phorbol esters is mediated by protein kinase C. First, only beta isomers of phorbol diesters were stimulatory, whereas the alpha derivatives were without effect. Second, stimulation was also observed with non-phorbol activators of the kinase. Finally, concentrations of staurosporine that inhibit protein kinase C inhibited the effect of phorbol esters. The physiological significance of the activation of the ATPase was highlighted by the finding that pumping can also be promoted by chemotactic peptides, such as formyl-methionyl-leucyl-phenylalanine.

As in macrophages, the cellular localization of the proton pumps responsible for the cytosolic alkalization is currently being investigated by biochemical and immunocytochemical means. Although the results of these studies are still pending, functional evidence is consistent with the hypothesis that at least some of the protons are removed from the cytosol by extrusion across the plasma membrane. Measurements of the rate of extracellular acidification in lightly buffered media indicate that accelerated acid extrusion occurs concomitantly with the alkalization of pHl. It is noteworthy that both the rate of pHl recovery after an acid load and the rate of extracellular acidification are substantially accelerated by cytochalasin B, an agent known to potentiate several responses in neutrophils, including degranulation. The latter observation may suggest that pumps are activated by insertion into the plasma membrane through an exocytic process and/or that acid is initially pumped into endomembrane organelles, which then secrete their contents from the cell. These alternatives remain to be resolved.

In summarizing this section, it can be concluded that regulation of pHl by V-ATPases is comparatively unimportant in resting neutrophils, but becomes more significant following stimulation of protein kinase C or by engaging the chemoattractant receptor. Activation of the pumps is accompanied by increased bafilomycin-sensitive acid extrusion from the cells, compatible with the hypothesis that at least some of the activated pumps reside in, or become inserted into, the plasma membrane. Together with the
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The proton pumps in phagocytic cells contribute to pH homeostasis when neutrophils are stimulated.

Mechanisms and regulation of organellar acidification

The pH of most organellar compartments has been determined to be acidic, yet quite heterogeneous, ranging from 7.0 to as low as 4.5. The maintenance of an acidic pH within these compartments is vital to several important cellular functions, including receptor–ligand dissociation, receptor recycling, protein sorting and trafficking, transport of solutes and intracellular degradation of various molecules (Forgac, 1989; Mellman et al. 1986). Over the past decade, various investigators have reported that active proton accumulation mediated by V-ATPases is the prime mechanism responsible for acidification of most intracellular organelles. However, the factors underlying the variation in pH among different organellar compartments remain poorly defined. Theoretically, these differences could be accounted for by factors such as the activity or number of proton pumps in the endomembrane, the passive proton permeability of the membrane, the buffering capacity of the organelle contents or the permeability of counterions across the membrane.

Previous investigations, performed mostly in isolated endosomal preparations, have identified counterion flux (particularly of chloride) as one of the major determinants of proton pump activity and thus vacuolar pH levels. Since subcellular fractionation might alter the ionic permeability or its regulation, the extrapolation of these concepts to the in vivo setting remains unproven. To overcome this potential shortcoming, we proposed to evaluate the mechanisms regulating the development and maintenance of acidic organellar pH levels using preparations of whole cells. In our initial studies, we investigated pH regulation within the phagosomal compartments of murine macrophages following ingestion of fluoresceinated Staphylococcus aureus. The advantages of this system were that the phagosomal pH could be monitored fluorimetrically throughout the acidification phase of the organelle and that dissipation of the pH gradient could be studied after steady-state phagosomal pH had been achieved. By manipulating the ionic composition of the media and using selective inhibitors and ionophores, we hoped to be able to dissect out the factors regulating the rate of acidification and steady-state pH within the phagosomal compartment. Based on substrate requirements and inhibitor profile, we first concluded that phagosomal acidification in macrophages (as well as in neutrophils) was mediated by V-ATPases (Lukacs et al. 1990). Subsequent studies, therefore, focused on the factors determining the steady-state intraphagosomal pH (approximately 5.8) of elicited murine peritoneal macrophages.

Passive H+ efflux following phagosomal acidification was evaluated by measuring the rate of change of the phagosomal pH (pH$_{p}$) upon complete inhibition of the proton pump with bafilomycin A$_1$. The proton permeability was found to be relatively low. Dissipation of the pH gradient was not limited by counterion permeability, since the addition of the protonophore carbonyl cyanide $p$-chlorophenylhydrazone (CCCP) induced a rapid H$^+$ efflux. Thus, phagosomes appear to exhibit relative ‘tightness’ to protons, at least in

Na$^+$/H$^+$ antiporter and the proton conductance, the pumps are likely to contribute to pH homeostasis when neutrophils are stimulated.
intact cells. Interestingly, phagosomes isolated by cavitation or studied in situ as permeabilized cells demonstrated markedly increased proton efflux following treatment with bafilomycin alone, suggesting that soluble cytosolic factors may contribute to the regulation of proton permeability.

The role of counterion conductance was next studied. If steady-state pH was determined by counterion flux, then an increase in ion permeability would facilitate acidification. However, increasing cation permeability with the ionophores valinomycin, nonactin or gramicidin had no effect on the rate of acidification or the steady-state pH developed. This finding is indicative of a low phagosomal membrane potential and suggests a high intrinsic conductance to ions other than protons. The finding that bafilomycin plus CCCP induced rapid phagosomal alkalization (as described above) provided support for this concept. Both cations and anions appear to contribute to this permeability, as shown by the ability of quinine (a blocker of K⁺ channels) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, a blocker of Cl⁻ channels) to reduce H⁺ efflux (Lukacs et al. 1991). Finally, since counterion conductance was shown to be high, the pH gradient across the phagosomal membrane was predicted to be the primary determinant of the rate of H⁺ pumping. This hypothesis was tested by measuring the rate of pumping as a function of pHp, or the pH gradient across the phagosomal membrane, at a constant cytosolic pH. As shown in Fig. 3, elevation of the pHp above the steady-state value using small doses of monensin caused a marked increase in the rate of pumping. These data suggest that pHp is the major determinant of the rate of proton pumping.

A similar general approach was also used to investigate the factors regulating the acidification of endosomal compartments. As pointed out above, most previous studies reported a central role for anion, specifically chloride, conductance in determining

Fig. 3. pH dependence of phagosomal H⁺ pump activity. The pH of the phagosomal compartment was monitored by the fluorescence of fluorescein-labelled and ingested *Staphylococcus aureus* (Lukacs et al. 1990, 1991). Phagosomes were allowed to acidify and the indicated concentrations of monensin (MON in μmol l⁻¹) were added. After a new steady-state pHp had been attained, the H⁺ pump was blocked with bafilomycin A₁ (BAF, 500 nmol l⁻¹). The bafilomycin-induced rate of net proton efflux was used to evaluate H⁺ pump activity.
organellar pH using isolated subcellular preparations. By using molecular biology techniques, we were able to examine the role of chloride movement in the acidification of endosomes in situ. Chinese hamster ovary (CHO) cells were stably transfected with the gene for the cystic fibrosis transmembrane regulator (CFTR). Based on purification and reconstitution experiments of the CFTR gene product, it has been proposed that CFTR is an anion-selective channel demonstrating a single-channel conductance of 7–9 pS and activation by protein kinase A (PKA)-mediated phosphorylation (Bear et al. 1992). We reasoned that if CFTR was present and functional in endosomes of transfected cells, the effect of chloride permeability on endosomal pH regulation could be assessed.

The expression and function of CFTR at the plasma membrane level was documented by biochemical and electrophysiological techniques (Tabcharani et al. 1991). To localize CFTR functionally in the endosomal compartment, the ionic conductance of the endosomal membrane, both in situ and in isolated endosomes, was determined using the same protocol as described for phagosomes, i.e. by monitoring dissipation of the organellar pH gradient following treatment with bafilomycin A1 and CCCP. The endosomal pH was measured fluorimetrically in cell suspensions or by video-imaging after selective labelling of the endosomes by fluid-phase endocytosis of the pH-sensitive dichlorofluorescein–dextran and/or fluorescein–dextran (G. L. Lukacs, X.-B. Chang, N. Kartner, O. D. Rotstein, J. R. Riordan and S. Grinstein, in preparation). The rate of endosomal alkalization was then determined in the presence of bafilomycin and CCCP. Stimulation of PKA with forskolin, deacetyl-forsklin or 8-(4-chlorophenylthio-adenosine 3',5'-cyclic monophosphate (CTP-cyclic AMP) increased the rate of endosomal alkalization by approximately 180%, indicating an increase in counterion conductance. By contrast, activation of PKA in wild-type or mock-transfected CHO cells failed to alter the conductance of endosomes. The conductance activated by PKA was further investigated in endosomes labelled in vivo and subsequently isolated by differential centrifugation. Acidification of endosomes required both ATP and Mg2+ and was highly sensitive to bafilomycin (apparent KIC = 0.2 nmol l−1), confirming the role of vacuolar-type proton-pump-mediated acidification. The ionic conductance of endosomes was estimated from the rate of pH gradient dissipation in N-methyl-d-glucammonium medium (to prevent cation uptake as counterion). Treatment of endosomes with the catalytic subunit of PKA increased the rate of alkalization by 190% in CFTR-transfected cells but not in wild-type or mock-transfected cells. The stimulatory effect of PKA was reversed by a synthetic peptide inhibitor of PKA (114%), by treatment with alkaline phosphatase (124%) and by the addition of a monoclonal antibody directed against the second nucleotide binding fold of CFTR. When considered together, these observations strongly suggest that CFTR mediates a PKA-activated chloride conductance in endosomes of CFTR-expressing cells, thereby validating the use of this approach to investigate the role of chloride conductance in the regulation of endosomal pH.

Activation of CFTR by forskolin or CTP-cyclic AMP had no effect on steady-state endosomal pH (6.1–6.3) in intact cells. Further, forskolin did not influence endosomal pH levels observed at earlier stages of endosomal trafficking. To examine the possibility that PKA stimulation accelerated the rate of acidification by increasing chloride permeability, the acidification of the endosomal compartment was monitored in CFTR-expressing cells...
Fig. 4. Diagrammatic representation of pHi regulatory mechanisms in phagocytic cells. These include the Na⁺/H⁺ antiporter, the HCO₃⁻/Cl⁻ exchanger, H⁺ channels and V-ATPases. V-ATPases also play an important role in organellar acidification.

in the presence or absence of forskolin after the pH gradient had been dissipated with monensin. These studies demonstrated that PKA stimulation had no effect on the rate of acidification (starting at pH 7.3) while, in the same experiment, dissipation of the pH gradient was markedly augmented, thereby proving that forskolin did indeed increase chloride conductance. These results, therefore, provide strong evidence that anion conductance is not the primary determinant of endosomal acidification and that other factors, such as differential pH sensitivity, altered stoichiometry or slippage of the proton pump, might play important roles in the pH regulation of acidic organelles.

Conclusions

Based on present evidence, four mechanisms of cytoplasmic pH regulation exist in phagocytic cells (Fig. 4). These are (1) the Na⁺/H⁺ antiporter, (2) the HCO₃⁻/Cl⁻ exchanger, (3) proton channels and (4) V-ATPases. The relative activity of each of these processes probably depends on the local microenvironment of the cell. For example, V-ATPases play a major role in pH homeostasis within an acidic milieu, reminiscent of that found within tumors and localized infection. V-ATPases are also responsible for the development and maintenance of acidic pH observed within organellar compartments. This process is central to numerous important cell functions, including protein trafficking, receptor-ligand interactions, antigen processing and microbial degradation. Further investigation into the mechanisms regulating these proton translocating processes will provide important information regarding cell function in normal and disease states.

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