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Review

# Charge compensation during the phagocyte respiratory burst

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**Abstract**

The phagocyte NADPH oxidase produces superoxide anion ($O_2^{\bullet -}$) by the electrogenic process of moving electrons across the cell membrane. This charge translocation must be compensated to prevent self-inhibition by extreme membrane depolarization. Examination of the mechanisms of charge compensation reveals that these mechanisms perform several other vital functions beyond simply supporting oxidase activity. Voltage-gated proton channels compensate most of the charge translocated by the phagocyte NADPH oxidase in human neutrophils and eosinophils. Quantitative modeling of NADPH oxidase in the plasma membrane supports this conclusion and shows that if any other conductance is present, it must be miniscule. In addition to charge compensation, proton flux from the cytoplasm into the phagosome (a) helps prevent large pH excursions both in the cytoplasm and in the phagosome, (b) minimizes osmotic disturbances, and (c) provides essential substrate protons for the conversion of $O_2^{\bullet -}$ to $H_2O_2$ and then to HOCl. A small contribution by $K^+$ or $Cl^-$ fluxes may offset the acidity of granule contents to keep the phagosome pH near neutral, facilitating release of bactericidal enzymes. In summary, the mechanisms used by phagocytes for charge compensation during the respiratory burst would still be essential to phagocyte function, even if NADPH oxidase were not electrogenic.
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*Keywords:* Respiratory burst; Phagocyte; Proton channel; NADPH oxidase; Electron current; pH

## 1. Introduction

Electrogenic membrane proteins are transporters, because their activity results in the net flux of one or more charged species across the membrane. The activity of an electrogenic transporter will change the membrane potential unless charge translocation is compensated. At first glance, it appears that there are two functionally distinct types of electrogenic transporters that we anthropomorphically designate as intentional and inadvertent.

(1) Many transport proteins "intentionally" change the membrane potential. For example, voltage-gated sodium and potassium channels exist in excitable cells for the purpose of generating action potentials [1]. They move cations across the membrane with the explicit intent of changing the membrane potential. A secondary consequence of their activity is to dissipate ionic gradients, albeit very slowly [2], with the result that the $Na^+$,$K^+$-ATPase must expend energy to restore the gradients.

(2) The main goal of the second category of transport protein is to move ions en masse across the membrane. For these transporters, electrogenicity is (at first glance, anyway) a nuisance. At least, this is what we imagine. Such molecules must have a charge compensation mechanism to avoid producing large membrane potentials that would oppose further transport, in a self-inhibitory manner. However, it may be that their electrogenicity serves some additional purpose. For example, the $Na^+$,$K^+$-ATPase mentioned above serves three anti-entropic functions: it continually restores the inwardly-directed sodium gradient and the outwardly-directed potassium gradient, and because it pumps three $Na^+$ out for every two $K^+$ it pumps in, it simultaneously hyperpolarizes the membrane. Any cell membrane "leak" will tend to dissipate both of the ion concentration gradients that are necessary for cellular life and to depolarize the membrane potential; the job of the $Na^+$,$K^+$-ATPase is to combat these consequences of entropy. The primary function of this pump is to restore ion concentration gradients, but as an added bonus, it contributes to the membrane potential [3].

In this review, we examine an enzyme that has traditionally been categorized in the second group as an electrogenic

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transporter that simply needs charge compensation to continue working. Upon closer examination, however, the processes by which charge is compensated are seen to serve several specific and crucial functions. We are left wondering whether the second type of electrogenic transporter exists at all, or whether the electrogenicity of all transporters might serve useful secondary functions.

Here, we consider charge compensation by the phagocyte NADPH oxidase, which is reviewed in more detail elsewhere [4–12]. This multi-component enzyme is crucial to innate immunity, and is expressed abundantly in neutrophils, eosinophils, and macrophages. NADPH oxidase or its homologs are also found in many other cells, where they serve signaling or oxygen-sensing functions, but these are not considered in this review. The primary function of NADPH oxidase in phagocytes is to produce superoxide anions ($O_2^{\bullet -}$) in the extracellular (or intraphagosomal) space (Fig. 1). The $O_2^{\bullet -}$ are precursor to a variety of reactive oxygen species that are generally believed to participate in the killing of bacteria, parasites, and other unpleasant invaders [13–19]. The electrons used by this enzyme to produce $O_2^{\bullet -}$ originate in NADPH inside the cell. The translocation of electrons from NADPH across the membrane is electrogenic, and this property, immediately upon its discovery, led Lydia Henderson, Brian Chappell, and Owen Jones [20] to propose that the charge movement must be compensated. Indeed, it was recently confirmed that the extreme depolarization that would occur without charge compensation is in itself sufficient to turn the enzyme off altogether [21]. Nevertheless, closer examination of the mechanisms used by phagocytes to compensate charge leads to the realization that these mechanisms serve valuable "secondary" purposes for these cells. In fact, Segal and colleagues proposed recently that the primary function of the NADPH oxidase is not to produce $O_2^{\bullet -}$ at all, that $O_2^{\bullet -}$ is simply a byproduct of the primary function, namely, creation of an electrical driving force sufficient to concentrate $K^+$ in the phagosome [22], where it has been proposed to serve important bactericidal functions [23,24]. Although we consider this view to be extreme, it seems clear that the actual charge compensation process does benefit the cell immeasurably. In fact, the oxidase would not be able to complete its bactericidal mission without the specific charge compensation mechanisms that it has adopted if, for example, it were miraculously able to move electrons across the membrane in an electrically silent manner.

After an overview of the structures involved in charge compensation (Section 2), we attempt to integrate existing knowledge by use of a model of electrical events at the plasma membrane during the respiratory burst (Section 3). We then briefly consider aspects of charge compensation in the

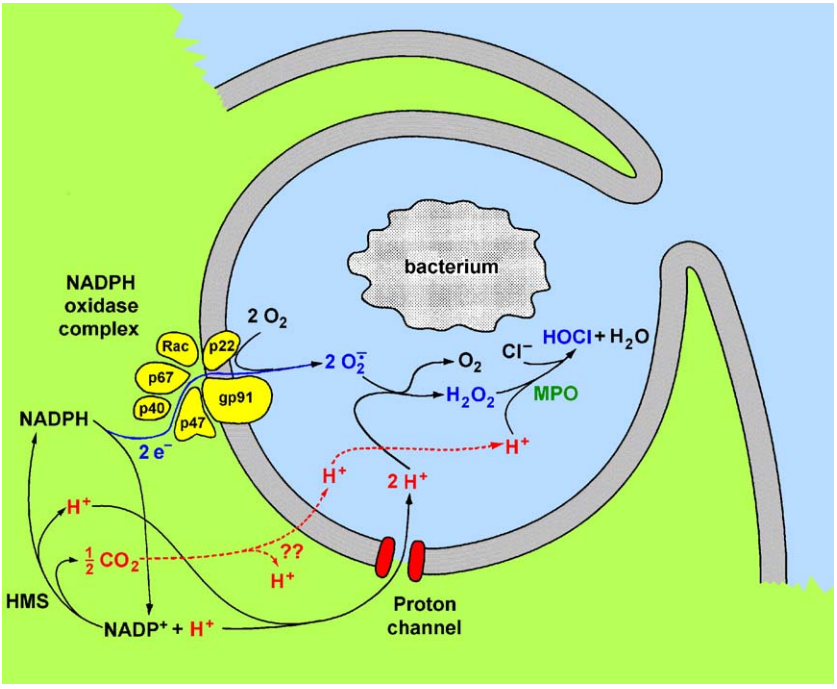


Fig. 1. Diagram of the main reactions that influence $pH_{phagosome}$ during the respiratory burst. During phagocytosis, a bacterium is engulfed and enclosed by plasma membrane (gray) that will form the phagosome. Initially, the phagosome contains extracellular fluid (blue), which is supplemented by fusion of several types of granules and vesicles that release their contents. The six components of the NADPH oxidase complex (yellow) assemble upon stimulation by bacteria, phorbol ester (PMA), chemotactic peptides, or other agonists. Electrons from NADPH in the cytoplasm (green) are translocated across the membrane to reduce $O_2$ to superoxide anion ($O_2^{\bullet -}$) in the phagosome or in the extracellular solution. In eosinophils and PMA-stimulated neutrophils, most of the NADPH oxidase complexes assemble in the plasma membrane and release $O_2^{\bullet -}$ into the extracellular fluid. The electron flux is balanced by proton flux through voltage-gated proton channels (red). In addition to the well-defined proton stoichiometry described in Fig. 6, the hexose monophosphate shunt (HMS) produces one $CO_2$ for every two NADPH regenerated. If the $CO_2$ forms $H_2CO_3$ that dissociates in the cell, the resulting proton may lower $pH_i$ or pass electrogenically into the phagosome. If molecular $CO_2$ diffuses through the phagosome membrane, this will lower $pH_{phagosome}$ in an electrically silent manner. The uncertainty regarding the fate of $CO_2$ is denoted by a dashed line. If the proton derived from $CO_2$ ends up in the phagosome, this will offset the alkalinization by myeloperoxidase (MPO), possibly resulting in a completely pH neutral situation. Modified from [6] with permission of the American Physiological Society.

phagosome (Section 4). Finally, we consider the consequences of various possible mechanisms of charge compensation (Section 5).

## 2. Structures and dimensions of charge compensation in phagocytes

Simultaneous with their discovery that the human neutrophil NADPH oxidase was electrogenic, Henderson and colleagues [20,25,26] also deduced that electrogenic proton efflux from the cytoplasm occurred when the oxidase was activated. The mechanism of charge compensation thus appeared to be proton efflux, which they hypothesized to be mediated by voltage-gated proton channels similar to those discovered 5 years earlier in snail neurons by Roger Thomas and Bob Meech [27]. This proposal has been strongly supported by various types of data, and we still believe that the bulk of charge compensation is mediated by voltage-gated proton channels, although a small fraction of the electronic charge may be compensated by other ions, with consequences that nevertheless may be significant and important.

We will summarize the existing evidence for each mechanism (Section 5) because their relative importance is not known yet with certainty. This uncertainty results in a large part from the inaccessibility of the phagosome to direct experimental examination. A phagosome containing an engulfed bacterium with a total volume 0.2–1.0 fl [7,28,29] may include only a minuscule volume besides the bacterium itself [29], and hence its contents (a) are hard to determine and (b) will change drastically when anything is added or removed. Cytoplasmic vesicles and granules of various types [30] fuse with the phagosome [31] and release their contents [32]. The specific and azurophilic granules in neutrophils are small, with diameters roughly 0.13 and 0.3 μm, respectively [33]; granules in eosinophils are larger, 7–20 fF (corresponding to spheres of diameter 0.47–0.8 μm) [34]. The process is highly dynamic and rapid; 99% of some strains of *Escherichia coli* are killed within 5 min of phagocytosis [35]. Finally, the NADPH oxidase generates $O_2^{\bullet -}$ into this tiny volume in enormous quantities, for which several estimates exist. Reeves et al. [23] calculated that cumulatively 4 M electron-equivalents are introduced into the phagosome. NADPH oxidase activity has been calculated to increase the phagosomal $O_2^{\bullet -}$ concentration (nominally) by 5–20 mM $s^{-1}$ [16,28], or 0.3–1.2 M $min^{-1}$. Another estimate is based on the assumptions of Hampton et al. [28] that maximal stimulation occurs with 20 bacteria each 0.5 fl, giving a total phagosomal volume of 10 fl. Each zymosan-stimulated neutrophil produces 130 fmol of $H_2O_2$ in 2 h [36], corresponding to a cumulative 260 fmol $O_2^{\bullet -}$ or 26 M electron-equivalents in the 10 fl phagosome volume. Although these calculations involve arbitrary assumptions and simplifications, it is clear that enormous quantities of $O_2^{\bullet -}$ are produced. The electrical properties of the phagosome are similarly inaccessible. To our knowledge, attempts to determine electrophysiological properties of phagosomes have been unsuccessful. Furthermore, the types of proteins in the phagosome membrane may change drastically when vesicles or granules fuse and add the constituents of their membranes. Specific granules contain 90% of the cytochrome $b_{558}$ (the membrane-bound part of NADPH oxidase) in resting neutrophils [31], which is inserted into the phagosome or plasma membrane when these granules fuse [37], which they do within seconds after phagocytosis [32]. In this dilemmatic situation, we appreciate the topological diversity of defense mechanisms in innate immunity. Although it is true that neutrophils normally phagocytose their prey and then kill them in secret, remote from our prying eyes, eosinophils boldly attack helminths many times their own size, and secrete $O_2^{\bullet -}$ into the extracellular solution. This behavior greatly facilitates the study of both NADPH oxidase and its charge compensation mechanisms. However, although the several components of the NAPDH oxidase complex (Fig. 1) are believed to be identical in neutrophils and eosinophils [38], there is no assurance that the mechanism of charge compensation will be the same. It is possible that some of the controversy that has arisen regarding charge compensation could result from differences in the particulars of the systems used by different cells.

## 3. Model of charge compensation across the phagocyte plasma membrane

Charge compensation in the phagosome is exceedingly complex because of many interacting factors that include phagosomal membrane potential, voltage- and time-dependent ion channels, a multiplicity of enzymatic and chemical reactions, $pH_i$ and $pH_{phagosome}$, osmotic strength, and phagosomal volume. Compounding this problem, many of these factors are very difficult to measure in situ. Because of the complexity of the phagosome, we model charge compensation across the phagocyte plasma membrane. Not only is this a much simpler situation, but far more extensive and direct experimental data are available for membrane potential and currents across the plasma membrane than for the phagosome membrane. As far as possible, we have used values reported in the literature. We use this model to answer several questions about various scenarios of charge compensation. This model is directly relevant to the eosinophil, which assembles NADPH oxidase in its plasma membrane and secretes $O_2^{\bullet -}$ into the extracellular solution [39]. We use cells stimulated with PMA (a widely-used phorbol ester that activates PKC and effectively activates NADPH oxidase) as a model for the activated state because a large body of data exists. The model may also apply to neutrophils stimulated with PMA or opsonized zymosan, in which a large fraction of NADPH oxidase activity occurs at the plasma membrane [31,40,41], and perhaps to some extent, the phagosome, in view of recent evidence suggesting that $pH_{phagosome}$ remains fairly constant [42,43].

### 3.1. Assumptions and methods

The model includes NADPH oxidase, voltage- and time-dependent proton channels, and eosinophil geometry

Table 1a
Equations

$$I_e = I_{e,max}(t)\left(1 - \frac{1}{1+e^{(V_{1/2}-V_m)/V_{slope}}}\right) \quad (1)$$

$$I_{e,max}(t) = I_{e,max}(\infty)\left(1 - e^{-t/\tau(I_e)}\right)^4 \quad (2)$$

$$I_H = g_{H,max}(t)(V_m - E_H)P_{open}^2 \quad (3)$$

$$dP_{open}/dt = \alpha(1-P_{open}) - \beta P_{open} \quad (4)$$

$$\alpha = \alpha_0(t)e^{k_\alpha V_m} \quad (5)$$

$$\beta = \beta_0(t)e^{-k_\beta V_m} \quad (6)$$

$$dV_m/dt = -(I_e + I_H)/4\pi r^2 c_m \quad (7)$$

$$g_{H,max}(t) = \left(g_{H,max}(\infty) - g_{H,max}(0)\right)\left(1 - e^{-t/\tau(g_H)}\right)^4 + g_{H,max}(0) \quad (8)$$

$$\alpha_0(t) = \left(\alpha_0(\infty) - \alpha_0(0)\right)\left(1 - e^{-t/\tau(\alpha)}\right)^4 + \alpha_0(0) \quad (9)$$

$$\beta_0(t) = \left(\beta_0(\infty) - \beta_0(0)\right)\left(1 - e^{-t/\tau(\beta)}\right)^4 + \beta_0(0) \quad (10)$$

(Tables 1a–1c). The outputs are membrane currents ($H^+$ and $e^-$) and membrane potential (Table 1a, Eqs. (3), (1), and (7), respectively). The proton conductance is regulated by membrane potential and both $pH_o$ and $pH_i$ [44]. We simplified the model by assuming that $pH_i = pH_o = 7.0$ because, although a small acidification followed by alkalinization occurs, $pH_i$ changes by <0.1 Unit during the respiratory burst [45]. We introduced opening and closing rates (Eqs. (4)–(6)) that approximate the behavior of proton channels in human eosinophils and neutrophils studied in perforated-patch configuration at room temperature [46,47]. A simple first-order gating mechanism did not result in a realistic voltage-dependence of the proton conductance, $g_H$, when the observed voltage dependencies of gating parameters were assumed, but a Hodgkin–Huxley type $n^2$ model [1] came closer to reality and was therefore adopted (Eq. (3)). The NADPH oxidase is assigned a maximum activity based on electron currents measured in human eosinophils [48] and neutrophils [46]. Because the NADPH oxidase is inhibited by membrane depolarization [21], we include its voltage dependence, modeled as a Boltzmann function (Eq. (1)) [49] adjusted so that $I_e$ is 24% smaller at +58 mV than at −60 mV, as observed in human neutrophils [21]. When the membrane depolarizes, the same number of active NADPH oxidase complexes generate less $I_e$.

### 3.2. Results of the eosinophil model

The model respiratory burst is driven by turning on NADPH oxidase. The time course reflects assembly and activation of NADPH oxidase. Although the real time course varies enormously depending on the type of agonist and other conditions [11], there is always a delay. We assigned a sigmoid set to reach 50% of its maximal value after 2.5 min (Fig. 2A; Table 1a, Eqs. (1), (2)), to match the time course of $I_e$ observed in eosinophils at room temperature (20–25 °C) [47]. Fig. 2B shows the calculated electron current, $I_e$, in an activated eosinophil ("PMA"), which turns on and reaches a maximum of −10 pA as observed at physiological pH [48]. The other curves are described later.

Fig. 2C shows the calculated proton current, which is equal in magnitude and of opposite sign, always balancing $I_e$ almost perfectly. Fig. 2D shows the calculated plasma membrane potential during these events. The initial resting potential is set at −33 mV ($V_m(0)$ in Table 1c) as reported in human eosinophils [50]. As soon as the NADPH oxidase starts to turn on, the membrane potential rapidly depolarizes to near the $H^+$ Nernst potential, $E_H$, from which voltage it is reluctant to leave. Only after $I_e$ becomes much larger (Fig. 2B) does further depolarization ensue. Eventually, the potential reaches +15 mV (Fig. 2D, PMA), close to the value of +17 mV reported in eosinophils [50].

### 3.3. Effect of proton channel "activation"

When NADPH oxidase is activated, the properties of voltage-gated proton channels change, such that the channels have a much greater tendency to open [46,51]. The voltage at which $H^+$ channels open is shifted by 40 mV to more negative voltages, opening ($\tau_{act}$) is faster, closing ($\tau_{tail}$) is slower, and the maximum conductance ($g_{H,max}$) doubles [46,47]. These kinetic parameters do not change immediately upon addition of PMA, but with variable time courses that are modeled to match the half-times reported in eosinophils (Table 1a, Eqs. (8)–(10)) [47]. In all parts of Fig. 2, curves labeled "rest" were calculated assuming that the gating properties of resting proton channels persist throughout the response, whereas "PMA" indicates the

Table 1b
Parameters common to both cell types

| Parameter | Value | Units |
|---|---|---|
| $H^+$ Nernst potential ($E_H$) | 0 | mV |
| $V_{½}$ | 80 | mV |
| $V_{slope}$ | 40 | mV |
| $k_\alpha$ | 0.025 | $mV^{-1}$ |
| $\alpha_0(0)$ | 0.06327 | $s^{-1}$ |
| $\alpha_0(\infty)$ | 0.2694 | $s^{-1}$ |
| $\alpha_0$(Zn) | $1.412\times10^{-6}$ | $s^{-1}$ |
| $k_\beta$ | 0.04167 | $mV^{-1}$ |
| $\beta_0(0)$ | 0.9106 | $s^{-1}$ |
| $\beta_0(\infty)$ | 0.2694 | $s^{-1}$ |
| $\beta_0$(Zn) | 0.001109 | $s^{-1}$ |
| Cell radius ($r$) | 4 | μm |
| Membrane capacitance ($c_m$) | 1 | μF $cm^{-2}$ |

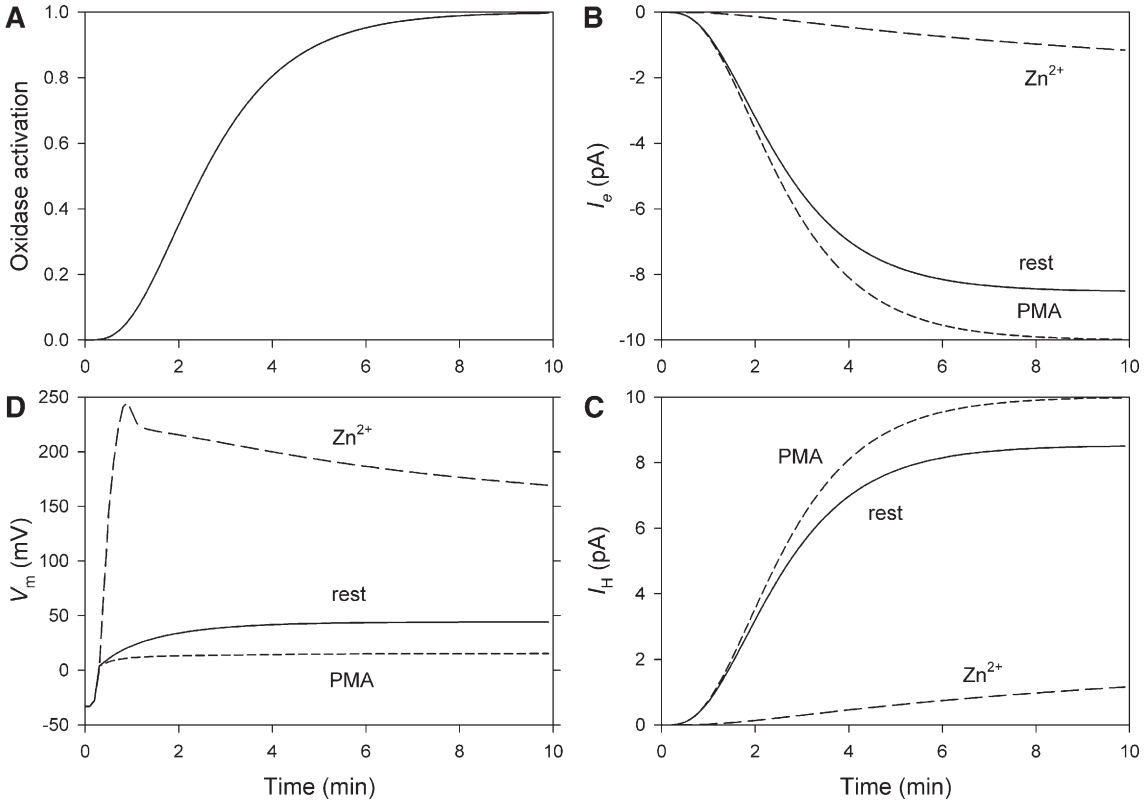


Fig. 2. Model of the respiratory burst in an eosinophil at room temperature. (A) The time course assumed for the turn-on of NADPH oxidase. (B) Calculated $I_e$ for the time course from A, assuming proton channel gating parameters from unstimulated cells ("rest"), PMA-stimulated cells ("PMA"), and in PMA-stimulated cells in the presence of 1 mM $Zn^{2+}$ ("$Zn^{2+}$"). (C) Calculated proton current, which is a mirror image of the $I_e$. (D) Calculated membrane potential under these conditions. The transient peak in the $Zn^{2+}$ curve results from extreme slowing of the proton channel opening rate. NADPH oxidase activity depolarizes the membrane to +245 mV before enough proton current activates to balance it. The potential after 10 min is +15 mV (PMA), +44 mV (rest), and +168 mV ($Zn^{2+}$). To fix the initial resting membrane potential at −33 mV in a steady-state condition, we balanced the tiny calculated inward proton current with an equal outward current of 1.16 fA. See text for further details.

response if the properties of activated proton channels develop after stimulation, as observed experimentally. The main effect of proton channel activation is to reduce the extent of the depolarization by nearly 30 mV (Fig. 2D); the depolarization reaches +44 mV using resting parameters. Because of the voltage sensitivity of NADPH oxidase [21], this depolarization would reduce $I_e$ from −10 pA to −8.5 pA. Thus, by enhancing the gating of proton channels, the eosinophil increases the efficiency of NADPH oxidase by 18%.

### 3.4. Effect of proton channel inhibition by $Zn^{2+}$

Inhibiting proton channels by divalent cations, usually $Cd^{2+}$ or $Zn^{2+}$, inhibits NADPH oxidase activity. It is clear that these metals do not inhibit the enzyme directly, because electron currents are not inhibited by millimolar $Zn^{2+}$ concentrations [21,49,52]. Instead, metals prevent proton channels from opening, thus preventing charge compensation. The resulting depolarization is the immediate inhibitor of NADPH oxidase [21]. The main effects of metals on most voltage-gated channels, including proton channels [53], are to shift the voltage dependence of opening toward more positive voltages and to slow the opening rate. We simulate the effects of $Zn^{2+}$ by shifting the voltage dependence of proton channels and slowing opening, as measured in eosinophils [47], based on a quantitative model of $Zn^{2+}$ effects in epithelial cells [53].

The "$Zn^{2+}$" curves in Fig. 2 indicate the effects of 1 mM $Zn^{2+}$. The profound shift of the voltage dependence and the slowing of the opening rate of proton channels combine to reduce the proton current drastically, such that $I_e$ is not balanced until the membrane potential has depolarized to extreme positive values >200 mV (Fig. 2D). It is well established that the membrane depolarization that occurs during the respiratory burst is exacerbated by $Zn^{2+}$ or $Cd^{2+}$ [20,51,54,55]. The extent of the depolarization would be attenuated by any leak or other ionic conductance not included in the model. Conversely, extreme depolarization can occur only if other conductances are small or absent.

In a recent unpublished observation, Gábor Petheõ and Nicolas Demaurex observed that NADPH oxidase activity in excised membrane patches from eosinophils depolarized the membrane to as high as +180 mV in the presence of $Zn^{2+}$ [55], in excellent agreement with the model predictions. Fig. 3 illustrates the membrane potential measured in an inside-out patch membrane exposed to NADPH to permit NADPH oxidase activity. In contrast to the model time course, which reflects agonist-activated turn-on of NADPH oxidase (Fig. 2A), the measurements in Fig. 3 were initiated by switching the

Table 1c
Cell-specific parameters

| Parameter | Eosinophils | Neutrophils | Units |
|---|---|---|---|
| $T$ | 21 | 37 | °C |
| $V_m(0)$ | −33 | −60 | mV |
| $I_{e,max}(\infty)$ | −12.0 | −26.5 | pA |
| $\tau(I_e)$ | 1.36 | 1.63 | min |
| $g_{H,max}(0)$ | 0.6 | 0.8 | nS |
| $g_{H,max}(\infty)$ | 1.2 | 1.376 | nS |
| $\tau(g_H)$ | 1.09 | 1.85 | min |
| $\tau(\alpha)$ | 0.54 | 0.27 | min |
| $\tau(\beta)$ | 0.87 | 2.34 | min |

Equations and parameters of the plasma membrane model. The voltage-gated $H^+$ channel is modeled as a homodimer in which each subunit can exist in two states (open and closed). $P_{open}$ is the probability that a subunit is in the open state, and $\alpha$ and $\beta$ are the opening and closing rate constants, respectively. Both subunits must be in the open state in order for the channel to conduct. $V_m$ is the membrane potential, $I_e$ and $I_H$ are the electron and proton currents, and $g_{H,max}$ is the maximum proton conductance. When simulating the effects of $Zn^{2+}$, $\alpha$ and $\beta$ were held constant; otherwise they were time ($t$) dependent, as were $g_{H,max}$ and the maximum electron current, $I_{e,max}$. For time dependent parameters, (0) indicates the resting value, and ($\infty$) indicates the final value after stimulation. $I_e$, $\alpha$ and $\beta$ were also voltage dependent. The mathematical model of the respiratory burst was realized as a system of ordinary differential equations, which were then solved with the function NDSolve in Mathematica ver. 4.2 (Wolfram Research, Champaign, IL).

amplifier to zero-current mode, to record the membrane potential. In the presence of NADPH (Fig. 3A), the membrane depolarized beyond +20 mV and relaxed to ~+10 mV, similar to the depolarization measured in activated eosinophils [50]. When 3 mM $Zn^{2+}$ was present, the depolarization exceeded +140 mV in the example in Fig. 3A, and then relaxed to a lower value. The large depolarization and even the subsequent relaxation strongly resemble the model predictions (Fig. 2D), although the explanation for the relaxation differs. In the model, the depolarization decays due to slow activation of proton currents, whereas in Fig. 3A, attenuation of the decay by NBT (nitroblue tetrazolium), a $O_2^{\bullet-}$ scavenger, suggests that product inhibition may be responsible. Fig. 3B shows control measurements in which the membrane potential equilibrated around −40 mV in the absence of NADPH (roughly corresponding to the membrane potential in a resting cell), and depolarized to +160 mV when NADPH was added. These experiments confirm that NADPH oxidase activity in the presence of $Zn^{2+}$ can produce extreme depolarization.

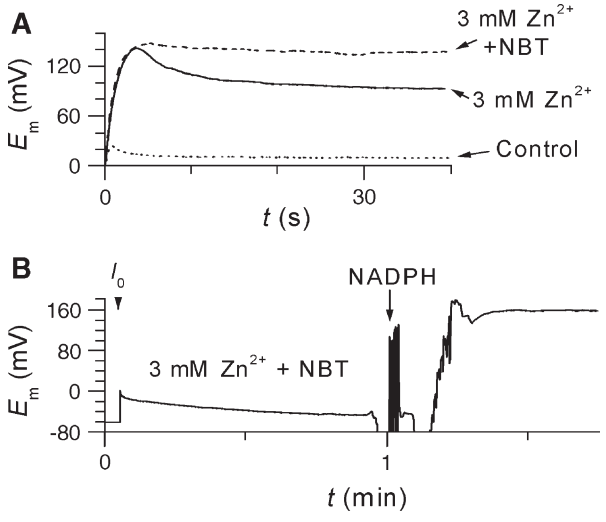


Fig. 3. Membrane potential changes in inside-out membrane patches from human eosinophils with an active NADPH oxidase. (A) Time-course of spontaneous membrane potential changes measured with pipette solutions containing 0 or 3 mM $Zn^{2+}$ (dotted and solid line, respectively) or 3 mM $Zn^{2+}$ together with 0.5 mg/ml NBT (dashed line). In inside-out recording configuration, the pipette solution faces the extracellular side of the membrane. The patch was previously exposed to 0.8 mM NADPH and then switched to zero-current ($I_0$) mode from a −60 mV holding potential at time 0. (B) Membrane potential changes elicited by the addition of 0.8 mM NADPH to a patch held in $I_0$ mode. The pipette solution contained 3 mM $Zn^{2+}$ and 0.5 mg/ml NBT. In other experiments (not shown), 6 μM DPI inhibited the NADPH induced depolarization. Experimental conditions were similar to those described in [49]. [Previously unpublished figure generously provided by Gábor Petheõ and Nicolas Demaurex.]

Extreme depolarization directly inhibits $I_e$ (Fig. 2B) by opposing electron transfer through NADPH oxidase [21]. The integral of $I_e$ (effectively reflecting cumulative $O_2^{\bullet-}$ production) over 10 min is reduced by $Zn^{2+}$ by 92% in the model, as observed experimentally [21]. To reiterate, $Zn^{2+}$ reduces $I_e$ by membrane depolarization, which in the model, as in real cells [21], reduces the turnover rate of NADPH oxidase. $Zn^{2+}$ does not directly affect NADPH oxidase; rather, its effects are mediated by proton channels.

### 3.5. Application to neutrophils

Both proton conductance ($g_{H,max}$) and NADPH oxidase activity ($I_e$) are several times larger in eosinophils than neutrophils [6,56]. The membrane potential in neutrophils during the respiratory burst is modeled at 37 °C in Fig. 4, because depolarization has been measured in neutrophils at 37 °C [54,57]. Adjustment of parameters for temperature increases $I_e$ somewhat more than $g_{H,max}$, because $I_e$ has

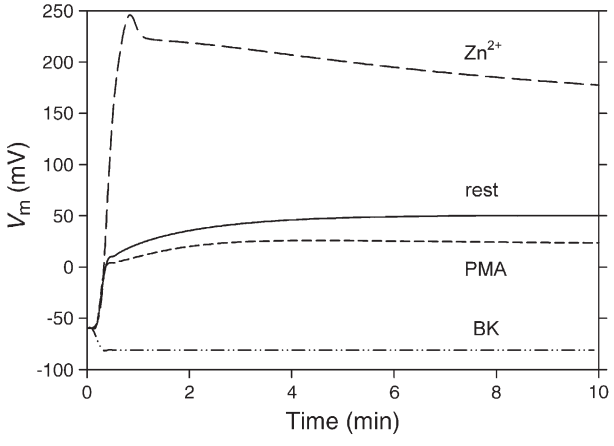


Fig. 4. Model of the respiratory burst in a neutrophil at 37 °C. Labels have the same meaning as in Fig. 2, with the addition of "BK" which includes a $g_K$ of 3.3 nS ($E_K$ is −89 mV) as reported in one study of human neutrophils [24]. Similar to Fig. 2, we forced the starting resting potential, in this case to −60 mV, by a tiny outward current of 77.5 aA. Membrane potential values 10 min after stimulation are −81 mV (BK), +24 mV (PMA), +50 mV (rest), and +176 mV ($Zn^{2+}$).

stronger temperature dependence [56,58]. The initial resting potential was set to −60 mV (Table 1c), typical of numerous studies of neutrophils [6]. As NADPH oxidase turns on, there is rapid depolarization as was seen in the eosinophil model (Fig. 2D). The enhanced activation of proton channels ("PMA") decreases the calculated peak depolarization by 24 mV relative to resting proton channels ("rest"). Similar to its effects in eosinophils, 1 mM $Zn^{2+}$ reduced the calculated $O_2^{\bullet -}$ production by neutrophils by 93%.

The depolarization measured during the respiratory burst in PMA-stimulated human neutrophils can exceed +50 mV [54,57,59]. The model generated a peak depolarization only to +26 mV using proton channel parameter values from PMA-activated neutrophils [46]. Several factors may contribute to this discrepancy. First, reducing the proton conductance increases the predicted depolarization. Depolarization to +50 mV could be produced in the model by reducing $g_{H,max}$ to 27% (not shown). Thus, our extrapolation of $g_{H,max}$ to 37 °C may have overestimated its actual value. Second, although we assumed that $pH_i$ and $pH_o$ are identical and unchanging, small pH changes, possibly occurring near the membrane, could significantly affect proton channels. Third, the oxidase might be activated in neutrophils before the proton channels are fully activated. The rate of activation of proton channels (here "activation" means conversion to the enhanced gating mode) by PMA is about twice as fast in eosinophils [47] as in neutrophils [46]. With resting proton channel gating kinetics, the model depolarization reached +50 mV (Fig. 4, "rest"), in agreement with experimental observations. Finally, the patch-clamp studies of proton currents included EGTA in all external solutions, which shifts proton channel gating negatively by 15–20 mV [44]. "Correcting" for the EGTA effects (which may or may not be more physiological) would increase the depolarization. Further study may resolve this question. In most respects, the predictions of the model closely resemble actual data.

This exercise brings into focus the idea that charge compensation is ultimately very simple. Given a particular $I_e$, the membrane will depolarize until an equal and opposite current is activated. The proton conductance is clearly sufficient to prevent excessive depolarization. If anything, it appears somewhat too large to reproduce the depolarization observed in neutrophils perfectly. Adding any other conductance, including nonselective channels (excepting $Ca^{2+}$ or $Na^+$, which have positive Nernst potentials), would exacerbate the discrepancy by coercing the membrane potential toward the relevant Nernst potential. This issue is addressed in the next section.

### 3.6. Are $K^+$ currents in phagocytes possible?

What would happen if there were a significant $K^+$ conductance ($g_K$)? Fig. 4 ("BK") illustrates the effect of introducing a $g_K$ of 3.3 nS that is activated over several minutes after PMA stimulation, as reported in neutrophils [24]. With this large $g_K$, there is no depolarization, but instead the membrane hyperpolarizes and is clamped near the Nernst potential for $K^+$, which does not change, because PMA stimulation does not alter the intracellular $K^+$ content [60]. In human neutrophils and eosinophils, depolarization well beyond 0 mV has been observed consistently [50,51,54,57,59]. Depolarization even to 0 mV cannot occur in the model if $g_K$ exceeds 240 pS (the conductance of a single BK channel). The proposed participation of BK channels [24] is also unlikely in view of evidence that BK channels are not expressed in human neutrophils or eosinophils [61]. Nevertheless, some other $K^+$ channel or transporter might exist, at least in neutrophils [62], and contribute to the observed $K^+$ flux [23,54]. However, any $g_K$ in the plasma membrane must be quite small.

Rada et al. [54] measured depolarization of the plasma membrane during the respiratory burst in neutrophils, and adjusted the activity of NADPH oxidase with variable amounts of the inhibitor DPI, diphenylene iodonium. A remarkable observation was that the relationship between NADPH oxidase activity and membrane depolarization was highly non-linear (Fig. 5). More than 50% of the stimulus-induced depolarization occurred when NADPH oxidase activity was <5% of maximal. The dashed curve in Fig. 5 shows that the depolarization predicted by the neutrophil model, as a function of $I_e$, closely resembles the data. Thus, this nonlinear behavior is completely predictable. Another manifestation of this phenomenon is evident in Fig. 2D where there is rapid depolarization to near 0 mV at a time when $I_e$ has just begun to activate. The nonlinear behavior results from the lack of any conductance at negative voltages that can counteract depolarization. Although eosinophils have a small inwardly rectifying $K^+$ conductance [63], this channel closes with depolarization, and hence will have little effect. The resistance of the entire plasma membrane of activated human eosinophils is very high, 53 GΩ measured

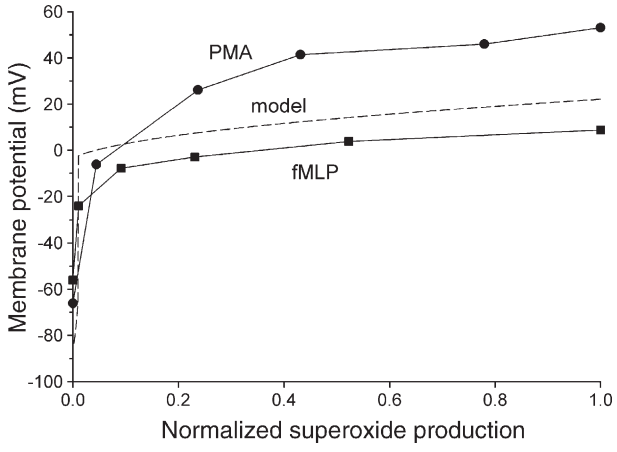


Fig. 5. Comparison of depolarization observed in human neutrophils by Rada et al. [54] and the predictions of the model of the respiratory burst in a neutrophil at 37 °C. The membrane potential measured 7 min after stimulation with PMA (●) or 1 min after fMetLeuPhe (■) is plotted against the normalized $O_2^{\bullet -}$ production [54]. The curve shows the membrane potential predicted from the neutrophil model, plotted against the normalized steady-state $I_e$, calculated by varying $I_{e,max}$. In order to "set" the initial resting potential at a negative value, a 10-pS $K^+$ conductance was included, roughly that of one inward rectifier $K^+$ channel [63]. In both data and model, a tiny activation of NADPH oxidase suffices to produce rapid depolarization toward the Nernst potential for protons, $E_H$. $E_H$ is 0 mV in the experimental conditions in which most proton channel parameters were measured ($pH_i$=$pH_o$), but is near −19 mV in intact neutrophils, where $pH_o$ is 7.4 and $pH_i$ is 7.1 [45].

between −60 mV and 0 mV with $K^+$ in the pipette solution [Morgan, D., Cherny, V.V. and DeCoursey, T.E., unpublished data]. Without a balancing conductance, it takes relatively few electrons crossing the membrane to produce rapid depolarization. Once the proton conductance turns on, the membrane potential rapidly approaches $E_H$, the $H^+$ Nernst potential.

The phenomenon in Fig. 5 may also explain reports that depolarization paradoxically preceded measurable $O_2^{\bullet -}$ release in neutrophils [64–67]. If depolarization is the result of NADPH oxidase activity, one might expect it to occur simultaneously with $O_2^{\bullet -}$ release. This phenomenon likely reflects the fact that depolarization requires only a miniscule flux of electrons. To depolarize the plasma membrane by ~100 mV requires extrusion of two million uncompensated electrons; less than 0.01% of the 30 billion electrons that are translocated by one eosinophil during the respiratory burst. An additional factor may be that the "activated" gating mode of proton channels facilitates their opening early in the respiratory burst, as suggested previously [6]. In fact, proton channel "activation" would drive the membrane toward $E_H$ even without $I_e$ if it resulted in inward $H^+$ current that was larger at the resting potential than any prevailing outward current (presumably through inward rectifier $K^+$ channels or the $Na^+$,$K^+$-ATPase). Most likely, both factors contribute to the predominance of depolarization.

### *3.7. Summary of the phagocyte plasma membrane model*

Using reasonable parameter values derived from measurements in human neutrophils and eosinophils, the model reproduces the experimentally observed plasma membrane depolarization during the respiratory burst. The known effects of $Zn^{2+}$ on proton channels result in extreme depolarization that inhibits $I_e$, also consistent with actual behavior. A striking result is that the depolarization that is observed experimentally during the respiratory burst cannot occur if there is any significant conductance other than the $g_H$. Furthermore, the model shows that the modulation of proton channel properties during the respiratory burst results in 30 mV less depolarization than would occur otherwise. This smaller depolarization increases the steady-state electron current by about 18%, significantly improving the efficiency of NADPH oxidase.

## 4. Charge compensation in the phagosome

Charge compensation in the phagosome is far more complicated than at the plasma membrane. A model would have to include a multiplicity of enzymatic reactions, variable $pH_{phagosome}$, buffering, osmotic strength, vesicle fusion with the phagosome, and changing phagosomal volume. Because of the complexity and because many of the important parameters have not been quantified, the results would be less definitive. It is important to recognize that although NADPH oxidase that is assembled on the plasma membrane, as in eosinophils or PMA-stimulated neutrophils, may be the best model we have for the phagosome, there are crucial differences. First, granules and vesicles fuse with the phagosome and add their contents to this small volume (Fig. 6), whereas the extracellular fluid is effectively infinite and unchanging. Second, membrane proteins might exist in the phagosome that are not present in the plasma

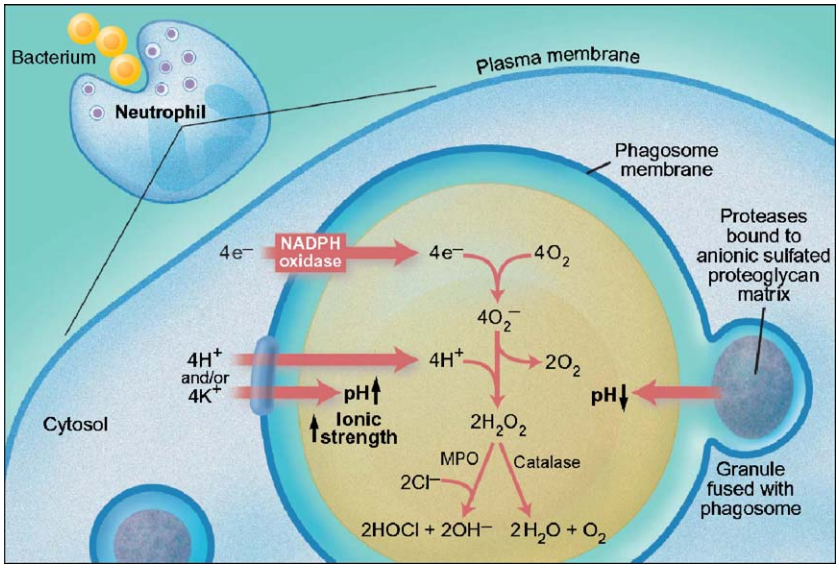


Fig. 6. Diagram of reactions occurring in the small volume inside the phagosome during the respiratory burst in a phagocyte. Electrons extracted from cytoplasmic NADPH are moved across the membrane where they reduce $O_2$ to $O_2^{\bullet -}$. Theoretically, the charge translocation due to electron flux could be compensated by $H^+$ or $K^+$ flux in the same direction, or by $Cl^-$ entry into the cell. Compensation by $K^+$ rather than by $H^+$ tends to increase $pH_{phagosome}$ and ionic strength. The $O_2^{\bullet -}$ rapidly and spontaneously disproportionates (dismutates) into $H_2O_2$ consuming one proton for each electron (or $O_2^{\bullet -}$). A large fraction of the $H_2O_2$ in neutrophils is converted to hypochlorous acid, HOCl, by MPO, myeloperoxidase. This reaction consumes $Cl^-$ and produces $OH^-$ leading to net alkalinization of the phagosome. Alternatively, $H_2O_2$ can be "disarmed" by catalase (MPO can also act as a catalase) into $H_2O$ and $O_2$, both of which are freely permeant through membranes. Granules that fuse with the phagosome release their acidic contents, lowering $pH_{phagosome}$ and increasing its volume. Reprinted with permission of the authors and AAAS from ref. [17]. Copyright 2002 AAAS.

membrane. Third, phagocytosed bacteria are present in the situation in which we are most interested. It is entirely possible that charge compensation in the phagosome differs drastically from that occurring at the plasma membrane.

### 4.1. Phagosomal membrane potential

We are unaware of any data on the phagosome membrane potential during the respiratory burst. In macrophages, the phagosome membrane appears to be permeable to monovalent cations and anions, and consequently, develops little membrane potential [68]. What would be the consequences if this were true of neutrophil phagosomes? First, the ionic composition of the phagosome would rapidly approach that of the cytoplasm: 138 mM $K^+$, 13.5 mM $Na^+$ [57], and 80 mM $Cl^-$ [69]. This equilibration would have little net osmotic consequence; $K^+$ influx and $Na^+$ efflux would be about equal and $Cl^-$ efflux would result in slight shrinkage. (Here, we refer to fluxes from the perspective of the phagosome, so that efflux is from phagosome to cytoplasm and influx is the reverse.) Despite the constraint of minimal membrane potential, there must still be charge compensation. The proton channel would have a moderate open probability at a 0 mV transmembrane potential (which might occur in a "leaky" phagosome), but only if it had "activated" properties, because the open probability of "resting" proton channels is very low at 0 mV. Thus, we may propose that the activation of proton channels plays a key role in the phagosome membrane, enabling a proton conductance capable of compensating charge at 0 mV.

If, instead, the phagosomal membrane in neutrophils or eosinophils is electrically tight, then charge translocation by any transporter, including NADPH oxidase, will profoundly affect the membrane potential. One expects that the dominant force will be $I_e$, which will tend to depolarize the membrane (i.e., the voltage in the cell becomes positive relative to that in the phagosome). It is almost certain that proton channels are present, because the phagosome is created by endocytosis of plasma membrane that contains abundant proton channels in all phagocytes [6]. No other ion channels have been identified in the phagosomal membrane. The density of NADPH oxidase complexes in the phagosome membrane is likely substantially greater (2- to 10-fold, depending on assumptions) than in the plasma membrane of PMA-stimulated neutrophils, based on geometrical and other considerations [7,29,31,70]. In contrast, there is no evidence that proton channels are concentrated. This imbalance of $I_e$ and $I_H$ ought to result in greater depolarization of the phagosomal membrane than the plasma membrane.

### 4.2. Charge compensation and phagosomal volume

Two major constraints on charge compensation mechanisms in the phagosome are its effects on phagosomal volume and on $pH_{phagosome}$. The phagosome volume depends on the size of the engulfed microbe, but will also increase dramatically over time. First specific granules, and shortly thereafter azurophilic granules fuse with the phagosome, increasing its size, and especially its volume, which, excluding the bacterium, is thought to be initially extremely small. Multiple phagosomes may coalesce, forming much larger structures [32].

Charge can be compensated by cation influx or anion efflux from the phagosome. Influx of cations other than $H^+$ would result in swelling to 10–20 times the original phagosome volume, assuming a total electron flux of ~2–4 M [16,23], even if one ignores the added volume due to vesicle fusion. The actual phagosome volume increases only ~3-fold in 15 min [23], and this increase can be accounted for by the addition of granule contents. Fusion of specific granules adds 0.26 fl and azurophilic granules add another 0.92 fl to the phagosome, which together would triple its volume if it started out at 0.6 fl, a reasonable value, depending on the strain of bacteria ingested. These estimates are based on the dimensions and numbers of granules in neutrophils, 4600 specific granules 0.13 μm in diameter and 1300 azurophilic granules 0.3 μm in diameter [33], and the assumption that all granules fuse with the first 20 phagosomes, which seems a reasonable synthesis of several observations [16,28,32]. Thus, it appears that the phagosomal volume changes that occur can be accounted for entirely by vesicle fusion, and consequently charge compensation must be essentially osmotically neutral. Anion efflux would rapidly deplete the phagosome (Section 5.6) and thus can account at most for a small fraction. In contrast, protons can compensate charge because they "disappear" osmotically via biochemical reactions once inside the phagosome (Section 5.3).

### 4.3. Charge compensation and $pH_{phagosome}$

The pH of neutrophil granules is low (5.6) [71], and this must present a challenge to the phagosome, because $pH_{phagosome}$ remains near neutral during the respiratory burst in human neutrophils [42,43]. Thus, the acid load presented by the granule contents must be neutralized, as has been discussed by Segal and colleagues [23,24]. Four possible mechanisms include: (1) charge compensation by ions other than protons, as proposed by Reeves et al. [23], (2) induction of a proton leak, as postulated by Jankowski et al. [42], (3) the MPO reaction (Fig. 1), or (4) electrically silent diffusion of HOCl out of the phagosome (Section 5.6).

At most only a small fraction of charge can be compensated by ions other than protons. If we postulate a $K^+$ influx that results in 200–300 mM $K^+$ in the phagosome [23], we must arbitrarily prevent osmotic volume changes. Observed $K^+$ flux was 5–6% of the total charge compensation [23]. A postulated $g_{Cl}$ could conceivably compensate charge only until phagosomal $Cl^-$ was dissipated, but this would preclude formation of HOCl by MPO (Section 5.6). For the purpose of charge compensation, we rule out the second possibility, conductive proton efflux from the phagosome to the cytoplasm, despite evidence that the phagosome membrane proton permeability increases upon activation of NADPH oxidase [42] because charge cannot be compensated by protons that later leak out to maintain $pH_{phagosome}$. The third possibility is that as azurophilic granules fuse with the phagosome and release MPO,

alkalinization will result. The MPO reaction initially consumes protons, then HOCl reactions will regenerate some or most of the protons, perhaps resulting in no further change in $pH_{phagosome}$. One predicts a rapid initial increase in $pH_{phagosome}$ followed by further alkalinization if HOCl diffuses out of the phagosome, the fourth option. As discussed in Section 5.6, diffusion of HOCl out of the phagosome would occur only with the neutral protonated form of this weak acid ($pK_a$ 7.53 [18]), and would therefore remove protons in an electrically silent manner, increasing $pH_{phagosome}$ and allowing recycling of protons for further charge compensation. These possibilities are considered in the next section.

## 5. The specific mechanisms of charge compensation are beneficial to phagocyte function

Charge compensation requires physical movement of charge (electrons or ions) across the phagosomal or plasma membrane and this movement will have consequences beyond electrical ones. Specifically, one must consider effects on the pH, osmolarity, and ion content of both the phagosome and the cytoplasm. As we will see, protons are the Renaissance ions of the respiratory burst—they do almost everything. Proton efflux compensates charge, maintains pH in the cytoplasm and phagosome, while minimizing changes in osmolarity, in addition to which, protons act as substrate for production of reactive oxygen species.

### *5.1. Protons compensate nearly all of the charge translocated by NADPH oxidase at the plasma membrane*

Of four possible mechanisms of acid extrusion, $Na^+/H^+$-antiport, the $H^+$-ATPase, $CO_2$ diffusion, and proton channels, only the $H^+$-ATPase and proton channels are electrogenic, and thus are capable of charge compensation (ignoring other ions for the present). Although itself electroneutral, $Na^+/H^+$-antiport could load the cell with $Na^+$, which might be extruded by the electrogenic $Na^+,K^+$-ATPase, which does contribute to the resting membrane potential of neutrophils [72,73]. If the goal is charge compensation, this mechanism would be inefficient because three $H^+$ must be exchanged in order to extrude one net charge, at the expense of ATP. Some $H^+$ is extruded via antiport, because antiport inhibitors result in cytoplasmic acidification upon stimulation [45]. However, eliminating $Na^+/H^+$-antiport reduced peak NADPH oxidase activity in PMA-stimulated neutrophils by only 11–14% [45,74]. This slight reduction could be accounted for by inhibition of the oxidase by low $pH_i$ [48,74], without need to postulate loss of charge compensation. Inhibiting the $H^+$-ATPase does not affect the respiratory burst [42,75,76]. In contrast, inhibiting proton channels abolishes the respiratory burst [21,26,77–81]. Stoichiometric measurements in human neutrophils indicate that the ratio of electrons transported to protons extruded is 1:1 [82–84]. When proton channels are inhibited by $Zn^{2+}$, partial restoration of function is seen upon addition of a protonophore, CCCP, which mediates charge compensation by protons [21,26]. Thus, substantial experimental evidence indicates that proton flux through voltage-gated proton channels compensates most of the charge across the plasma membrane. The practical significance of charge compensation is to limit the extent of the large, rapid depolarization that would otherwise occur when NADPH oxidase is active.

### *5.2. Proton flux offsets the changes in pH that would otherwise result from electron flux*

NADPH oxidase activity tends to acidify the cytoplasm and alkalinize the phagosome. Opposing the latter tendency, neutrophil lysosomes or granules that fuse with and release their contents into the phagosome are acidic, pH 5.6 [71]. The cytosolic pH ($pH_i$) must be kept near neutral to optimize NADPH oxidase activity [48]. In contrast, the oxidase is insensitive to extracellular pH ($pH_o$) and therefore presumably also to the phagosomal pH ($pH_{phagosome}$) [48]. In early studies, $pH_{phagosome}$ was reported to change biphasically, first becoming alkaline then acidic [85,86]. It was concluded that $pH_{phagosome}$ first alkalinizes to release proteolytic enzymes [23] and to optimize the pH for certain enzymes that prefer neutral or alkaline pH [14,32], and then acidifies to the advantage of antimicrobial enzymes that kill or degrade bacteria most effectively at low pH [14,32,87–89]. Despite this comfortable scenario, the extent of phagosomal acidification in neutrophils, but not macrophages [89], may be much less than previously thought. Many fluorescent pH indicators are chemically modified by HOCl, compromising their pH sensitivity [90]. In fact, more recent studies found no change in $pH_{phagosome}$ during the respiratory burst in neutrophils, neither alkalinization nor acidification [42,43]. In summary, previously reported pH changes in the neutrophil phagosome appear to be largely artifactual, and recent evidence supports little change in $pH_{phagosome}$.

The cytoplasmic pH ($pH_i$) in neutrophils first decreases and then increases above resting values [45,91]. The decrease reflects intracellular acid production by NADPH oxidase activity (Fig. 1), and the secondary alkalinization reflects activation of $Na^+/H^+$-antiport. $Na^+/H^+$-antiport appears to play a role in vivo because inhibiting the antiporter results in a monotonic decrease in $pH_i$ during the respiratory burst [45,74,92]. The voltage-gated proton channel evidently has a lower $pH_i$ set-point than does the $Na^+/H^+$-antiporter, whose set-point is increased during the respiratory burst (i.e., it activates at a higher $pH_i$ range than in resting cells) [92–94]. However, the majority of proton efflux persists in the presence of the $Na^+/H^+$-antiport inhibitor amiloride [84,95]. The activity of NADPH oxidase represents a huge challenge to pH homeostasis. Because the $Na^+/H^+$-antiporter is electroneutral [96,97], it cannot contribute directly to charge compensation. Thus, nearly all of the 4 M electrons are compensated by protons exiting through proton channels, with a small and ill-determined contribution by the $H^+$-ATPase. $Na^+/H^+$-antiport can only fine-tune the process of acid extrusion across the plasma membrane. The $Na^+/H^+$-antiporter likely extrudes enough protons to increase $pH_i$ to its new higher set-point,

as well as any intracellular protons "left behind" by the small fraction of charge compensation contributed by ions other than protons.

### 5.3. Proton flux minimizes osmotic swelling of the phagosome

If all the electronic charge were compensated by $K^+$ movement from cytoplasm into the phagosome, the volume of the phagosome would obligatorily increase roughly 10- to 20-fold due to osmotic swelling, as was discussed in Section 4.2. In fact, the phagosome swells about 3-fold over 15 min in neutrophils [23], and most or all of this is attributable to the addition of specific granule contents, which are already present in 50% of phagosomes within 30 s after ingestion of bacteria [32], and later of azurophil granule contents. In this context, $K^+$ flux can contribute at most a small fraction of charge compensation. In contrast, protons are ideally suited to this function. The end products of the protons and electrons that enter the phagosome include membrane permeable substances like $H_2O$ and $O_2$ formed by MPO when acting as a catalase or HOCl and $OH^-$ by the alternative pathway (Fig. 6). The $H_2O$ formed directly or by neutralization of $OH^-$ may help to solubilize granule contents. Some of the HOCl attacks groups on bacterial proteins [98,99], but it is membrane permeable [100] and thus, like $O_2$, can disappear without osmotic consequence. Thus, charge compensation by protons produces minimal osmotic effects.

### 5.4. Protons are required substrates for the production of reactive oxygen species

Most of the $O_2^{\bullet -}$ produced by NADPH oxidase in phagocytes is converted to $H_2O_2$ by spontaneous disproportionation (dismutation) [14,101]. As evident in Fig. 6, one $H^+$ is consumed for each $O_2^{\bullet -}$ converted to $H_2O_2$. A substantial fraction of the $H_2O_2$ is then converted to HOCl (hypochlorous acid). Although some early studies suggested that relatively small amounts of HOCl were produced in the phagosome [102], most studies find this pathway to be significant [16,18,98,103,104]. Estimates of the percent of $O_2$ consumed that is converted to HOCl are 40% [36], >28% [105], 31–55% with PMA and 72% with OPZ as the stimulus [106], >11% which the authors regarded as several-fold too low [15], and 70% [99]. The MPO catalyzed reaction that produces HOCl (Fig. 1) also consumes protons [18]. (This reaction can be written without consuming a proton (Fig. 6), but in this case, $OH^-$ is produced, which amounts to the same thing.) Thus, although the extrusion of $H^+$:$e^-$ in 1:1 stoichiometry is formally pH neutral, as has been pointed out [23], the subsequent activity of MPO consumes protons and will tend to increase the phagosomal pH. HOCl is highly reactive (which is the reason it kills bacteria), and it rapidly reacts with proteins from both bacteria and the neutrophil itself [99]. Extensive evidence indicates chlorination of a variety of molecules of the ingested organism [18]. Nevertheless, in at least some cases, HOCl reactions may regenerate protons and $Cl^-$ as in the following examples [Winterbourn, C., and Kettle, A.J., personal communication]:

$$HOCl + 2RSH \rightarrow RSSR + H_2O + H^+ + Cl^-$$

$$HOCl + Met \rightarrow Met-sulfoxide + H^+ + Cl^-.$$

Chloramine formation [106] can regenerate protons, but consumes $Cl^-$, at least temporarily:

$$HOCl + RNH_3^+ \rightarrow RNHCl + H_2O + H^+.$$

Thus, the extent to which MPO reactions consume protons may be limited and will depend on reaction kinetics. In addition, the fusion of granules that release their acidic contents into the phagosome would constitute a strong acidifying force. A conservative estimate that 50% of the $H_2O_2$ is converted into HOCl implies that one additional proton is consumed in this reaction per 4 electrons moved by NADPH oxidase. This is precisely the rate of proton generation by $CO_2$ from the hexose monophosphate shunt (HMS, Fig. 1), although the $CO_2$ might simply diffuse out of the cell. It appears that the proton and electron fluxes are almost perfectly matched, and that the NADPH oxidase, in combination with the proton husbandry of various reactions in the cytoplasm and in the phagosome, can operate in a pH neutral manner. This capability would permit the extremely high level of activity that occurs during the respiratory burst, without the risk of severe perturbation of pH. Because the voltage-gated proton channel is regulated by voltage and the pH gradient, without obligatory coupling to NADPH oxidase, it extrudes protons at whatever rate is required.

### 5.5. Potassium flux may facilitate release of proteolytic enzymes

Potassium flux from the cytoplasm into the phagosome has been estimated to account for 5–6% of total charge compensation [23]. PMA stimulates $K^+$ efflux across the plasma membrane in neutrophils [54]. Because PMA induces a substantial fraction of the NADPH oxidase complexes to assemble on the plasma membrane [107], it is assumed that this $K^+$ efflux to the extracellular space is a model for $K^+$ flux into the phagosome. The phagosomal $K^+$ concentration increases during the respiratory burst to 200–300 mM [23]. Two teleological arguments have been put forward in support of a fraction of charge compensation by ions other than $H^+$. First, 1:1 $H^+$:$e^-$ extrusion is approximately pH neutral [23], whereas $pH_{phagosome}$ by some early accounts does change, with a small alkalinization during the first few minutes, followed by substantial acidification [85,86]. Secondly, the increased phagosomal $K^+$ content and ionic strength during the respiratory burst are hypothesized to facilitate release of proteolytic enzymes [23].

Several aspects of the $K^+$ hypothesis are problematic. The lack of change in $pH_{phagosome}$ during the respiratory burst

[42,43] seems to compromise the first argument, in which biphasic $pH_{phagosome}$ changes are assigned teleological roles. Nevertheless, it remains true that any charge that is compensated with $K^+$ rather than $H^+$ would tend to alkalinize the phagosome, and might help maintain $pH_{phagosome}$ as acidic granule contents are released (Section 4.3). If 0.5 g/ml acidic granule proteins are released in to the phagosome, and these proteins require 400 μmol KOH per gram of protein to neutralize their pH from pH 5 to 8 [23], then entry of 200 mM $K^+$ into the phagosome would completely restore $pH_{phagosome}$. Because the required charge compensation is 4 M, the reported $K^+$ flux compensates 5% of the total charge. The remaining 95% of charge compensation must be via $H^+$ flux in order to keep $pH_{phagosome}$ near neutral. The release and solubilization of granule contents by hypertonic solution requires the additional proposal that phagosome swelling is prevented by encasing the phagosome in a mesh of cytoskeletal fibers [7,23]. The extent to which the phagosome becomes hypertonic and the plausibility of the ad hoc mechanism by which cytoskeleton prevents swelling have both been questioned [108]. The pathway that mediates putative $K^+$ fluxes has not been identified. Although small outward $K^+$ currents were reported in human neutrophils [62], no other study has detected outward $K^+$ current of any type in human eosinophils [56,63,81,109]. In human eosinophils, the membrane potential approaches the Nernst potential for protons when NADPH oxidase is active, and replacing intracellular $K^+$ with $Cs^+$ has no effect [51], demonstrating that the proton conductance is much greater than the $K^+$ conductance in the relevant voltage range. The large maxi-$K^+$ (BK) currents reported recently in both cell types [24] have no precedent in the literature, and their amplitude is quantitatively incompatible [110] with the profound depolarization of the plasma membrane potential that occurs during the respiratory burst in neutrophils [54,57,59] and eosinophils [50], as we confirm here by modeling (Fig. 4). Finally, a recent study demonstrated that BK channels are not expressed in human neutrophils or eosinophils [61]. Thus, it is not surprising that the respiratory burst is not affected by inhibitors of BK channels [24] nor of inward rectifier channels [63] that close during depolarization. Nevertheless, it cannot be excluded that $K^+$ flux mediated by an as-yet-unidentified transporter might compensate up to 5% of the charge translocated during the respiratory burst, at least in neutrophils.

### 5.6. Could chloride compensate charge?

Based on inhibition of the respiratory burst by anion transport inhibitors, it has been proposed that $Cl^-$ flux might compensate charge [22,111]. In addition, reducing the cytosolic $Cl^-$ concentration appears to favor NADPH oxidase activity [112–114]. Two factors argue strongly against charge compensation by $Cl^-$ flux across the plasma membrane. First, activation of the respiratory burst is associated with large $Cl^-$ efflux across the plasma membrane [60,112,115]. $Cl^-$ efflux is in the wrong direction to compensate charge, and therefore would comprise additional charge that must be compensated, if its transport were electrogenic. Second, it is clear from the calculations for $K^+$ (Section 3.6) that introducing even a small conductance with a Nernst potential negative to 0 mV ($E_{Cl}$ is −16 mV [69] and would become more negative if cytosolic $Cl^-$ were reduced) is not compatible with the observed membrane depolarization beyond 0 mV. Any such conductance must be exceedingly small.

$Cl^-$ efflux from the phagosome into the cytoplasm via glycine-activated, strychnine-sensitive $Cl^-$ channels was recently proposed to account for 90% of charge compensation [22]. The source of the required $Cl^-$ is said to be from granules. This scenario puts the neutrophil in an untenable position. Because a large fraction of the $O_2^{\bullet -}$ is converted via $H_2O_2$ into HOCl [15,18,105,106], $Cl^-$ is needed as a substrate for this MPO-catalyzed reaction (Figs. 1 and 6). Any $Cl^-$ that is used for charge compensation must be extruded from the phagosome into the cytoplasm, diminishing the $Cl^-$ available for production of HOCl. Because the MPO reaction continues throughout the respiratory burst (up to 2 h) [36,98], this reaction evidently is not limited by depletion of $Cl^-$ from the phagosome, as would rapidly occur if $Cl^-$ compensated charge. As discussed above, the best estimate for $Cl^-$ consumed by MPO in the phagosome is 1 $Cl^-$ per 4 electrons moved by NADPH oxidase (assuming that half the $H_2O_2$ is converted into HOCl; see Fig. 6). Given the estimates of 4 M electrons introduced into the phagosome during the respiratory burst, up to 1 M phagosomal $Cl^-$ might be consumed cumulatively by MPO. Weiss et al. [36] detected 50 fmol HOCl per neutrophil over a 2-h period, corresponding with a cumulative total requirement for 5 M $Cl^-$ as substrate for MPO, assuming a 10 fl total volume of all phagosomes [28]. However, these estimates may be decreased by recycling of $Cl^-$ after HOCl reacts with proteins, as mentioned above (Section 5.4). On the other hand, a significant fraction of the proteins chlorinated by HOCl are neutrophil, not bacterial proteins (94% for *S. aureus* [99], ~15% for *E. coli* [98]), presumably because HOCl is membrane permeable [100] and diffuses freely and extensively out of the phagosome. HOCl diffusion out of the phagosome will obligatorily remove protons as well as $Cl^-$, leading to alkalinization of $pH_{phagosome}$ as well as $Cl^-$ depletion, without contributing to charge compensation. The $Cl^-$ consumed by MPO seems to exceed the $Cl^-$ concentration initially present in the phagosome, roughly 0.1–0.15 M [29,104], which is derived from endocytosed extracellular solution, and then supplemented with granule contents. How much $Cl^-$ is released from granules that fuse with the phagosome is not known.

If $Cl^-$ efflux from the phagosome compensated 90% of the charge during the respiratory burst [22], this would amount to extrusion of 3.6 M $Cl^-$. It is difficult to imagine that sufficient $Cl^-$ exists in phagosomes, even with the addition of granule contents, to compensate this charge as well as act as a substrate for MPO. According to the calculations for $K^+$ (Section 5.5), no more than 5% of charge compensation can be ascribed to any ion besides protons, given that $pH_{phagosome}$ remains near neutral. Up to 10% of charge could be

compensated by a $H^+/Cl^-$ antiporter with 1:1 stoichiometry [116], because half of its contribution to charge compensation would reflect proton flux. If the $H^+/Cl^-$ ratio is 1/2 [117], <8% of the charge can be compensated. The constraint of essentially constant $pH_{phagosome}$ (Section 4.2) means that the sum of $K^+$ and $Cl^-$ contributions to charge compensation cannot exceed 200 mM (5% of the total).

Indirect support for the $Cl^-$ hypothesis are studies that show the existence of $Cl^-$ currents in the plasma membrane of neutrophils [62,118] and eosinophils [111], that some inhibitors of anion transport partially inhibit $O_2^{\bullet -}$ production [111,112,119,120], that intracellular $Cl^-$ depletion activates the respiratory burst [112–114], and that the burst is reduced in ClC-3 knockout mice [120]. However, inhibition is often incomplete, and the anion transport inhibitors SITS or DIDS either have no effect on $O_2^{\bullet -}$ production [121], or stimulate the respiratory burst [112,122]. An additional complication is that the inhibition of $Cl^-$ flux that impaired $O_2^{\bullet -}$ production was $Cl^-$ efflux across the plasma membrane [112], not across the phagosome membrane. As discussed above, $Cl^-$ efflux is in the wrong direction to compensate charge. Finally, the pharmacology of $Cl^-$ transport is messy. Few inhibitors are selective, and some have toxic effects at the same concentrations that block $Cl^-$ flux [123]. In any case, a requirement for $Cl^-$ channels would not establish that their function is charge compensation; preventing $Cl^-$ flux also impairs degranulation [121], adherence [112,114], volume regulation [124], chemotaxis, phagocytosis, and killing [125].

## 6. Conclusions

The preponderance of evidence indicates that voltage-gated proton channels compensate most of the charge translocated by NAPDH oxidase in human neutrophils and eosinophils. About 5% of the charge may be compensated by $K^+$ or $Cl^-$ flux, which might prevent acidification of the phagosome by granule contents and thereby facilitate release of proteolytic enzymes. The massive proton flux into the phagosome (a) compensates charge and thus sustains NADPH oxidase activity by preventing extreme membrane depolarization, (b) helps prevent large pH excursions both in the cytoplasm and in the phagosome, keeping $pH_i$ near optimal for NADPH oxidase activity, (c) minimizes osmotic disturbances, and (d) provides essential substrate protons for the conversion of $O_2^{\bullet -}$ to $H_2O_2$ and then to HOCl.

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