The pH dependence of NADPH oxidase in human eosinophils

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NADPH oxidase generates reactive oxygen species that are essential to innate immunity against microbes. Like most enzymes, it is sensitive to pH, although the relative importance of pHₐ and pHᵢ has not been clearly distinguished. We have taken advantage of the electrogenic nature of NADPH oxidase to determine its pH dependence in patch-clamped individual human eosinophils using the electron current to indicate enzyme activity. Electron current stimulated by PMA (phorbol myristate acetate) was recorded in both perforated-patch configuration, using an NH₄⁺ gradient to control pHᵢ, and in excised, inside-out patches of membrane. No electron current was detected in cells or excised patches from eosinophils from a patient with chronic granulomatous disease. When the pH was varied symmetrically (pHₐ = pHᵢ) in cells in perforated-patch configuration, NADPH oxidase-generated electron current was maximal at pH 7.5, decreasing drastically at higher or lower values. Varying pHₐ and pHᵢ independently revealed that this pH dependence was entirely due to effects of pHᵢ and that the oxidase is insensitive to pHₐ. Surprisingly, the electron current in inside-out patches of membrane was only weakly sensitive to pHᵢ, indicating that the enzyme turnover rate per se is not strongly pH dependent. The most likely interpretation is that assembly or deactivation of the NADPH oxidase complex has one or more pH-sensitive steps, and that pH-dependent changes in electron current in intact cells mainly reflect different numbers of active complexes at different pH.

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NADPH oxidase is a multi-component enzyme complex in phagocytes that plays a crucial role in innate immunity. Physically dissociated in resting cells, upon activation the complex assembles and produces superoxide anion, O₂⁻, which is a precursor to a variety of reactive oxygen species thought to be toxic to microbes (Klebanoff, 2005). Hereditary dysfunction of NADPH oxidase results in chronic granulomatous disease, a susceptibility to recurrent infections that is generally lethal if not treated (Dinauer et al. 2001). NADPH oxidase is electrogenic (Henderson et al. 1987), because it translocates electrons from intracellular NADPH across the plasma membrane, which then reduce extracellular (or intraphagosomal) O₂ to O₂⁻. The activity of NADPH oxidase can be measured directly as an inward electron current, Iₑ (that reflects outward movement of electrons) in eosinophils (Schrenzel et al. 1998; Báafi et al. 1999; DeCoursey et al. 2001a; Cherny et al. 2001), neutrophils (DeCoursey et al. 2000), PLB-985 cells (DeCoursey et al. 2001b), and osteoclasts (Mori et al. 2003). Eosinophils are favoured cells for studying Iₑ because most of the NADPH oxidase complexes assemble in the surface membrane rather than in phagosome membranes (Lacy et al. 2003), and because the respiratory burst in eosinophils is more vigorous than in other cells (Giembycz & Lindsay, 1999). Electron currents measured at room temperature are −6 to −15 pA in human eosinophils (Schrenzel et al. 1998; DeCoursey et al. 2001a; Cherny et al. 2001; Morgan et al. 2003), −2.3 pA in human neutrophils (DeCoursey et al. 2000), −2.4 pA in differentiated PLB-985 cells (DeCoursey et al. 2001b), and −8.4 pA in large, multinucleated osteoclasts (Mori et al. 2003).

The pH sensitivity of NADPH oxidase is a crucial issue. Not only is this enzyme sensitive to pH, but also its activity has profound consequences for local pH. Superoxide anion, O₂⁻, is produced by the translocation of electrons across the plasma membrane. These electrons tend to alkalinize the external (or intraphagosomal) solution, and the protons left behind simultaneously acidify the cytoplasm. In a human eosinophil at body temperature, the NADPH oxidase extrudes the equivalent of ∼30–40 pA of pure electrons (Morgan et al. 2003). This enormous
electron efflux is possible only because ~94% is paralleled by proton efflux which compensates both charge and pH (Reeves et al. 2002). The phagosomal pH strongly influences both the release of antimicrobial enzymes into the phagosome as well as the activity of these enzymes (Odeberg & Olsson, 1975; Elsbach et al. 1979; Segal et al. 1981).

A number of studies have reported various effects of pH on NADPH oxidase activity. Many of these results are difficult to interpret and several are qualitatively inconsistent. These complexities result mainly from the techniques used in previous studies. When NADPH oxidase activity is measured at different pH values, most, but not all, studies report profound inhibition at low pH, (Gabig et al. 1979; Suzuki & Lehrer, 1980; Simchowitz, 1985; Leblebicioglu et al. 1996; Bankers-Fulbright et al. 2001). Two studies reported no effect of lowering pH to 6.0 (Reeves et al. 2002) or 5.5 (Rotstein et al. 1987). Trevani et al. (1999) observed enhanced H2O2 production by several agonists at low pH, and ascribed the contradictory results of previous studies to more rapid spontaneous dismutation of O2− at low pH. It is clear that lowering pH also lowers pH in intact phagocytes (Molski et al. 1980; Simchowitz, 1985; Rotstein et al. 1987), and it is difficult to separate what part of the effect is due to pH, and what to pHi. Increasing pHi above 7.4 has been reported to increase (Simchowitz, 1985; Gyllenhammar, 1989; Bidani et al. 2000), decrease (Suzuki & Lehrer, 1980; Light et al. 1981; Leblebicioglu et al. 1996), or not change NADPH oxidase activity (Reeves et al. 2002). Reports that pH effects differ with the nature of the agonist (Gyllenhammar, 1989; Leblebicioglu et al. 1996; Trevani et al. 1999) suggest pH sensitivity of signalling pathways rather than of NADPH oxidase activity per se. For example, fMetLeuPhe-stimulated O2− production increases monotonically with pH up to 8.0 (Simchowitz, 1985) or even 8.6 (Gyllenhammar, 1989). Attempts to dissociate effects of pHo and pHi include changing pHi by exposing the cells to weak acids (Rotstein et al. 1987; Liberek et al. 1993) or varied Na+ concentration (Simchowitz, 1985) and studies in cell-free or broken-cell assays. Most of these studies report inhibition of NADPH oxidase activity by high or low pH and a pH optimum at 6.5–7.5 (Babior et al. 1976; Suzuki & Lehrer, 1980; Light et al. 1981; McPhail et al. 1985; Clark et al. 1987; Cox et al. 1987). However, pHi independence between pH 6.0 and 7.5 was reported in fractionated neutrophils (Gabig et al. 1979) and Simchowitz (1985) reported maximal activity at pHo = pHi = 8.0. Another approach is to stimulate cells at various pH values and subsequently assay NADPH oxidase activity under identical conditions. This approach is susceptible to detecting pH effects on receptor binding or signalling pathways.

Here we report studies of pH effects on NADPH oxidase using two approaches – perforated-patch and excised-patch techniques – that differ profoundly from previous studies. Although these methods have certain complexities of their own, they provide information that was not obtainable in previous studies. First, we measure NADPH oxidase activity as electron current, Ic, which is a direct reflection of enzyme function (namely, the movement of electrons across the membrane through the electron transport chain), whereas previous studies mainly used methods that detected the enzyme product, O2−, or the product of its dismutation, H2O2. Some assays for products of NADPH oxidase are themselves pH dependent (Suzuki & Lehrer, 1980; Gyllenhammar, 1989), whereas Ic is measured directly without any intermediary. Perforated-patch measurements are done in single intact cells, rather than in populations of cells, or in biochemical concoctions that are devoid of actual cells. The Ic is a direct, real-time measure of enzyme activity in a single intact cell. Because we measure Ic after activation in a single cell at several different pH values, each cell is its own control, and we avoid complications due to pH-dependent early steps in signalling pathways. However, because the cytoplasm is intact, it is possible that assembly and deactivation of NADPH oxidase may still occur.

The second approach is to record electron current in excised, inside-out patches of membranes from activated eosinophils (Petheo et al. 2003). Here we further validate this approach by showing that patches excised from eosinophils that lack functional NADPH oxidase, obtained from a patient with chronic granulomatous disease, do not respond to NADPH. The excised patch is in a sense a cell-free system, but it differs significantly from the usual ‘cell-free system’ which is a mixture of cytoplasmic components of NADPH oxidase and permeabilized membrane vesicles that contain cytochrome b558 (the gp91phox and p22phox heterodimer). In such a system, assembly and recycling of oxidase components is possible. In contrast, the excised patch is exposed to a saline solution that lacks cytoplasmic oxidase components, and therefore assembly is not possible. Electron current measured when NADPH is supplied to the bath (facing the cytoplasmic side of the membrane patch) therefore reflects only the activity of NADPH oxidase complexes that were assembled before the patch was excised. This approach therefore provides novel information. The results provide a clearer view of the effects of pH on NADPH oxidase function.

**Methods**

**Isolation of human eosinophils**

Venous blood was drawn from healthy adult donors who gave informed written consent in accordance with the procedures outlined by the Rush University Institutional Review Board and Federal regulations, and in conformance to the standards set by the Declaration of Helsinki, the Medical Research Council’s online guidelines, and the
guidelines specified by The Journal of Physiology. Blood from a patient with chronic granulomatous disease was obtained with informed written consent in accordance with the procedures outlined by the Children’s Research Center Institutional Review Board. The diagnosis of this patient was made initially by a flow cytometry test (O’Gorman & Corrochano, 1995). Blood was mixed into a sterile 0.9% sodium chloride solution with 3 mM EDTA and layered onto Lymphocyte Separation Medium (LSM, Cambrex Bioscience, Walkersville, MD, USA). The blood was then separated by centrifugation at room temperature (21–24°C) for 30 min. Mononuclear cells and plasma were aspirated off and the pellet resuspended in ice-cold distilled water for 20–30 s to lyse the red blood cells. The water–cell mixture was diluted with ice-cold 2× PBS and mixed with an equal volume (10 μl) of MACS CD16 MicroBeads (Miltenyi Biotec, Auburn, CA, USA). The resulting mixture was incubated at 4°C for 45 min, mixing by pipette every 10 min. The beads–cells mixture was diluted with 0.5 ml PBS–EDTA–BSA (with EDTA and BSA as described above), then added to a magnetized column. The column was washed twice with PBS–EDTA–BSA. Cells were kept on ice in this solution or resuspended in RPMI media with GM-CSF (at a working concentration of 1 ng ml⁻¹) and stored in an incubator.

**Electrophysiology**

We used freshly isolated eosinophils cells kept on ice overnight in phosphate buffered saline (Gibco, Grand Island, NY, USA) supplemented with 2 mM EDTA (Sigma) and 0.5% bovine serum albumin (BSA, Sigma Chemical Co.) and mixed with an equal volume (10 μl) of MACS CD16 MicroBeads (Miltenyi Biotec, Auburn, CA, USA). The resulting mixture was incubated at 4°C for 45 min, mixing by pipette every 10 min. The beads–cells mixture was diluted with 0.5 ml PBS–EDTA–BSA (with EDTA and BSA as described above), then added to a magnetized column. The column was washed twice with PBS–EDTA–BSA. Cells were kept on ice in this solution or resuspended in RPMI media with GM-CSF (at a working concentration of 1 ng ml⁻¹) and stored in an incubator.

solution (composition (mM): 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 5 Hepes, pH 7.4). The current signal from the patch clamp (EPC-7, List Electronic, Darmstadt, Germany; EPC-9, HEKA Elektronik, Lambrecht/Pfalz, Germany; or Axopatch 200B, Axon Instruments, Foster City, CA, USA) was recorded and analysed using an Indec laboratory data acquisition and display system (Indec Corporation, Sunnyvale, CA, USA), in-house software, PULSE software (HEKA), or pCLAMP software supplemented by Microsoft Excel and SigmaPlot (SPSS Inc., Chicago, IL, USA). Seals were formed with Ringer solution in the bath, and the potential was zeroed after the pipette was in contact with the cell. No liquid junction potential correction was applied to current records. However, values for reversal potential of proton currents, V<sub>rev</sub>, were corrected for liquid junction potentials measured as described elsewhere (Neher, 1992). Compounds such as PMA or DPI (diphenylene iodonium) were introduced by complete bath changes. Experiments were done at room temperature (generally 21–25°C), with the temperature recorded by a resistance temperature detector in the bath.

Perforated-patch recording was done generally as previously described (DeCoursey et al. 2000). Seals were formed on non-adherent eosinophils using the amphotericin-containing pipette solution, with Ringer solution in the bath. After seal formation, the patch was held at −60 mV relative to the resting potential of the cell, and depolarizing test pulses were applied at 30 s intervals, usually in pH 7.0 TMA (tetramethylammonium) solution containing 50 mM NH₄⁺. When the resulting H⁺ currents had stabilized, the cytosol and pipette solution were assumed to have equilibrated. Cells were then stimulated with 60 nM PMA. Pulses to 40 or 60 mV were applied at 30 s intervals to monitor the activation of proton currents. The holding current was also monitored for the appearance of inward electron current that reflects NADPH oxidase activity in perforated-patch configuration (DeCoursey et al. 2000). Families of 8 s pulses from −50 to +20 mV in 10 mV steps were usually applied to evaluate proton currents before and after stimulation by PMA, or any change in bath solution.

The reversal potential of proton currents, V<sub>rev</sub>, was estimated by three methods. (1) In cells in which V<sub>rev</sub> was negative to the threshold for activating proton current, the standard ‘tail current’ technique (Hodgkin & Huxley, 1952) was used routinely. A prepulse to a voltage at which substantial proton current was activated was followed by repolarization to a range of voltages that encompassed V<sub>rev</sub> and the zero-current intercept was calculated from the initial amplitude of the tail current. (2) In some cases, V<sub>rev</sub> was estimated by ‘interpolation’ between the final amplitude of the outward proton current and the initial value of the tail current, assuming a linear instantaneous current–voltage relationship (Humez et al. 1995). (3) In cells in which the threshold for activating proton current
was negative to $V_{rev}$, the latter was estimated by interpolation of the amplitudes of current activated during test pulses immediately above and below $V_{rev}$, after scaling the current according to the subsequent tail current amplitude to correct for the different proton conductance activated at different voltages. The latter technique has the advantage of avoiding large proton efflux during prepulses, which spuriously increases pH by depleting cytoplasmic protons (DeCoursey, 1991; Katus et al. 1993; Gordienko et al. 1996).

For excised-patch studies, eosinophils were activated with 60–200 nM PMA either before or after forming a seal to establish cell-attached patch configuration. The patch was then excised by lifting the pipette briefly out of the bath solution. The patches were generally held at $-60 \text{ mV}$. Voltages are given in the normal sense as the voltage at the intracellular side of the membrane minus the extracellular voltage. Thus, applying $+60 \text{ mV}$ to the pipette produces a membrane potential of $-60 \text{ mV}$, and the latter is what we report. In most experiments, NADPH was added to the bath solution directly (rather than by complete bath change) and then the bath was stirred.

**Results**

**Effect of symmetrical pH changes on electron current generated by NADPH oxidase**

Stimulation of eosinophils with PMA results in activation of the NADPH oxidase complex, which transports electrons across the cell membrane. This outward flow of electrons comprises an inward electron current ($I_e$) that is a direct, real-time indication of NADPH oxidase activity in the plasma membrane. $I_e$ can be measured as an increase in the inward current after stimulation of cells in the perforated-patch configuration (DeCoursey et al. 2000). Figure 1A illustrates that pH had little effect on the leak current in unstimulated eosinophils. The cell in Fig. 1B was voltage clamped at $-60 \text{ mV}$ and stimulated by 60 nM PMA in pH 7.0 TMAMeSO$_3$ solution. After stimulation, the inward current increased over several minutes reaching $-18 \text{ pA}$ after 4 min. The net electron current ($I_e$) was determined by subtracting the current before the addition of PMA, typically $\sim 2 \text{ pA}$ (assumed to be non-specific leak current), from the steady-state current. For this cell, $I_e$ was $-16 \text{ pA}$ at symmetrical pH 7.0. The bath was then changed to a series of solutions of different pH. All solutions contained 50 mM NH$_4^+$, as did the pipette solution (referred to as 'symmetrical' pH), which ideally would set pH$_o$ to pH$_i$ (Grinstein et al. 1994). Changing to pH 8.0 reduced $I_e$ by about one-half to $-8 \text{ pA}$. Restoring the bath to pH 7.0 restored the $I_e$ to its initial value, showing reversibility of the inhibition by high pH. Lowering pH decreased $I_e$ to $-13.8$ at pH 6.5, $-6.0 \text{ pA}$ at pH 6.0, and $-2.5 \text{ pA}$ at pH 5.5. Returning to pH 7.0 again restored $I_e$. The experiment ended when the patch ruptured, resulting in whole-cell configuration, in which $I_e$ turns off rapidly (Morgan et al. 2003). The residual current was similar to that at the start of the experiment before addition of PMA. In other experiments (e.g. Fig. 3B), DPI (diphenylene iodonium) was introduced to verify that the PMA-activated inward current was indeed electron current generated by NADPH oxidase. DPI is a classical NADPH oxidase inhibitor (Cross & Jones, 1986) that also interacts with certain other flavin-containing enzymes (O’Donnell et al. 1994; Chakraborty & Massey, 2002). DPI has no effect on the current at $-60 \text{ mV}$ in unstimulated human eosinophils in the perforated-patch configuration (Cherny et al. 2001).

Data from a number of experiments are summarized in Fig. 1C. The average $I_e$ at pH 7.0 was $-11.8 \pm 0.6 \text{ pA}$ (mean $\pm$ s.e.m., $n = 34$) at an average temperature of 23.5°C, comparable to $-9 \text{ pA}$ measured at similar temperature in a previous study (Morgan et al. 2003). At the optimal pH of 7.5, $I_e$ averaged $-14.5 \pm 1.7 \text{ pA}$ ($n = 13$). In agreement with many previous studies, increasing or decreasing pH reduced NADPH oxidase activity drastically. The reduction of $I_e$ by higher or
lower pH was reversible, except when a cell was exposed to low pH (pH 5.5) for an extended period of time (e.g., > 6 min). This observation is strikingly reminiscent of the irreversible inhibition of NADPH oxidase reported in succinate-treated neutrophils at symmetrical pH 5.5 (Rotstein et al. 1987).

Figure 1D confirms that symmetrical 50 mM NH4+ solutions controlled pHi well. The reversal potential of voltage-gated proton currents, \( V_{\text{rev}} \), was measured (Methods) and used to calculate pHi, assuming that pHo is well controlled. Under these conditions, there appears to be some deviation of pHi from ideal at low pH (6.0 or 5.5), with excellent control over the rest of the range studied.

The average time for \( I_e \) to reach 90% of its peak value after challenge with PMA was 3.9 ± 0.6 min in 18 cells in which \( I_e \) appeared to reach a period of stability. Some cells displayed ‘rundown’ – a gradual decline in \( I_e \) over the course of the experiment. Rarely, rundown of \( I_e \) was reversed by addition of glucose, a precursor to NADPH, but generally, glucose did not prevent or reverse rundown. Thus, we do not know its cause. Cells exhibiting pronounced rundown were excluded from analysis. In most cells, \( I_e \) was relatively stable over 15–30 min.

When pH was changed, \( I_e \) typically did not change immediately, but required up to several minutes to reach a new steady-state level. It takes several seconds to change

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**Figure 1. The effect of symmetrical pH changes on \( I_e \) in PMA-stimulated eosinophils**

*Panel A:* changing pH has little effect on leak currents in unstimulated eosinophils. This cell was studied in perforated-patch configuration in the same NH4+ containing solutions used for stimulated cells. The average deviation of the current recorded at −60 mV from that measured at pH 7.0 was +0.01 ± 0.10 pA (mean ± S.E.M., n = 4 cells) at pH 5.5, −0.22 ± 0.32 pA (n = 3) at pH 6.5, and −0.62 ± 0.15 pA (n = 3) at pH 8.5. *Panel B:* current in an eosinophil voltage clamped at −60 mV in the perforated-patch configuration and stimulated with 60 nM PMA. The pipette solution contained KMMeSO3 and 50 mM NH4+ at pH 7.0. Arrows indicate a change in the bath to a solution at different pH, as indicated, all with 50 mM NH4+. The experiment was terminated when spontaneous patch rupture resulted in whole-cell configuration, which results in rapid loss of oxidase activity (Morgan et al. 2003).

*Panel C:* average net \( I_e \) (mean ± S.E.M.) plotted against pHo in experiments with symmetrical 50 mM NH4+. Numbers of experiments, from low to high pH, respectively, are 6 (at pH 5.5), 10, 12, 34, 13, 13, and 8 (at pH 8.5). \( I_e \) was determined by subtracting the initial leak before the addition of PMA from the total current recorded after solution changes and \( I_e \) had reached steady state. For pH 8.5, an additional −0.62 pA was subtracted (see A). *Panel D:* symbols show pHi (mean ± S.E.M.; numbers of experiments, from low to high pH, respectively, are 6 (at pH 5.5), 10, 10, 32, 11, 10 and 9 (at pH 8.5)) calculated from the measured \( V_{\text{rev}} \) plotted against pHo. The line shows the Nernst prediction if the NH4+ gradient perfectly clamped pHi to pHo.
the bath solution and the applied NH₄⁺ gradient requires a finite time to establish a new pHᵢ. The time course of the change in pH could be determined from the \( V_{\text{rev}} \) of proton currents, estimated roughly by the ‘interpolation’ method (Methods), using test pulses applied every 30 s. In a number of cells, the change in \( V_{\text{rev}} \) was complete or nearly so by the time of the first test pulse after the solution change. Therefore, slower equilibration times reflect a slow adjustment to the pH change, rather than the rate at which the solution was changed or the rate the applied NH₄⁺ gradient established the new pHᵢ.

**NADPH oxidase activity decreases pHᵢ**

In intact phagocytes, the charge translocated by electron efflux is compensated mainly by proton efflux through voltage-gated proton channels (Henderson et al. 1987; DeCoursey, 2004). In voltage-clamped cells, the depolarization that under physiological conditions activates proton current does not occur. Thus, NADPH oxidase activity would be expected to lower pHᵢ if the symmetrical 50 mM NH₄⁺ gradient does not clamp pHᵢ perfectly. To determine whether this occurred, in Fig. 2A the small deviations from the ideal pH that were calculated from \( V_{\text{rev}} \) are replotted from Fig. 1D on an expanded scale. Unexpectedly, the error did not simply increase as pH was driven above or below neutral, but instead the relationship was non-monotonic. The actual pHᵢ was higher than ideal at pH ≤ 6.0 and was lower than ideal in the neutral to alkaline pH range, but the deviation decreased again at high pH. The average \( I_e \) from these experiments is plotted in Fig. 2B. Comparison of these graphs suggests that NADPH oxidase activity, which is directly reflected as \( I_e \), contributes to the pH dependence of the deviation. In the pH range that is optimal for NADPH oxidase activity, the actual pHᵢ is more acidic than ideal.

**Effect of asymmetrical pH on \( I_e \)**

The profound effects of symmetrical pH changes on \( I_e \) could reflect effects of pHᵢ or pHᵢ or both. In order to distinguish the effects of pHᵢ and pHᵢ, we varied the NH₄⁺ gradient. In practice, we kept the pipette [NH₄⁺] constant at 50 mM, and adjusted [NH₄⁺]ᵢ. Three pH ranges were explored, pH 6.0 (or 6.3) to 7.0, pH 7.0–8.0, and pH 7.5–8.5. Ideally, the NH₄⁺ gradient and the H⁺ gradient are identical:

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\frac{[\text{NH}_4^+]_o}{[\text{NH}_4^+]_i} = \frac{[\text{H}^+]_o}{[\text{H}^+]_i}.
\]

We estimated the actual pHᵢ by measuring the reversal potential \( (V_{\text{rev}}) \) of proton currents. By this criterion, the control of pHᵢ was less effective when large NH₄⁺ gradients were applied, as found previously (Grinstein et al. 1994; DeCoursey et al. 2000).

Figure 3 shows two experiments in which we adjusted the NH₄⁺ gradient to vary pHᵢ and pHᵢ independently. The intent was to change pHᵢ while keeping pHᵢ approximately constant, and to change pHᵢ while keeping pHᵢ constant. In Fig. 3A, the cell was activated with PMA at symmetrical pH 7.0. Changing the bath solution to pH 6.3 with 50 mM NH₄⁺ (nominally pHᵢ = pHᵢ = 6.3) decreased \( I_e \) by one-half, as shown previously (Fig. 1C). Based on \( V_{\text{rev}} \) measured during the gap in the record (~29–33 min), pHᵢ was 6.4. Next, the bath was changed to a pH 7.0 solution with 3 mM NH₄⁺, which had little effect on \( I_e \) or pHᵢ, which was measured to be 6.5. Returning to pH 7.0 with 50 mM NH₄⁺ (symmetrical conditions) restored \( I_e \) to the original level. Finally, the patch ruptured, resulting in whole-cell configuration, which abolishes \( I_e \) (Morgan et al. 2003). Similar experiments in seven cells produced similar results; varying pHᵢ between 6.0 and 7.0 at roughly constant pHᵢ had little effect on \( I_e \). Conversely, decreasing pHᵢ at constant pHᵢ, 7.0 inhibited \( I_e \) profoundly. Average values from these experiments are plotted in Fig. 7 (▽) for comparison with data from experiments with

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**Figure 2. Complex pH dependence of the deviation of pHᵢ from ideal parallels the pH dependence of \( I_e \)**

A, the deviation from the ideal of mean ± S.E.M. values of pHᵢ calculated from \( V_{\text{rev}} \) in symmetrical 50 mM NH₄⁺ solutions is plotted against pHᵢ. The numbers of measurements from low to high pH are 8, 10, 4, 8, 69, 13, 15, and 9, respectively. In B, \( I_e \) is replotted from Fig. 1C. Both data sets are fitted arbitrarily with quadratic functions (lines).
symmetrical 50 mM NH$_4^+$ (○). The inhibition when pH$_i$ was changed at constant pH$_o$ is comparable with that seen with symmetrical pH changes, suggesting that the pH sensitivity in the latter experiments is ascribable entirely to the effect of pH$_i$.

Changing pH$_o$ between 7.0 and 8.0 with pH$_i$ held roughly constant had little effect on $I_e$. The average $I_e$ at pH$_o$ 8.0 (3 mM NH$_4^+$) where pH$_i$ was measured to be 7.0 was not significantly different from than at pH$_o$ 7.0 (symmetrical 50 mM NH$_4^+$) where pH$_i$ was 6.9 (12% larger in three cells, $P = 0.45$). In contrast, increasing pH$_i$ from 7.0 to 7.9 at constant pH$_o$ 8.0 (by increasing NH$_4^+$ from 3 to 50 mM) reduced $I_e$ by 71 and 74% in two cells. Thus, increasing pH$_o$ from 7.0 to 8.0 had little effect on $I_e$, but increasing pH$_i$ from 7.0 to 7.9 markedly reduced $I_e$.

Figure 3B illustrates the effect of increasing pH$_o$ above 7.5 at approximately constant pH$_i$. The cell was activated in symmetrical pH 7.5 solution (50 mM NH$_4^+$), then the bath solution was changed to pH 8.5 with 3 mM NH$_4^+$. Calculated from the measured $V_{rev}$, pH$_i$ changed from 7.2 to 7.4. Thus, increasing pH$_o$ from 7.5 to 8.5 at constant pH$_i$ had little effect on $I_e$. Next, at constant pH$_o$, 8.5, NH$_4^+$ was increased from 3 to 50 mM. As pH$_i$ increased to 8.4, $I_e$ was rapidly inhibited. Returning the bath to pH 7.5 with symmetrical 50 mM NH$_4^+$ reversed the inhibition by high pH$_i$ and restored $I_e$ to its previous value at symmetrical pH 7.5. This experiment shows that increasing pH$_o$ from 7.5 to 8.5 has little effect when pH$_i$ is kept constant, but increasing pH$_i$ from 7.2 to 8.4 reduced $I_e$ drastically. Average values from four such experiments plotted in Fig. 7 (△), exhibit similar inhibition by high pH$_i$ as for symmetrical pH changes. Therefore, the strong inhibition of $I_e$ at high pH in symmetrical solutions (Fig. 1D) is due to the inhibition by high pH$_i$.

**Effect of pH$_o$ on $I_e$ in inside-out patches**

To examine direct effects of pH$_i$ on NADPH oxidase, we studied $I_e$ due to NADPH oxidase activity in the inside-out patch configuration (Petheö et al. 2003). It is necessary to activate NADPH oxidase before excising the patch. In cell-attached patch configuration,
unstimulated cells exhibited a range of behaviours. There were proton currents in some patches, others had very little conductance over the voltage range explored (as in Fig. 4A), many had a weakly voltage- and time-dependent conductance that was active at the holding potential. In the latter case, deactivation observed upon large depolarization and reactivation after repolarization were reminiscent of volume-gated anion currents in neutrophils (Stoddard et al. 1993). After stimulation with PMA, two responses observed in many cell-attached patches indicated that NADPH oxidase was activated. First, inward current at the holding potential appeared, presumably reflecting electron current (Fig. 4B versus 4A). Second, the properties of proton currents in the cell-attached patch changed dramatically (Fig. 4B), indicating ‘activation’ of these channels. Proton currents in neutrophils or eosinophils with active NADPH oxidase are larger, activate upon depolarization more rapidly and at 40 mV more negative voltages, and deactivate more slowly (Bánfi et al. 1999; DeCoursey et al. 2000). The similar time course of activation of NADPH oxidase and proton channels means that the latter can be used as an indicator that NADPH oxidase is probably activated as well. After the appearance of clear signs of activation (we typically waited ~5–10 min), the patch was excised into inside-out configuration, so that the bath solution faced the intracellular side of the membrane. Proton currents were observed consistently in inside-out patches (Fig. 4C), and were the main conductance present in these conditions. There is no $I_e$ in Fig. 4C because no NADPH was present.

Figure 5A illustrates $I_e$ in an excised patch. Addition of 2.5 mM NADPH rapidly produced an inward current,
which was abolished immediately upon washout. Re-introduction of the same NADPH concentration restored the inward current to roughly the same level. Finally, DPI was added, and then, \( \sim 50 \) s later, NADPH added and the bath stirred. The inward current initially was restored to its previous level, but immediately began to decay, with a time constant of 30 s.

Seven factors support the identification of the NADPH-dependent inward current in patches as \( I_e \) mediated by NADPH oxidase. First, the PMA-stimulated inward current in cell-attached patches disappeared when the patch was excised into a bath lacking NADPH (e.g. Fig. 4B and C). Second, NADPH-induced inward current was not observed in patches that were excised from unstimulated cells. The mean change in current with 2 mM NADPH present was \(+0.036 \pm 0.085\) pA (mean \pm s.e.m.) in three patches. Third, NADH could also act as a substrate (data not shown) (Babior et al. 1976; Suzuki & Lehrer, 1980; Light et al. 1981; Clark et al. 1987). Fourth, the current was inhibited by DPI. Fifth, inhibition by DPI was not immediate, but progressed slowly, with a time constant in agreement with the previously reported value of 25.5 s in intact eosinophils (Morgan et al. 2003). Sixth, DPI is believed to inhibit NADPH oxidase only under reducing conditions, such as occurs when the oxidase is activated in the presence of NADPH (Doussièrè & Vignais, 1992; O’Donnell et al. 1994; Doussiere et al. 1999). In the experiment in Fig. 5A, the patch was exposed to DPI for 50 s, yet the initial \( I_e \) upon introduction of NADPH to the bath was fully restored to its previous level, indicating that no inhibition occurred until NADPH was added. Seventh, as will be shown in Fig. 6, no inward current appeared when NADPH was added to inside-out patches excised from eosinophils treated identically to control cells, but from a patient with chronic granulomatous disease.

The experiment in Fig. 5B illustrates measurement of the pH\(_i\) dependence of NADPH oxidase activity in an inside-out patch. The cell was activated with PMA prior to excising the patch, and then the excised patch was placed into a pH 7.5 solution without NADPH. Adding NADPH to the bath (with stirring) immediately produced an inward current that we consider to be \( I_e \). Subsequently, the NADPH was washed out of the bath with a solution at different pH, and this process was repeated. Whenever NADPH was washed out of the chamber, the inward current rapidly decreased to near the original value. Addition of the same concentration of NADPH to the pH 8.5 solution, followed immediately by stirring (blanked from the record), restored \( I_e \) to essentially the same value seen at pH 7.5. Returning to pH 7.5 restored \( I_e \) with no indication of rundown. Patches typically survived only 10–15 min, but \( I_e \) appeared stable in most cases. Exposure to pH 6.5 and then 5.5 clearly reduced \( I_e \), but to a much smaller extent than was observed in intact cells when pH\(_i\) was lowered by NH\(_4^+\) gradients.

Figure 6 illustrates the absence of detectable \( I_e \) in an inside-out patch from an eosinophil from a patient with chronic granulomatous disease. The lack of functional NADPH oxidase in these cells was confirmed by measuring H\(_2\)O\(_2\) production (which was absent), using the Amplex red method (data not shown). These cells were treated identically to the control cells in this study, first stimulated with PMA in cell-attached patch configuration, and then inside-out patches were excised and exposed to NADPH. Excision of the patch resulted in a small inward current that decayed over several minutes in this patch as well as all others. Whether this is a genuine conductance or

![Figure 6. Absence of \( I_e \) in an excised, inside-out membrane patch from a CGD patient](image)

Currents recorded at nominally –60 mV in a patch of membrane in an eosinophil from a CGD patient. Solution changes produce large artifacts; the current during families of voltage pulses is blanked, but small spikes remain. At the first arrow PMA was added to the cell-attached patch. Excision of the patch resulted in an inward current that decayed over several minutes. At 'wash' the bath was stirred without any addition, resulting in a slowly decaying outward transient current that probably reflects a capacitance change due to an altered interfacial area between bath solution and the pipette. Addition of 4 mM NADPH to the bath, followed by stirring, produced another transient but no evidence of inward current (i.e. no electron current). Washout of NADPH did not change the patch current. Similar results were obtained in 3 cells.
simply non-specific leak current is not clear; in other patches this current appeared to reverse near 0 mV. Three patches failed to respond to 4 mM NADPH. Three other cells from this patient were studied in perforated-patch configuration. No $I_e$ was elicited by PMA, but the proton current was enhanced, as previously described (DeCoursey et al. 2001b). These results confirm that the $I_e$ measured in both perforated-patch and excised-patch configuration is generated by NADPH oxidase.

In Fig. 7 the average $I_e$ measured in inside-out patches of membrane is plotted (●), normalized to its value at pH$_i$ 7.5. Only the value at pH$_i$ 5.5 is significantly lower than the maximal value at pH$_i$ 7.5. $I_e$ in excised patches was much less sensitive to pH$_i$ than $I_e$ in perforated-patch configuration (○), which is replotted in Fig. 7 to facilitate comparison. Mean normalized data from measurements with asymmetrical NH$_4^+$ gradients like those in Fig. 3 are also plotted (▽ and △). These data, in which pH$_o$ was held constant and pH$_i$ was changed by creating a large NH$_4^+$ gradient, directly reflect the effect of changes in pH$_i$. The excellent concordance of these data with the data at symmetrical pH supports the conclusion that NADPH oxidase is sensitive only to pH$_i$, with no effect of pH$_o$.

Discussion

NADPH oxidase activity in intact eosinophils is sensitive to pH$_i$ but not pH$_o$

We find that NADPH oxidase activity in intact eosinophils is highly sensitive to pH and has an optimum near pH 7.5. This result is in general agreement with most previous studies in neutrophils (mostly in cell-free systems, in which the membranes presumably are permeabilized so that pH$_i$ = pH$_o$) in which high or low pH inhibited NADPH oxidase, although the reported pH optimum ranged from 6.5 to 8.0. Previous results for pH$_o$ were qualitatively inconsistent. In the present study, NADPH oxidase activity was evaluated by varying pH$_i$ and pH$_o$ independently in individual PMA-activated eosinophils. The present data indicate that the pH sensitivity of NADPH oxidase is due entirely to pH$_i$ and that pH$_o$ has no detectable effect, at least over the range pH$_o$ 6.3–8.5. This result suggests that any effect of pH$_o$ on NADPH oxidase activity reported previously was probably due to changes in pH$_i$ or other complicating factors mentioned in the Introduction. That pH$_o$ does not affect NADPH oxidase activity seems reasonable when one considers that most of the events leading to NADPH oxidase activity occur inside the cell. Among the few major events that occur in or near the external solution are O$_2$ binding and O$_2$•− release.

Electron current in excised patches is insensitive to pH$_i$

Despite the strong sensitivity of $I_e$ to pH$_i$ in perforated-patch studies, as well as in previous studies in which pH$_i$ sensitivity can be inferred (Suzuki & Lehrer, 1980; Light et al. 1981; McPhail et al. 1985; Ligeti et al. 1988), direct measurement of $I_e$ in patches of membrane containing active NADPH oxidase complexes revealed surprisingly weak pH$_i$ dependence (●, Fig. 7). We used high NADPH concentrations to saturate the system; the affinity of NADPH for the oxidase is highly pH sensitive (Babior et al. 1976). As a frame of reference, assuming a turnover rate of 294 s$^{-1}$ (Cross et al. 1985), 1 pA of $I_e$ reflects the activity of ∼21 000 NADPH oxidase complexes. Compared with its maximal value at pH 7.5, $I_e$ in patches decreased only by 13% at pH 8.5 and by 32% at pH 5.5 (only the latter difference was significant). In contrast, in perforated-patch configuration at symmetrical pH, $I_e$ was reduced from its value at pH 7.5 by 74% at pH 8.5 and by 85% at pH 5.5.
Studying NADPH oxidase in an excised-patch of plasma membrane approaches the ideal of studying the enzyme itself, in isolation from the assembly process. In contrast, the cell-free system apparently falls between the level of organization found in intact phagocytes and the minimal system present in a patch of membrane. The most significant difference between the excised-patch and intact cell studies is that NADPH oxidase assembly is virtually impossible in excised membrane patches. The solution that the inner side of the NADPH oxidase complex faces lacks the cytosolic oxidase components, ATP, GTP, arachidonic acid, and any other cofactor that may be needed for assembly or function (Lu & Grinstein, 1990; Henderson et al. 1993; Park et al. 1997). Furthermore, regulatory interaction of oxidase components with cytoskeleton (Vignais, 2002; DeCoursey & Ligeti, 2005) may be lost. The interaction of oxidase components with cytoskeleton of this molecule makes this idea unlikely. The entire course of changes in cytoplasmic NADPH concentration (50–100 µM) must turn over 4–8 times per second to sustain a 10 pA Ie; hence, any pH-dependent step in biosynthesis would manifest itself almost immediately. In addition, most studies of NADPH oxidase activity in cell-free systems, under conditions of constant exogenously applied NADPH concentration, report similar pH dependence to that in intact cells (Suzuki & Lehrer, 1980; Light et al. 1981; McPhail et al. 1985; Ligeti et al. 1988). Alternatively, the pH-sensitive process may be a reversible mechanism that activates or deactivates the oxidase complex itself. Because of the similar pH dependence found here and reported in cell-free system studies, the latter evidently provide an environment in which this pH-sensitive step can occur. By activating cells at various pH values and then assaying activity in a cell-free system, Gabig et al. (1979) similarly concluded that assembly (‘activation of the O2−-forming system’) was sensitive to low pH, whereas enzyme turnover was less sensitive. Numerous studies provide evidence that during the respiratory burst, there is continual turnover of NADPH oxidase complexes (Akard et al. 1988; Heyworth & Badwey, 1990; Quinn et al. 1993; van Bruggen et al. 2004; Gillibert et al. 2005), presumably reflecting dynamic balance between assembly or activation and an intrinsic deactivation process (DeCoursey & Ligeti, 2005). Evidently, one or more pH-dependent processes critically affect the balance between assembly and deactivation of the oxidase.

NADPH oxidase activity generates intracellular protons

In intact phagocytes, the charge translocated by electron efflux is compensated mainly by proton efflux through voltage-gated proton channels (Henderson et al. 1987; DeCoursey, 2004). In voltage-clamped cells, the depolarization that under physiological conditions activates proton current does not occur; hence, NADPH oxidase activity (Ie) would be expected to lower pH. In a previous study, activation of Ie shifted Vrev negatively at symmetrical pH 7.0, consistent with the idea that NADPH oxidase generates intracellular protons (DeCoursey et al. 2001a). As shown in Fig. 2, the pH dependence of Ie mirrored that of the deviation of pH from its ideal, suggesting that Ie contributes to the pH dependence of the deviation. One might have expected that the error in pH would be minimal near physiological pH; and increase monotonically at extreme pH, as found in simple whole-cell recording (Byerly & Moody, 1986; Cherny et al. 1995). The observed non-monotonic pH dependence supports the interpretation that the magnitude of Ie directly affects pH. The actual pH was consistently lower than ideal in the neutral to alkaline pH range, but was higher than ideal at pH ≤ 6.0. Thus, the actual pH was more acidic than ideal in the optimal pH range for NADPH oxidase, where Ie was large. Although it is generally accepted that NADPH oxidase activity generates intracellular protons, this result corroborates this viewpoint.

In summary, the NADPH oxidase in intact cells has optimal activity near pH 7.5 and is strongly inhibited at higher or lower pH, emphasizing the importance of pH regulation during the respiratory burst. This pattern is entirely the result of sensitivity to pH; the oxidase is insensitive to pHo. However, the assembled, active NADPH oxidase complex itself is only weakly sensitive to pHi. Thus, the pHi sensitivity in intact cells is most likely attributable to a process in the cytoplasm that plays a crucial role in the balance between activation and deactivation of NADPH oxidase.

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pH dependence of NADPH oxidase


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