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 pH_o and pH_i 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 NH_4^+ gradient to control pH_i, 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 ($pH_o = pH_i$) 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 pH_o and pH_i independently revealed that this pH dependence was entirely due to effects of pH_i and that the oxidase is insensitive to pH_o. Surprisingly, the electron current in inside-out patches of membrane was only weakly sensitive to pH_i, 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, $O_2^{\bullet -}$, 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) O_2 to $O_2^{\bullet -}$. The activity of NADPH oxidase can be measured directly as an inward electron current, I_e (that reflects outward movement of electrons) in eosinophils (Schrenzel *et al.* 1998; Bánfi *et al.* 1999; DeCoursey *et al.* 2001*a*; Cherny *et al.* 2001), neutrophils (DeCoursey *et al.* 2000), PLB-985 cells (DeCoursey *et al.* 2001*b*), and osteoclasts (Mori *et al.* 2003). Eosinophils are favoured cells for studying I_e 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.* 2001*a*; 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.* 2001*b*), 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, $O_2^{\bullet -}$, 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

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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_o (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_o to 6.0 (Reeves *et al.* 2002) or 5.5 (Rotstein *et al.* 1987). Trevani *et al.* (1999) observed enhanced H_2O_2 production by several agonists at low pH_o, and ascribed the contradictory results of previous studies to more rapid spontaneous dismutation of $O_2^{\bullet-}$ at low pH. It is clear that lowering pH_o also lowers pH_i 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_o and what to pH_i. Increasing pH_o 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_o 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 $O_2^{\bullet-}$ production increases monotonically with pH_o up to 8.0 (Simchowitz, 1985) or even 8.6 (Gyllenhammar, 1989). Attempts to dissociate effects of pH_o and pH_i include changing pH_i 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, pH_i independence between pH 6.0 and 7.5 was reported in fractionated neutrophils (Gabig *et al.* 1979) and Simchowitz (1985) reported maximal activity at $pH_o = pH_i = 8.0$. 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, I_e, 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, $O_2^{\bullet-}$, or the product of its dismutation, H_2O_2. Some assays for products of NADPH oxidase are themselves pH dependent (Suzuki & Lehrer, 1980; Gyllenhammar, 1989), whereas I_e 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 I_e is a direct, real-time measure of enzyme activity in a single intact cell. Because we measure I_e 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 (Petheö *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 b_{558} (the $gp91^{phox}$ and $p22^{phox}$ 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 × Hanks' Balanced Salt Solution (HBSS) with 5 mM Hepes (adjusted to pH 7.4) and centrifuged at 800 g for 10 min at 4°C, and then this process was repeated. The pellet was then resuspended in 1 × HBSS with 2.5 mM Hepes. After counting, 1×10^7 cells were seeded in 10 μl PBS with 2 mM EDTA 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.

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 (Sigma), or cells that had been incubated at 37°C for up to 2 days after isolation in RPMI containing 25 mM Hepes and L-glutamine (Gibco) supplemented with 10% fetal bovine serum, 200 U ml^{-1} streptomycin (Sigma) and 1 ng ml^{-1} recombinant human GM-CSF (R & D Systems, Inc., Minneapolis, MN, USA). No difference was observed between cells at different times after isolation.

Micropipettes were pulled using a Flaming Brown automatic pipette puller (Sutter Instruments, San Rafael, CA, USA) from 7052 glass (Garner Glass Co., Claremont, CA, USA) coated with Sylgard 184 (Dow Corning Corp., Midland, MI, USA). Pipettes were polished to a resistance of 2–10 MΩ. Electrical contact with the pipette solution was achieved by a chlorided silver wire. A reference electrode made from a sintered Ag–AgCl pellet (In Vivo Metric Systems, Healdsburg, CA, USA) was connected to the bath solution through an agar bridge made with Ringer solution (composition (mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 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_{rev}, 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 NH_4^+. 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_{rev}, was estimated by three methods. (1) In cells in which V_{rev} 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_{rev}, and the zero-current intercept was calculated from the initial amplitude of the tail current. (2) In some cases, V_{rev} 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_i by depleting cytoplasmic protons (DeCoursey, 1991; Kapus *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 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 mV to the pipette produces a membrane potential of −60 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.

Solutions

For perforated-patch recording, the pipette solutions contained 1 mg ml^{-1} amphotericin, 128 mM $KMeSO_3$, 50 mM NH_4^+ in the form of 25 mM $(NH_4)_2SO_4$, 1 mM EGTA, 5 mM Bes and 1 mM $MgCl_2$. Bath solutions varied with the required pH_o and pH_i. Bath solutions were comprised of 100 mM $TMAMeSO_3$, 1 mM EGTA, 1.5 mM $CaCl_2$, 2 mM $MgCl_2$ and a buffer appropriate for the pH; Mes for pH 5.5–6.0, Bis-Tris (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane) for pH 6.5, Bes for pH 7.0–7.5, and Tricine (*N*-tris[hydroxymethyl]methylglycine) for pH 8.0–8.5. In some experiments, 1–2 mg ml^{-1} glucose was added to all bath solutions. The pH dependence of I_e appeared similar whether glucose was present or not. The pH_i was controlled by the external NH_4^+ concentration and pH_o (Grinstein *et al.* 1994). Ideally, pH_i is given by:

$$pH_i = pH_o - \log\left(\left[NH_4^+\right]_i / \left[NH_4^+\right]_o\right).$$

Actual pH_i was determined by monitoring V_{rev} and calculating pH_i from the Nernst equation.

For inside-out patches, the pipette solution contained 200 mM Hepes, 2 mM EGTA and 2 mM $MgCl_2$, and was titrated to pH 7.5 with TMAOH. Bath solutions were essentially the same but with different buffers for the corresponding pH range. Mes was used for pH 5.5 and 6.5, Hepes was used for pH 7.5, and Tricine was used for pH 8.0 and 8.5.

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 1*A* illustrates that pH had little effect on the leak current in unstimulated eosinophils. The cell in Fig. 1*B* was voltage clamped at −60 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 pA after 4 min. The net electron current (I_e) was determined by subtracting the current before the addition of PMA, typically ∼–2 pA (assumed to be non-specific leak current), from the steady-state current. For this cell, I_e was −16 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_i to pH_o (Grinstein *et al.* 1994). Changing to pH 8.0 reduced I_e by about one-half to −8 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 pA at pH 6.0, and −2.5 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. 3*B*), DPI (diphenylene iodinium) 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 mV in unstimulated human eosinophils in the perforated-patch configuration (Cherny *et al.* 2001).

Data from a number of experiments are summarized in Fig. 1*C*. The average I_e at pH 7.0 was -11.8 ± 0.6 pA (mean ± S.E.M., $n = 34$) at an average temperature of 23.5°C, comparable to −9 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 ± 1.7 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 1*D* confirms that symmetrical 50 mM NH_4^+ solutions controlled pH_i well. The reversal potential of voltage-gated proton currents, V_{rev}, was measured (Methods) and used to calculate pH_i, assuming that pH_o is well controlled. Under these conditions, there appears to be some deviation of pH_i 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

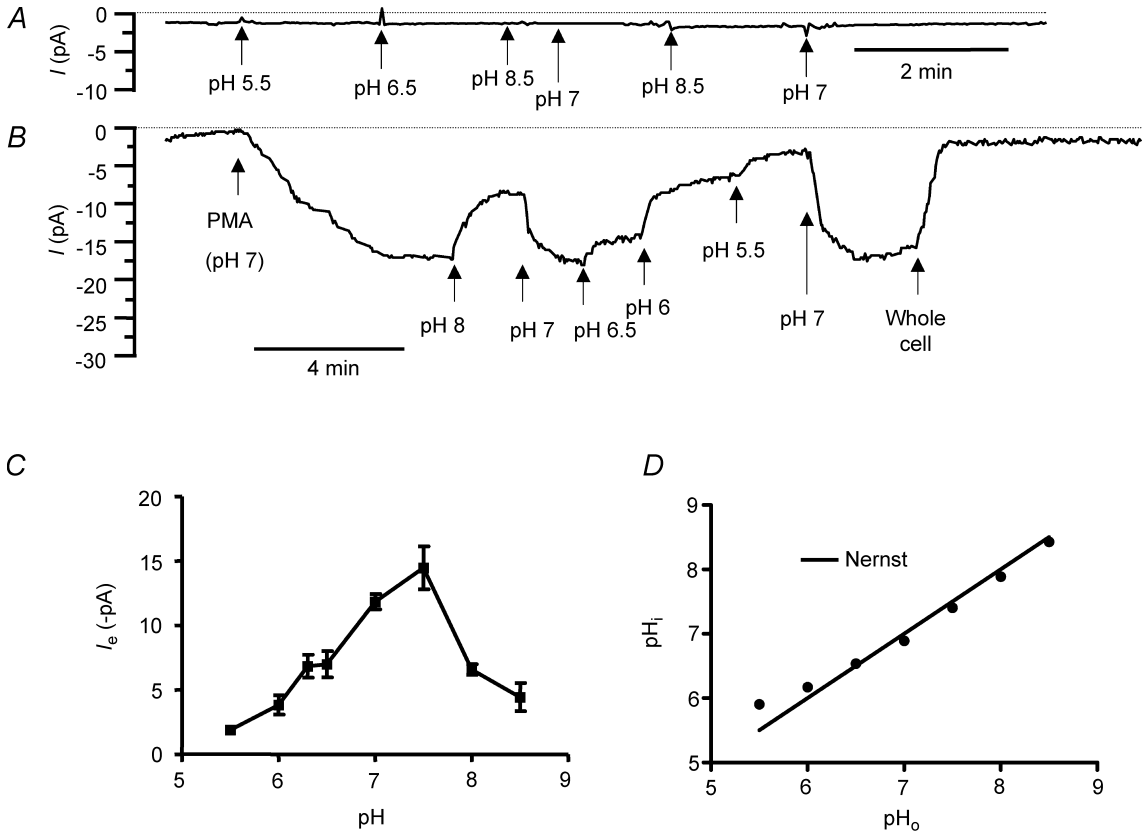

Figure 1. The effect of symmetrical pH changes on I_e in PMA-stimulated eosinophils
A, changing pH has little effect on leak currents in unstimulated eosinophils. This cell was studied in perforated-patch configuration in the same NH_4^+-containing solutions used for stimulated cells. The average deviation 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. *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 $KMeSO_3$ and 50 mM NH_4^+ at pH 7.0. Arrows indicate a change in the bath to a solution at different pH, as indicated, all with 50 mM NH_4^+. 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). *C*, average net I_e (mean ± S.E.M.) plotted against pH_o in experiments with symmetrical 50 mM NH_4^+. Numbers of experiments, from low to high pH, respectively, are 6 (at pH 5.5), 10, 4, 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*). *D*, symbols show pH_i (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_{rev} plotted against pH_o. The line shows the Nernst prediction if the NH_4^+ gradient perfectly clamped pH_i to pH_o.

the bath solution and the applied NH_4^+ gradient requires a finite time to establish a new pH_i. The time course of the change in pH_i could be determined from the V_{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_{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 NH_4^+ gradient established the new pH_i.

NADPH oxidase activity decreases pH_i

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 pH_i if the symmetrical 50 mM NH_4^+ gradient does not clamp pH_i perfectly. To determine whether this occurred, in Fig. 2*A* the small deviations from the ideal pH_i that were calculated from V_{rev} are replotted from Fig. 1*D* 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 pH_i 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. 2*B*. 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 pH_i is more acidic than ideal.

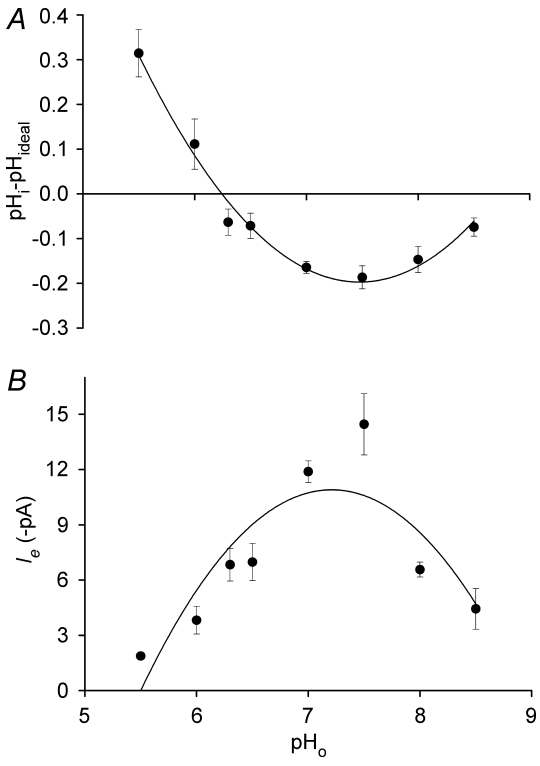

Figure 2. Complex pH dependence of the deviation of pH_i from ideal parallels the pH dependence of I_e
A, the deviation from the ideal of mean ± S.E.M. values of pH_i calculated from V_{rev} in symmetrical 50 mM NH_4^+ solutions is plotted against pH_o. The numbers of measurements from low to high pH are 8, 10, 4, 8, 69, 13, 15, and 9, respectively. In *B*, I_e is replotted from Fig. 1*C*. Both data sets are fitted arbitrarily with quadratic functions (lines).

Effect of asymmetrical pH on I_e

The profound effects of symmetrical pH changes on I_e could reflect effects of pH_o or pH_i or both. In order to distinguish the effects of pH_o and pH_i, we varied the NH_4^+ gradient. In practice, we kept the pipette $[NH_4^+]$ constant at 50 mM, and adjusted $[NH_4^+]_o$. 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 NH_4^+ gradient and the H^+ gradient are identical:

$$\frac{[NH_4^+]_o}{[NH_4^+]_i} = \frac{[H^+]_o}{[H^+]_o}.$$

We estimated the actual pH_i by measuring the reversal potential (V_{rev}) of proton currents. By this criterion, the control of pH_i was less effective when large NH_4^+ gradients were applied, as found previously (Grinstein *et al.* 1994; DeCoursey *et al.* 2000).

Figure 3 shows two experiments in which we adjusted the NH_4^+ gradient to vary pH_o and pH_i independently. The intent was to change pH_o while keeping pH_i approximately constant, and to change pH_i while keeping pH_o constant. In Fig. 3*A*, the cell was activated with PMA at symmetrical pH 7.0. Changing the bath solution to pH 6.3 with 50 mM NH_4^+ (nominally $pH_o = pH_i = 6.3$) decreased I_e by one-half, as shown previously (Fig. 1*C*). Based on V_{rev} measured during the gap in the record (~29–33 min), pH_i was 6.4. Next, the bath was changed to a pH 7.0 solution with 3 mM NH_4^+, which had little effect on I_e or pH_i, which was measured to be 6.5. Returning to pH 7.0 with 50 mM NH_4^+ (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 pH_o between 6.0 and 7.0 at roughly constant pH_i had little effect on I_e. Conversely, decreasing pH_i at constant pH_o 7.0 inhibited I_e profoundly. Average values from these experiments are plotted in Fig. 7 (▽) for comparison with data from experiments with

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 3*B* 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. 1*D*) is due to the inhibition by high pH_i.

Effect of pH_i 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,

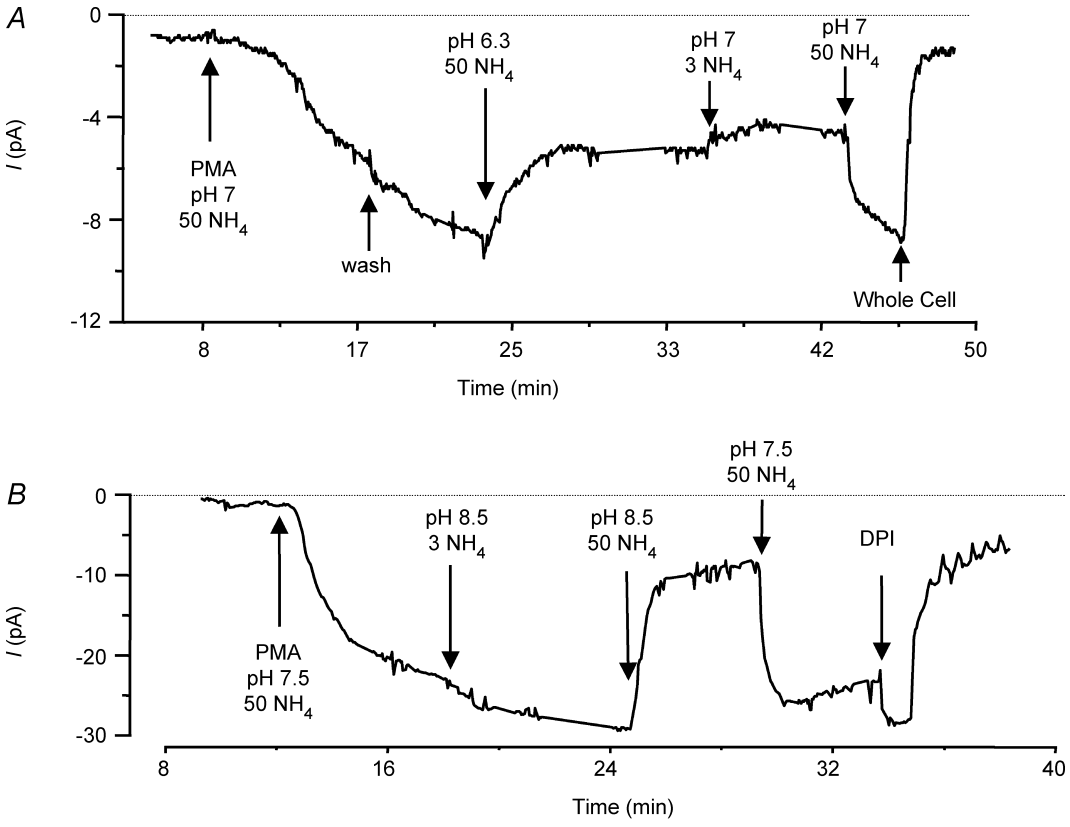

Figure 3. Differential sensitivity of I_e to pH_i and pH_o in asymmetrical NH_4^+ gradients
Eosinophils voltage-clamped at −60 mV in the perforated-patch configuration were stimulated with 60 nM PMA, and then exposed to solutions at the indicated pH and NH_4^+ concentrations (mM). The current at the holding potential of −60 mV is plotted; currents during pulses to other voltages (e.g. to measure V_{rev}) have been removed and replaced by straight lines. The experiment in *A* ended when the patch ruptured into whole-cell configuration, eliminating I_e. In *B*, 12 μM DPI was added at the end of the experiment, transiently increasing I_e (probably by increasing the temperature) and then inhibiting it.

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. 4*A*), 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. 4*B* *versus* 4*A*). Second, the properties of proton currents in the cell-attached patch changed dramatically (Fig. 4*B*), 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 (DeCoursey *et al.* 2000, 2001*a*; Cherny *et al.* 2001) means that the latter can be used as an indictor 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. 4*C*), and were the main conductance present in these conditions. There is no I_e in Fig. 4*C* because no NADPH was present.

Figure 5*A* illustrates I_e in an excised patch. Addition of 2.5 mM NADPH rapidly produced an inward current,

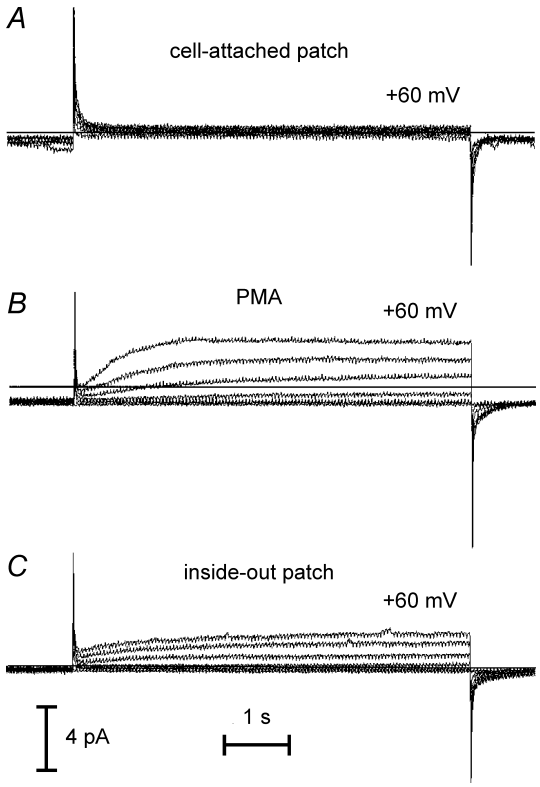

Figure 4. Currents in membrane patches from an eosinophil
Families of currents during pulses from a holding potential 60 mV negative to the resting potential of the cell, in 20 mV increments up to +60 mV. *A* is from a cell-attached patch in an unstimulated cell in Ringer solution. Note that neither pH_i nor the resting potential is known in this configuration. *B* is after stimulation with 80 nM PMA, with pH 7.5 $TMAMeSO_3$ solution in the bath. *C* is the same patch after excision into pH 7.5 $TMAMeSO_3$ solution. The horizontal line shows the nominal zero current.

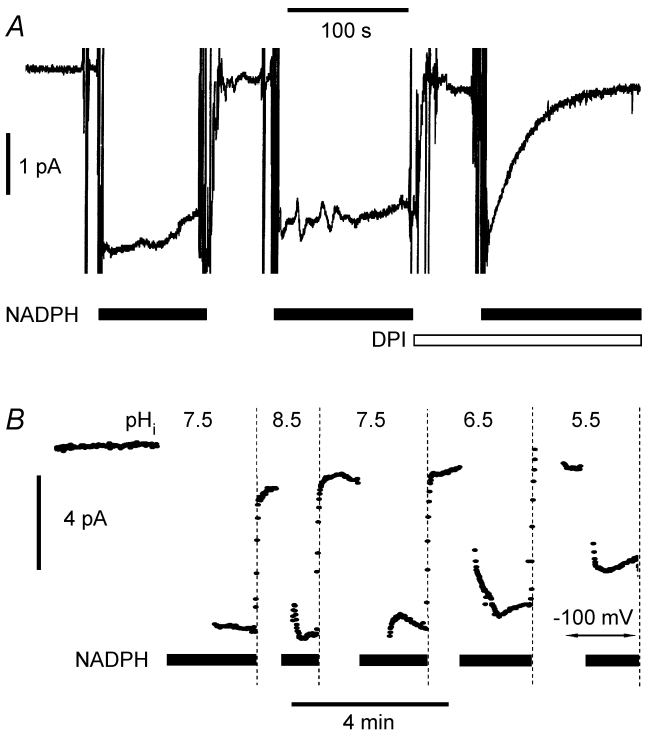

Figure 5. Sensitivity of I_e to pH_i in excised, inside-out membrane patches
A, electron currents in an inside-out patch of membrane excised from an eosinophil stimulated with 60 nM PMA. The filled bars indicate the presence of 2.5 mM NADPH, which was added to the bath followed by stirring (resulting in noisy artifacts). The open bar indicates 12 μM DPI, which was introduced by a complete bath change, simultaneously with the washout of NADPH. *B*, The pH_i dependence of I_e in an inside-out patch excised from an eosinophil stimulated with 80 nM PMA. The bars at the bottom of the trace indicate the presence of 3.2 mM NADPH in the bath solution. Portions of the current with mechanical noise generated by stirring were deleted. At the dashed vertical lines, the bath solution was replaced with an NADPH-free solution at different pH, and then the same amount of NADPH was added to the bath and stirred. The holding potential was −60 mV, except in the pH 5.5 bath solution (double-headed arrow). This low pH_i shifted the threshold for activating proton current negative to −60 mV, therefore the holding potential was reset to −100 mV. After washout of NADPH, residual baseline current of uncertain origin was sometimes observed. The net I_e was determined by subtracting the residual current after washout of NADPH from the current in the presence of NADPH at the same pH.

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, ∼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. 4*B* 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 ± 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 (Doussière & Vignais, 1992; O'Donnell *et al.* 1994; Doussiere *et al.* 1999). In the experiment in Fig. 5*A*, 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. 5*B* 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_2O_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

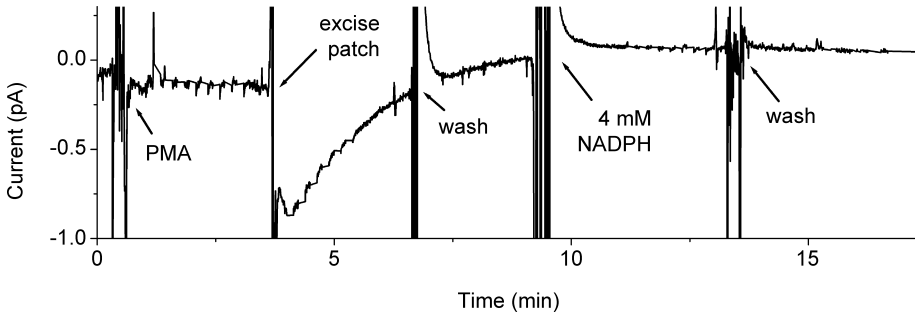

Figure 6. Absence of I_e in an excised, inside-out membrane patch from a CGD patient
Currents recorded at nominally −60 mV in a patch of membrane in an eosinophil from a CGD patient. Solution changes produce large artifacts; the current during families of voltage pulses is blanked, but small spikes remain. 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.* 2001*b*). 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.

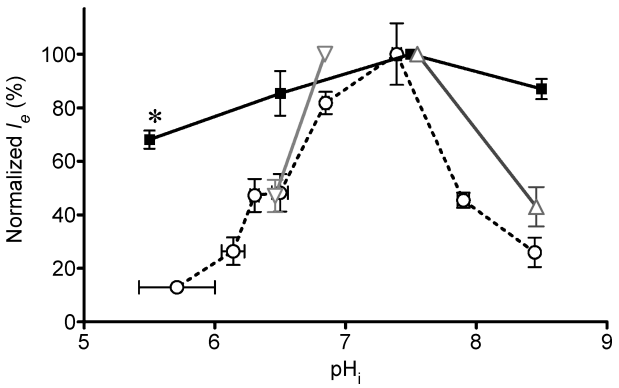

Figure 7. Summary of pH_i dependence of NADPH oxidase activity

Average values of I_e in inside-out patches excised from PMA-stimulated eosinophils (as in Fig. 5), normalized to I_e measured in the same patch at the same NADPH concentration at pH_i 7.5 (■). Data are the mean ± S.E.M. from 5 patches (pH 8.5 and 6.5) and 3 patches (pH 5.5). *Significant difference ($P < 0.05$) from the control value at pH_i 7.5. For comparison, the average I_e measured in cells in perforated-patch configuration at symmetrical pH (derived from the data in Fig. 1*C*) is also plotted (○). Within each experiment, all I_e values were normalized to the value at pH 7.0, the most frequently used initial pH. Then all data points were scaled so that the pH 7.5 value was 100%, to facilitate comparison with the excised-patch data. The data are plotted according to the actual pH_i values calculated from the V_{rev} of proton currents measured directly in each solution in each cell (horizontal bars indicate S.E.M. of the calculated pH_i values). Mean normalized data from measurements like those in Fig. 3 are also plotted (▽: pH_o 7.0 with 50 or 3 mM NH_4^+, $n = 7$; and △: pH_o 8.5 with 50 or 3 mM NH_4^+, $n = 4$) at the mean pH_i calculated from V_{rev} measurements, both normalized to the value at symmetrical 50 mM NH_4^+.

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 $O_2^{\bullet -}$ 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_i 8.5 and by 32% at pH_i 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. Therefore, I_e in patches is a nearly pure reflection of enzyme turnover kinetics. We conclude therefore, that the actual operation of the assembled and activated NADPH oxidase complex is only weakly sensitive to pH.

Nevertheless, in the intact cell, NADPH oxidase activity is strongly dependent on pH_i. The slow time course of changes in I_e often observed when pH_i was changed supports the conclusion that some process other than enzyme function *per se* is sensitive to pH. Conceivably, this could be a step in the synthesis of NADPH, although the high rate of consumption of this molecule makes this idea unlikely. The entire cytoplasmic NADPH concentration (50–100 μM) must turn over 4–8 times per second to sustain a 10 pA I_e; 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 $O_2^{\bullet -}$-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 (I_e) would be expected to lower pH_i. In a previous study, activation of I_e shifted V_{rev} negatively at symmetrical pH 7.0, consistent with the idea that NADPH oxidase generates intracellular protons (DeCoursey *et al.* 2001*a*). As shown in Fig. 2, the pH dependence of I_e mirrored that of the deviation of pH_i from its ideal, suggesting that I_e contributes to the pH dependence of the deviation. One might have expected that the error in pH_i would be minimal near physiological pH_i 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 I_e directly affected pH_i. The actual pH_i was consistently lower than ideal in the neutral to alkaline pH range, but was higher than ideal at $pH \leq 6.0$. Thus, the actual pH_i was more acidic than ideal in the optimal pH range for NADPH oxidase, where I_e 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_i; the oxidase is insensitive to pH_o. However, the assembled, active NADPH oxidase complex itself is only weakly sensitive to pH_i. Thus, the pH_i 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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