Abstract

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an enzyme of phagocytes that produces bactericidal superoxide anion ($O_2^-$) via an electrogenic process. Proton efflux compensates for the charge movement across the cell membrane. The proton channel responsible for the $H^+$ efflux was thought to be contained within the gp91$^{phox}$ subunit of NADPH oxidase, but recent data do not support this idea (DeCoursey, T.E., V.V. Cherny, D. Morgan, B.Z. Katz, and M.C. Dinauer. 2001. J. Biol. Chem. 276:36063–36066). In this study, we investigated electrophysiological properties and superoxide production of COS-7 cells transfected with all NADPH oxidase components required for enzyme function (COS$^{WT}$). The 7D5 antibody, which detects an extracellular epitope of the gp91$^{phox}$ protein, labeled 96–98% of COS$^{WT}$ cells. NADPH oxidase was functional because COS$^{WT}$ (but not COS$^{WT}$) cells stimulated by phorbol myristate acetate (PMA) or arachidonic acid (AA) produced superoxide anion. No proton currents were detected in either wild-type COS-7 cells (COS$^{WT}$) or COS$^{WT}$ cells studied at pH 7.0 and pH 5.5 or 7.0. Anion currents that decayed at voltages positive to 40 mV were the only currents observed. PMA or AA did not elicit detectable $H^+$ current in COS$^{WT}$ or COS$^{WT}$ cells. Therefore, gp91$^{phox}$ does not function as a proton channel in unstimulated cells or in activated cells with a demonstrably functional oxidase.

Key words: phagocytes • gp91$^{phox}$ • $H^+$ channels • superoxide • respiratory burst

Introduction

Nicotinamide adenine dinucleotide phosphate (NADPH)* oxidase is an important antibacterial enzyme of granulocytes that produces superoxide anion (Babior, 1999). This multicompartment complex comprises several cytosolic subunits and the membrane-bound cytochrome $b_558$, which is composed of two subunits (gp91$^{phox}$ and p22$^{phox}$) that coordinate FAD and two heme moieties (Yu et al., 1998). Upon stimulation, the cytosolic components, p67$^{phox}$, p47$^{phox}$, and Rac, a small GTP binding protein, translocate to the membrane and interact with cytochrome $b_558$ (DeLeo and Quinn, 1996). The assembled complex catalyses the oxidation of NADPH and the transfer of electrons across the membrane to extracellular oxygen, which is an electrogenic process (Henderson et al., 1987). The enzyme can be activated by the phorbol ester PMA and by arachidonic acid (Babior, 1999). Voltage-gated proton currents are also greatly enhanced by these stimuli (DeCoursey et al., 2000, 2001a; Cherny et al., 2001). $H^+$ efflux is thought to compensate electrically for electron movement through NADPH oxidase (Henderson et al., 1987, 1988).

It has been proposed that a proton channel exists within the gp91$^{phox}$ subunit of the NADPH oxidase. This conclusion was based originally on studies of CHO cells transfected with gp91$^{phox}$ in which $H^+$ channel function was deduced from pH changes monitored by fluorescent pH dyes (Henderson et al., 1995, 1997; Henderson, 1998). Using only indirect measurements it is difficult to assess the existence and properties of $H^+$ channels and to rule out the involvement of other mechanisms that transport $H^+$ equivalents. Two recent studies provide direct voltage-clamp evidence of $H^+$ currents in CHO, HEK-293, and COS-7 cells heterologously transfected with gp91$^{phox}$ (Henderson and Meech, 1999; Maturana et al., 2001). However, neither study is convincing. Transfection of gp91$^{phox}$ into HEK-293 cells simply increased the endogenous $H^+$ currents fourfold (Maturana et al., 2001). The putative $H^+$ cur-

Address correspondence to Tom DeCoursey, Department of Molecular Biophysics and Physiology, Rush Presbyterian St. Luke’s Medical Center, 1750 W Harrison, Chicago, IL 60612. Fax: (312) 942-8711; E-mail: tdecours@rush.edu

Abbreviations used in this paper: AA, arachidonic acid; CGD, chronic granulomatous disease; DPI, diphenylene iodonium chloride; NADPH, nicotinamide adenine dinucleotide phosphate; pH, intracellular pH; pHi, extracellular pH; PMA, phorbol 12-myristate 13-acetate; SITS, 4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid; SOD, superoxide dismutase; $V_{rev}$, reversal potential; WT, wild-type.

Portions of this work were previously published in abstract form (Morgan, D., V.V. Cherny, M.C. Dinauer, M.O. Price, and T.E. DeCoursey, 2002. Biophys. J. 82:625a).
MATERIALS AND METHODS

Cell Preparation and Maintenance

Green monkey kidney cells (COS\textsubscript{WT}) were obtained from American Type Culture Collection (CRL-1651). COS-7 cells stably transfected with the four main subunits of NADPH oxidase: gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox} (COS\textsubscript{phox} cells) were developed by Price and colleagues (Price et al., 2002). First, COS-7 cells were stably transfected with p22\textsuperscript{phox} and gp91\textsuperscript{phox}. These cells were then further modified by stable transfection with p47\textsuperscript{phox} and p67\textsuperscript{phox} to produce COS\textsubscript{phox} cells. Cells were maintained in Dulbecco’s MEM (Sigma-Aldrich) as described previously (Yu et al., 1997), with the exception that media for COS\textsubscript{phox} cells also contained three antibiotics to ensure selection of transformed cells: 2 μg ml\textsuperscript{-1} puromycin (Sigma-Aldrich), 0.8 mg ml\textsuperscript{-1} G418 (Sigma-Aldrich), and 175 μg ml\textsuperscript{-1} hygromycin (Sigma-Aldrich). COS\textsubscript{phox} cells were split when 70% confluent. COS-7 cells are adherent and were harvested by incubation with 0.25% trypsin in 1 mM EDTA (GIBCO-BRL) for 5 min at 37°C. For electrophysiological recordings, cells were allowed to attach to small glass coverslips. The cells were kept in suspension for the cytochrome c assays.

Electrophysiology

Whole cell and permeabilized patch recordings were performed as described previously (Cherny and DeCoursey, 1999; DeCoursey et al., 2000) using micropipettes pulled from 7052 glass (Garnet Glass). Although COS cells tend to flatten in culture, we selected rounded cells for recording, because voltage-clamp control is better and because the superoxide assays were done with nonadherent (and hence rounded) cells. Some cells were nearly spherical and others were irregular in shape; we did not attempt to record from flat cells. The cell diameter was ~15–25 μm, and the capacity in whole-cell measurements was 14.4 ± 4.5 pF (mean ± SD) in 20 sequentially studied COS\textsubscript{phox} cells. Whole-cell solutions (pipette and bath solutions) contained 100 mM buffer, 1 mM EGTA, 70 mM tetramethylammonium (TMA\textsuperscript{+}) methane sulfonate (MeSO\textsubscripts{4}\textsuperscript{–}), and 2 mM MgCl\textsubscript{2} or 3 mM CaCl\textsubscript{2}, with an osmolality of ~300 mOsm. Buffers were Mes for pH 5.3 and BES (N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) for pH 7.0 solutions. In some experiments the Cl\textsuperscript{–} concentration was increased by replacing TMAeSO\textsubscript{4} with TMACl or with Ringer’s solution (in mM: 160 NaCl, 4.5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 5 HEPES). Permeabilized patch recordings were performed in solutions containing 100 mM TMAMeSO\textsubscript{4}, 25 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 mM MgCl\textsubscript{2}, 5 mM BES, 1 mM EGTA titrated to pH 7.0 with TMAOH. The purpose of the 50 mM NH\textsubscript{4}Cl in the bath and pipette solutions was to clamp pHi near pH\textsubscript{i} (pH 7.0) (Grinstein et al., 1994; DeCoursey et al., 2000). Pipette solutions also contained 0.5–1 mg ml\textsuperscript{-1} amphotericin B (Sigma-Aldrich). Pipettes were filled after dipping the tip in amphotericin free solution. Experiments were done at 21°C or at room temperature (21–24°C). Currents were amplified using either Axopatch 200B (Axon Instruments, Inc.) or List EPC-7 (List Electronic) amplifiers and filtered at 100 or 200 Hz. PClamp8 (Axon Instruments, Inc.) or lab-generated software and an Indec laboratory acquisition and display system (Indec Corporation) were used for data acquisition and manipulation.

Superoxide Anion (O\textsubscript{2}\textsuperscript{–}) Production

Cells were harvested by incubating the adherent cells in trypsin/EGTA at 5°C for 10 min. They were centrifuged for 5 min at 2,000 g and resuspended in Hanks’ Balanced Salt Solution (HBSS) without phenol red at 5 × 10\textsuperscript{5} cells ml\textsuperscript{-1}. The assays were

...in COS-7 cells transiently transfected with gp91\textsuperscript{phox} and studied in whole-cell configuration (Maturana et al., 2001) appear to reverse >100 mV positive to the Nernst potential for H\textsuperscript{+}, and thus were not proton selective. The currents in CHO cells reported by Henderson and Meech (1999) differ profoundly from H\textsuperscript{+} currents in all phagocytes studied to date: they are much less sensitive to inhibition by Zn\textsuperscript{2+}, Zn\textsuperscript{2+} fails to slow \(\tau_{\text{act}}\), no H\textsuperscript{+} current was detectable at pH\textsubscript{i} 7.5 even though the currents at pH 6.9 were larger than native voltage-gated proton currents reported in any cell, and activation was at least an order-of-magnitude more rapid.

Nanda et al. (1994) found normal voltage-gated proton currents in human monocytes from chronic granulomatous disease (CGD) patients who lacked cytochrome b\textsubscript{558}, strong evidence that neither gp91\textsuperscript{phox} nor p22\textsuperscript{phox} is a proton channel in unstimulated phagocytes. We demonstrated recently that gp91\textsuperscript{phox} does not mediate voltage-gated proton currents in the myelocytic PLB-985 cell line (DeCoursey et al., 2001b). Upon induction by dimethylformamide, PLB-985 cells express functional NADPH oxidase and are capable of producing superoxide anions (Zhen et al., 1993). PLB-985 cells in which gp91\textsuperscript{phox} was knocked out by gene targeting had large voltage-gated proton currents that were normal in every respect. Furthermore, PMA stimulation increased the H\textsuperscript{+} conductance to a similar extent in wild-type (WT) and knockout cells. Similar results were obtained in neutrophils from CGD patients lacking gp91\textsuperscript{phox} expression. Therefore, gp91\textsuperscript{phox} does not mediate H\textsuperscript{+} currents in resting or activated cells. However, these results do not completely exclude the possibility that gp91\textsuperscript{phox} might function as a proton channel. Because of the endogenous H\textsuperscript{+} currents in PLB-985 cells and CGD neutrophils, a small contribution by gp91\textsuperscript{phox} might have been missed. Furthermore, one might propose that some other molecule might be up-regulated to compensate for the loss of gp91\textsuperscript{phox} as frequently occurs in genetic knockout studies (Yang et al., 2001). Therefore we performed the converse of the knockout experiment by introducing gp91\textsuperscript{phox} into COS-7 cells, which have no endogenous H\textsuperscript{+} currents (Maturana et al., 2001). Although the goal of this study was to search for proton currents, there were large anion currents in most COS-7 cells and these are described briefly. We report here that neither WT COS-7 cells (COS\textsubscript{WT}) nor COS-7 cells transfected with the four main NADPH oxidase components, including gp91\textsuperscript{phox} (COS\textsubscript{phox}), have detectable voltage-gated proton currents, nor were any induced by PMA stimulation. We conclude that under conditions in which NADPH oxidase activity can be demonstrated, gp91\textsuperscript{phox} does not function as a proton channel.
performed by incubating cells (2.5 × 10^6 cells well^-1) at 37°C in 250 μl HBSS containing 75 μM cytochrome c. Kinetic assays were performed on a Ceres UV9000 plate reader (Biotek Instruments) and superoxide production was quantified using an extinction coefficient of 21.1 mM cm^-1 for cytochrome c. The superoxide assays were performed on the same batches of cells and during the same time period that the electrophysiological studies were done.

7D5 Antibody Staining

Cells were harvested by brief trypsinization, pelleted (1,200 rpm for 6 min at 4°C), resuspended in PBS as a wash, and pelleted. Cells were resuspended in PBS containing 10% normal goat serum and incubated for 30 min on ice to block nonspecific binding sites. Cells were pelleted again then resuspended in PBS at 10^6 cells/ml. One aliquot of cells (100 μl) was incubated with 100 μl 7D5 supernatant for 30 min on ice while a second aliquot was incubated with 2 μl of isotype control CD-117 antibody. PBS containing 0.1% BSA (1 ml) was added as a wash and the cells were pelleted. Cells were resuspended in PBS and incubated for 30 min on ice with FITC conjugated goat-anti-mouse secondary antibody (1:100 dilution). PBS containing 0.1% BSA (1 ml) was added as a wash, and the cells were pelleted and then resuspended in 500 μl PBS for fluorescence-activated cell sorter analysis using a FACS Calibur machine (Becton Dickinson) equipped with CellQuest software. A total of 10,000 cells were counted for each condition. Cells with very low fluorescence (comprising ≤7% of the total, and falling below the lower limit of the graph) were considered dead and were gated out. Staining with 7D5 was compared with staining with isotype control. Also, levels of 7D5 staining were compared in COS_plus cells and COS_WT cells.

RESULTS

COS-7 Cells Have Cl^- Currents

COSWT and COS^plus cells were studied in whole-cell configuration in solutions lacking most permeant ions at pH 5.5 and pH_o 7, conditions favorable for detecting proton currents. Many cells appeared to become “leaky” within a few seconds of breaking the membrane patch. This “leak” reflected the appearance of a large time-independent conductance at the holding potential (–60 mV) and at all potentials negative to 40 mV. The properties of this conductance were similar in COSWT (Fig. 1, A–C) and COS^plus cells (Fig. 1, D–F).

![Figure 1](image_url)

**Figure 1.** Rundown of whole-cell currents in COS WT (A–C) and COS^plus (D–F) cells. Families of currents were recorded at various times after establishing whole cell configuration at pH 5.5 and pH_o 7.0 in TMAMeSO₄ solutions. Insets illustrate the voltage pulses, which were applied at an interval of 15 s. Results are representative of 6 COS WT cells and 22 COS^plus cells. The current at the holding potential decreased progressively during most of the families of pulses illustrated.
During large depolarizing pulses the currents turned off with time. This decay reflected a genuine decrease in conductance because it was reversed upon repolarization; the inward currents were initially small and turned on with time. As evident in Fig. 1, the conductance decreased progressively after establishing whole-cell configuration in both COS<sub>WT</sub> and COS<sub>phox</sub> cells. The rundown produces a progressive decrease in holding current that is evident in some of the families. Eventually, only very small time-independent currents remained (Fig. 1, C and F).

The behavior of the currents in COS<sub>WT</sub> and COS<sub>phox</sub> cells, including the rundown phenomenon, was reminiscent of Cl<sup>−</sup> currents in Chinese hamster ovary cells studied in similar solutions (Cherny et al., 1997). These currents resemble volume-regulated anion currents in several other epithelial cell types (McCann et al., 1989; Solc and Wine, 1991; Arreola et al., 1995). Fig. 2 shows the inhibition of whole-cell currents in a COS<sub>phox</sub> cell by the traditional Cl<sup>−</sup> channel blocker 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). The conductance decreased substantially at all potentials upon addition of SITS (Fig. 2 B), and was at least partially restored after wash out (unpublished data). To confirm the Cl<sup>−</sup> selectivity of the conductance we ramped the voltage from −100 to 80 mV in solutions with different Cl<sup>−</sup> concentrations (Fig. 2 C). Trace 1 shows ramp currents in symmetrical 5 mM Cl<sup>−</sup>/H<sub>11002</sub> where the reversal potential (V<sub>rev</sub>) was near 0 mV. Substituting the bath solution with 166 mM Cl<sup>−</sup>/H<sub>11002</sub> increased the outward current at depolarizing voltages and shifted V<sub>rev</sub> negative by 31 mV. Upon returning to the original solution, V<sub>rev</sub> shifted back and the currents were reduced below the original levels, probably due to rundown (compare Fig. 1). The shift in V<sub>rev</sub> was often less pronounced than in Fig. 2 C, but the shift in V<sub>rev</sub> was larger when the anion conductance was large (i.e., shortly after establishing whole-cell configuration). The predominant conductance in COS-7 cells was anion selective.
COS-7 Cells Lack H⁺ Currents

To identify putative voltage-gated proton currents, we waited for the anion conductance to subside and then looked for currents that increased with time during depolarizing pulses. In some cells time-dependent outward currents were seen, although typically this behavior was observed only after the input resistance had decreased markedly. An example is shown in Fig. 3. Normal behavior was observed early in this experiment on a COS_WT cell. The family of currents in Fig. 3 A was recorded starting 4 min after whole-cell configuration was established. Anion currents that are time independent except at large positive voltages were observed. The current at $V_{\text{hold}} = -60 \text{ mV}$ decreased progressively, in part because of progression of the rundown of anion currents during the 3.5 min required to record this family of long pulses. After the pulses to 60 and 80 mV that elicited decaying outward currents, the inward current upon repolarization was greatly reduced, reflecting the channel closing at positive voltages that is responsible for the current decay. The family of currents in Fig. 3 B was recorded in the same cell immediately after the previous family. By this time, the anion conductance had run down substantially and the currents at all voltages were scaled down, but otherwise exhibited similar time and voltage dependence. Near the end of the pulse to 60 mV (pulse 6), the current suddenly increased. The current at $V_{\text{hold}}$ increased as well, and no recovery was observed before the next pulse, to 80 mV. During this last pulse (pulse 7), the current was much larger and there was a time-dependent increase in outward current during the pulse. Under normal circumstances, we would interpret the sudden increase in conductance to indicate damage to the cell membrane or loss of the seal. Such cells are usually considered to be “dead” and are discarded. However, because the time-dependent increasing outward currents superficially resemble voltage-gated proton currents, we continued recording. In cells with currents like these, changing pH, did not change either $V_{\text{rev}}$ or the threshold voltage for activating time-dependent outward current. These currents thus were not H⁺ selective, nor did they display the pronounced pH sensitivity of gating of all voltage-gated proton channels (Eder and DeCoursey, 2001). Addition of 100 μM ZnCl$_2$ did not inhibit the time-dependent outward currents. Although such currents bear a superficial resemblance to voltage-gated proton currents, they are artifacts.

**Figure 3.** Sudden appearance of time-dependent outward current in a COS_WT cell, studied at pH$_{\text{e}}$ 7.0 and pH$_{\text{i}}$ 5.5. (A) A family of currents recorded starting 4 min after establishing whole-cell configuration displays behavior typical of the anion conductance. The pulses were applied in 20-mV increments up to 80 mV, at 30-s intervals. The entire family required $\sim$5.5 min to complete. The first and last pulses are numbered, showing the progressive decrease in current at $-60 \text{ mV}$. (B) A family recorded in the same cell shortly after the end of the previous family. The currents at all voltages including $V_{\text{hold}}$ are scaled down, reflecting the rundown typically observed in these cells. In this cell during the pulse to 60 mV (pulse 6), there was a sudden catastrophic increase in leak current. The inward current upon repolarization was greatly increased. Interpolation of the current at the end of the pulse to 60 mV and that immediately after repolarization to $-60 \text{ mV}$ gives an estimated $V_{\text{rev}}$ of 4 mV, suggesting a nonselective leak conductance. The subsequent pulse to 80 mV elicited an outward current that increased with time. Subsequent pulse families evoked time-independent leak current at all voltages, with a time-dependent increasing current at large positive voltages. Changing pH$_{\text{e}}$ from 7.0 to 5.5 did not shift $V_{\text{threshold}}$ or elicit changes in gating kinetics normally seen in cells with voltage-gated H⁺ currents. Addition of 100 μM ZnCl$_2$ did not inhibit the time-dependent outward currents. Although such currents bear a superficial resemblance to voltage-gated proton currents, they are artifacts.
gated proton currents in any COS\textsubscript{WT} or COS\textsubscript{phox} cell studied.

To investigate whether stimuli that activate NADPH oxidase might induce or enhance proton currents, as has been observed in human neutrophils and eosinophils (DeCoursey et al., 2000, 2001a; Cherny et al., 2001) we used the permeabilized patch configuration, which preserves NADPH oxidase function (DeCoursey et al., 2000). Many unstimulated COS\textsubscript{phox} cells in permeabilized patch configuration exhibited Cl\textsuperscript{-}/H\textsubscript{11002} currents that disappeared with time (Fig. 4, A–C), similar to the currents in whole-cell studies. Increasing [Cl\textsuperscript{-}]/H\textsubscript{11002} increased the outward current and tended to shift \( V_{\text{rev}} \) to more negative voltages (unpublished data). No time-dependent currents were seen in COS\textsubscript{phox} cells after the Cl\textsuperscript{-}/H\textsubscript{11002} currents ran down. In about one-third of COS\textsubscript{phox} cells the anion conductance was small or absent throughout the experiment, and the currents were similar to those in Fig. 4 C.

Fig. 4, D and E, respectively, show families of currents at high gain before and 10 min after the addition of 60 nM PMA. No time-dependent currents appeared at any voltage after the addition of PMA in this cell. In Fig. 4, D and E, discrete current steps consistent with a single channel conductance of \(~30\text{ pS}\) can be seen at some voltages. These currents are most likely conducted through anion channels similar to volume-sensitive anion channels reported previously (Solc and Wine, 1991). Single voltage-gated proton channel currents are too small to record directly (Byerly and Suen, 1989; DeCoursey and Cherny, 1993). Furthermore, 100 \(\mu\text{M} \text{Zn}^{2+} \) did not inhibit any component of the currents in PMA-treated COS\textsubscript{phox} cells. Thus, both unstimulated (31 cells studied in whole-cell or permeabilized-patch configuration) and PMA- or AA-stimulated COS\textsubscript{phox} cells (\( n = 45 \)) lack detectable proton currents.

**COS\textsubscript{phox} Cells Express gp91\textsubscript{phox} and Produce Superoxide Anion \((O_2^-)\)**

To assess the efficiency of gp91\textsubscript{phox} expression in COS\textsubscript{phox} cells, we used the 7D5 antibody, which is directed
against an extracellular epitope of the gp91<sub>phox</sub> molecule (Nakamura et al., 1988; Yu et al., 1998). Expression was tested in freshly thawed cells and passaged cells, in COS<sub>WT</sub> and COS<sub>phox</sub>, and with the CD-117 antibody as an isotype control. Fig. 5 illustrates histograms of the fluorescence intensity in COS<sub>WT</sub> (open histogram) and COS<sub>phox</sub> cells (solid histogram). 98% of the freshly thawed COS<sub>phox</sub> cells (Fig. 5) and 96% of COS<sub>phox</sub> cells maintained in culture for several weeks (unpublished data), as during the course of these experiments, stained positively for the 7D5 antibody. If cells with very low fluorescence (which presumably are dead cells) are included, then the 7D5-positive fraction is 95% of the freshly thawed COS<sub>phox</sub> cells and 93% of COS<sub>phox</sub> cells maintained in culture. Thus, the vast majority of COS<sub>phox</sub> cells express gp91<sub>phox</sub> at the plasma membrane.

Superoxide anion (O<sub>2</sub><sup>-</sup>) production by COS<sub>phox</sub> cells was assessed by their ability to reduce ferricytochrome c. Fig. 6 A shows the average time courses of cumulative O<sub>2</sub><sup>-</sup> release by four batches of COS<sub>phox</sub> cells challenged with different concentrations of PMA. O<sub>2</sub><sup>-</sup> production above control levels was seen at 6 nM PMA. PMA at 60 and 300 nM, respectively, produced similar levels of O<sub>2</sub><sup>-</sup>. The O<sub>2</sub><sup>-</sup> release time course was characterized by a delay of several minutes after the addition of PMA, followed by a rapid increase between 5 and 20 min, after which the rate of O<sub>2</sub><sup>-</sup> production diminished. Fig. 6 B summarizes the average maximal rate of O<sub>2</sub><sup>-</sup> production in these experiments. The highest maximal rate, observed at 300 nM PMA, was 26 ± 6 nmol min<sup>-1</sup> (10<sup>7</sup> cells)<sup>-1</sup>. The response was almost completely inhibited by 100 µg/ml superoxide dismutase (SOD), showing that ferricytochrome c reduction was due to the release of O<sub>2</sub><sup>-</sup> and not peroxides or other oxidizing agents. Diphenylene iodonium (DPI) at 6 µM also inhibited O<sub>2</sub><sup>-</sup> production, implicating a flavoenzyme (O’Donnell et al., 1994), most likely NADPH oxidase (Cross and Jones, 1986). As reported previously (Price et al., 2002), COS<sub>WT</sub> cells studied under identical conditions did not produce detectable O<sub>2</sub><sup>-</sup> (unpublished data).

Arachidonic acid (AA) can stimulate COS<sub>phox</sub> cells to produce O<sub>2</sub><sup>-</sup> at a higher rate than that stimulated by PMA (Price et al., 2002). We examined the time course of AA-stimulated O<sub>2</sub><sup>-</sup> production in six assays (unpublished data). The onset of O<sub>2</sub><sup>-</sup> production stimulated by 10–100 µM AA was faster than when PMA was used. However, the AA-stimulated respiratory burst was more transient, with little additional O<sub>2</sub><sup>-</sup> released after 10–15 min, whereas PMA-stimulated O<sub>2</sub><sup>-</sup> release continued over 30 min.

Because NADPH oxidase is electrogenic (Henderson et al., 1987), its activity can be detected electrically as a small inward current under favorable conditions.
Discussion

Although the goal of this study was to search for voltage-gated proton currents in cells expressing gp91phox, the only conductance found in COSphox cells was anion selective. Cl−-selective currents similar to volume-sensitive Cl− currents in many other epithelial cells (McCann et al., 1989; Solc and Wine 1991; Arreola et al., 1995; Cherny et al., 1997) were present shortly after establishing whole-cell configuration in most COSWT and COSphox cells. In an earlier study of CHO cells, small voltage-gated proton currents were revealed after the anion conductance ran down (Cherny et al., 1997), but in COSWT and COSphox cells no proton currents were ever observed. These results are consistent with the observation of Maturana et al. (2001) that COSWT cells lack endogenous proton channels.

We saw no H+ currents in COS-7 cells stably transfected with all four major components of the NADPH oxidase, including gp91phox (COSphox cells). In some COSphox and COSWT cells we saw outward currents that increased during large depolarizing pulses. These currents, seen mainly in "leaky" cells, were not inhibited by 100 μM Zn2+, a potent, classical inhibitor of voltage-gated proton channels (Cherny and DeCoursey, 1999), and neither Vrev nor the threshold potential was sensitive to pH, contrary to the properties of voltage-gated proton channels in all cells studied to date (Eder and DeCoursey, 2001). Thus, no credible evidence of voltage-gated proton currents was seen in any COSWT or COSphox cell. The maximum proton current in most phagocytes is ~100 times greater than that required to fully compensate for the electron flux through NADPH oxidase (Eder and DeCoursey, 2001). If a comparable relationship existed in COSphox cells, one would predict ~50 pA of proton current. Even a few picoamperes of H+ current would have been evident in COSphox cells after rundown of the anion conductance.

In previous studies, evidence of proton flux was reported when gp91phox was expressed without any other NADPH oxidase components in CHO, HEK-293, and COS-7 cells (Henderson et al., 1995, 1997; Henderson and Meech, 1999; Maturana et al., 2001). However, the putative proton currents reported in patch-clamp studies of gp91phox expressed in the CHO and COS-7 heterologous expression systems (Henderson and Meech, 1999; Maturana et al., 2001) differ significantly from proton currents endogenous to phagocytes (see Introduction). It is conceivable that gp91phox expressed alone might function as an ion channel, and that this function is absent when it is co-expressed with p22phox. If so, then gp91phox does not function as an ion channel under physiological conditions, because gp91phox is detectable in phagocyte membranes only together with p22phox in the flavocytochrome b558 heterodimer (Babior, 1999). The expression of gp91phox and p22phox are closely linked in neutrophils, where stable expression of either appears to be favored strongly by heterodimer formation, i.e., assembly to form flavocytochrome b558 (Roos et al., 1996; Yu et al., 1997; DeLeo et al., 2000).

The COSphox cells in this study were transfected with all the components necessary for a fully functional oxidase. COSphox cells released significant O2− production at 37°C (26 nmol min−1(107 cells))−1, comparable with the rate we reported previously in these cells (Price et al., 2002). The minimum efficiency of simultaneous expression of all four NADPH oxidase components was 69%, because this fraction was nitroblue tetrazolium (NBT)-positive in COSphax cells (Price et al., 2002). NBT reduction indicates O2− production, and all four NADPH oxidase components are required for this function (Babior, 1999). Expression of gp91phox in COSphox cells in the plasma membrane was confirmed by 7D5 antibody staining. The 7D5 antibody is directed against an extracellular epitope of gp91phox (Nakamura et al., 1988; Yu et al., 1998). That 96–98% of COSphox cells were stained positively by the 7D5 antibody indicates that essentially all cells expressed gp91phox at the surface membrane.

Although COSphox cells express a functional NADPH oxidase complex, attempts to detect electron currents that reflect the function of this enzyme were frustrated...
by the presence of background (leak) currents, and by the tiny amplitude of these currents. The maximum rate of $O_2\,^-$ production in COS$_{phox}$ cells is less than one-fourth that in human neutrophils stimulated with PMA (Price et al., 2002). The electron current averages only $-2.3$ pA in human neutrophils (DeCoursey et al., 2000) and can be detected only because the input resistance in neutrophils is very high (usually >70 GΩ), and the background leak current was small (usually $-0.6$ pA or less) and stable. If electron currents in COS$_{phox}$ were proportionately smaller they would be approximately $-0.5$ pA and consequently very difficult to detect; we did not detect electron currents in PMA-stimulated COS$_{phox}$ cells. AA also stimulates $O_2\,^-$ production in COS$_{phox}$ cells (Price et al., 2002). Although AA induced small inward currents in some COS$_{phox}$ cells, DPI reversed only a fraction of the current and thus the identification of these currents as genuine electron currents remains uncertain.

Bánfi et al. (1999) proposed that there were two types of H$^+$ channels in granulocytes; one in the resting cells and another that is active only when NADPH oxidase is active. Using the permeabilized patch configuration allowed us to investigate whether activating the oxidase might induce the appearance of H$^+$ currents in COS$_{phox}$ cells. We have shown previously (DeCoursey et al., 2000, 2001a,b) that PMA stimulation of human neutrophils or eosinophils or PLB-985 cells in these conditions results in H$^+$ currents that closely resemble the NADPH oxidase–related H$^+$ currents described by Bánfi et al. (1999). AA also greatly enhances voltage-gated proton currents (Cherny et al., 2001). No time-dependent currents appeared after addition of PMA or AA to COS$_{phox}$ cells. Evidently the enhancement of H$^+$ currents during the respiratory burst occurs only in cells that express voltage-gated proton channels in the resting state. This pattern is consistent with the idea that respiratory burst agonists modify the properties of preexisting H$^+$ channels (DeCoursey et al., 2000), rather than inducing the appearance of a new type of channel (Bánfi et al., 1999).

A curious point is that COS$_{phox}$ cells devoid of proton channels are still capable of producing substantial $O_2\,-$. The main function of voltage-gated proton channels in phagocytes is thought to be to provide a mechanism of charge compensation for the electron efflux through NADPH oxidase (Henderson et al., 1987; Eder and DeCoursey, 2001). Inhibiting H$^+$ currents reduces superoxide production in human neutrophils (Henderson et al., 1988) and eosinophils (Bankers-Fulbright et al., 2001) and in PLB cells (Lowenthal and Levy, 1999). Because anion currents were prominent in COS-7 cells, it is tempting to speculate that Cl$^-$ influx may serve this function in lieu of H$^+$ efflux. In preliminary experiments SITS did not inhibit $O_2\,-$ release, but SITS did not completely inhibit Cl$^-$ current (Fig. 2 A). The anion channel inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) partially reduced $O_2\,-$ production in eosinophils (Schwingshackl et al., 2000). Alternatively, COS$_{phox}$ cells may have some other mechanism of charge compensation.

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