Dual role of phagocytic NADPH oxidase in bacterial killing

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The classical model of bacterial killing by phagocytic cells has been recently challenged by questioning the toxic effect of oxygen products and attributing the fundamental role to K⁺ ions in releasing antimicrobial proteins within the phagosome. In the present study we followed O₂⁻ production, changes of membrane potential, K⁺ efflux, and bacterial killing in the presence of increasing concentrations of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor diphenylene iodonium. Efficiency of bacterial killing was assessed on the basis of bacterial survival measured by a new semiautomated method. Very low rates of O₂⁻ production were accompanied by significant membrane depolarization and K⁺ release and parallel improvement of bacterial killing. When O₂⁻ production exceeded 20% of its maximal capacity, no further change was detected in the membrane potential and only minimal further K⁺ efflux occurred, yet bacterial survival decreased parallel to the increase of O₂⁻ production. The presented results indicate that both electrophysiological changes (depolarization and consequent ion movements) and the chemical effect of reactive oxygen species play a significant role in the killing of certain pathogens. The observation that an increase of membrane depolarization can compensate for decreased O₂⁻ production may be important for potential therapeutic applications. (Blood. 2004;104:2947-2953)

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Introduction

Phagocytic cells play a fundamental role in the antimicrobial defense by engulfing and killing various potentially pathogenic microorganisms. Patients with chronic granulomatosus disease (CGD) suffer repeated, often fatal infections due to severe impairment of the antimicrobial defense mechanisms.¹ The discovery that CGD is the consequence of a genetic defect in one of the essential subunits of the O₂⁻-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase provided strong support for the role of O₂⁻ and other reactive oxygen species (ROS) in the mechanism of bacterial killing.²

When activated, NADPH oxidase transfers electrons from intracellular NADPH to extracellular (or intraphagosomal) O₂⁻, forming O₂⁻ anions. The enzyme carries out an electrogenic function (ie, with each turn one negative charge is removed from the cell).³ This charge movement is reflected in depolarization of the plasma membrane that was shown to accompany O₂⁻ production. In fact, intensive O₂⁻ generation is able to reverse the polarity of the plasma membrane: in resting polymorphonuclear neutrophils (PMNs), values around −60 mV have been measured,⁴ whereas upon phorbol myristate acetate (PMA) stimulation the membrane potential approached +60 mV.⁵ The electromotive force of the enzyme is similar to that of mitochondrial cytochromes: electron current via the phagocytic NADPH oxidase ceases only around +200 mV.⁶

Decrease or reversal of the plasma membrane potential alters the driving force for all other charged particles, favoring outward movement of cations and inward movement of anions. Indeed, compensating H⁺ efflux has been demonstrated by many laboratories⁷-¹² although the underlying transport pathway(s) is still the subject of intensive investigations.⁴ More recently K⁺ efflux has also been reported from activated PMNs.⁸,¹³,¹⁴ On the other hand, plasma membrane depolarization impedes Ca²⁺ influx induced either by store depletion or by stimulation of chemotactic receptors.¹⁵,¹⁶

In view of all these ion movements initiated by the charge separation via the NADPH oxidase, we had proposed that alteration of the ionic composition of the intraphagosomal space may contribute to the impairment of bacterial killing in CGD.¹⁷ Recently Reeves and coworkers questioned the toxic effect of various ROS under the conditions of the phagosome.¹⁸ Instead, they suggested a specific role for K⁺ ions enriched in the intraphagosomal space in the release of granule enzymes from their polyanionic support and thereby in their activation. In fact, this hypothesis reduces the role of O₂⁻ generation to providing a driving force for K⁺ movement into the phagosomes.¹³,¹⁴

The aim of the present study was to gain insight into the relative importance in bacterial killing of (1) O₂⁻ as a reactive molecule and (2) membrane potential changes initiated by electron transfer via the O₂⁻-generating NADPH oxidase. Our approach consisted of a stepwise decrease of the rate of O₂⁻ production by applying increasing concentrations of the inhibitor diphenylene iodonium (DPI) and relating O₂⁻ generation to changes of the plasma membrane potential, K⁺ release, and bacterial killing. Our quantitative analysis provides experimental support that both O₂⁻ and ion movements contribute to the killing of some microorganisms (eg, Staphylococcus aureus), whereas in other cases (eg, Escherichia coli) none of them seem to be decisive.

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Materials and methods

Materials

Ficoll was from Pharmacia (Peapack, NJ); 3′-dipentyloxacarbocyanine (di-O-C3) from Molecular Probes (Eugene, OR); saponin from Calbiochem (Darmstadt, Germany); and valinomycin, cytochrome c, lucigenin, cytochalasin B, phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), and DPI, from Sigma (St Louis, MO). 86RbCl was purchased from the Isotop Íntézet (Budapest, Hungary). All other reagents were of research grade. The extracellular medium (called H-medium) contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.8 mM CaCl2, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 5 mM glucose, pH 7.4. The Luria-Bertani (LB) medium contained 10 g/L tryptone, 5 g/L yeast extract, 80 mM NaCl, and 1 mM NaOH.

Preparation of neutrophil granulocytes from human blood

Human neutrophils were obtained from blood of healthy volunteers by dextran sedimentation followed by Ficoll-Paque gradient centrifugation using endotoxin-free solutions and sterile conditions at 4°C to prevent premature activation of the cells. Contaminating red blood cells were removed by hypotonic lysis. Cells were finally resuspended in sterile endotoxin-free H-medium and kept on ice until use. Preparations contained more than 95% neutrophils; their viability, as determined by trythrosin B dye exclusion, exceeded 95%.

Measurement of O2− production

Superoxide production of the cells was tested with the superoxide dismutase–inhibitable cytochrome c reduction or the lucigenin-based chemiluminescence. To measure superoxide production in the extracellular medium with cytochrome c, cells (105/mL) were suspended in H-medium containing 100 μM cytochrome c. Control samples contained 12.5 μg/mL superoxide dismutase. Aliquots (200 μL) of the suspension were added into wells of a 96-well plate and prewarmed at 37°C for 5 minutes in a shaking enzyme-linked immunosorbent assay (ELISA) reader (Labsystems iEMS Reader MF, Helsinki, Finland). The cells were stimulated with the appropriate stimuli by the addition of 5 μL of the stimulus solution. The changes in the absorption were recorded at 550 nm for 10 minutes with 2 measurements per minute at 37°C with gentle shaking. After subtracting the background values, superoxide production was calculated with the use of an absorption coefficient of 21 mM−1 cm−1 for cytochrome c.

To measure reactive oxygen species production with lucigenin-based chemiluminescence, the cells (105/mL) were suspended in H-medium containing 5.1 mg/mL lucigenin (dissolved in dimethyl sulfoxide [DMSO]). Aliquots (200 μL) of the suspension were added into wells of a 96-well plate and prewarmed at 37°C for 5 minutes in a shaking Fluoroskan Ascent FL lumimeter (Labsystems). Cells were stimulated with the appropriate stimuli by the addition of 5 μL of the stimulus solution. Changes in the luminescence were recorded for 20 minutes at 37°C with gentle shaking. In 6 independent experiments linear correlation was obtained between values of cytochrome c reduction and chemiluminescence; thus, relative fluorescence units (RFU) were converted to nanomoles of superoxide on the basis of parallel measurement of PMA-stimulated O2− production by cytochrome c reduction.

Measurement of membrane potential changes

Changes in the membrane potential were followed by the potential-sensitive fluorescent dye 3′-dipentyloxacarbocyanine, di-O-C3, as described by Seligmann and Gallin.19 Cells (105/mL) were suspended in H-medium in a stirred cuvette at 37°C, and the changes in fluorescence were followed in a Deltascan dual-wavelength spectrofluorimeter (Photon Technology International, South Brunswick, NJ) with 484 nm as excitation and 510 nm as emission wavelengths. The cells were preincubated for 5 minutes before 100 nM di-O-C3 was added. Fluorescence reached a constant value in 6 to 7 minutes. Thereafter, the cells were stimulated with different stimuli (fMLP, PMA), and the changes in the fluorescence were monitored. The obtained fluorescence data were calibrated to membrane potential values (millivolts [mV]) using 2 μg/mL valinomycin in a suspension of cells with different external K+ concentrations as described by Geisz et al.15 The values attained at 1 or 2 minutes after stimulation with fMLP or PMA, respectively, were used for further calculations.

Measurement of the 86Rb release from neutrophils

Neutrophils (107/mL) were incubated in H-medium with 86RbCl (0.25 μCi [0.0093 MBq]) for 30 minutes at 37°C, washed twice in cold phosphate-buffered saline (PBS), and resuspended in H-medium at the density of the killing assay (107/mL). After incubation with or without stimuli at 37°C for 5 minutes, the cells were immediately centrifuged (4 minutes, 500g). Radioactivity was determined in an aliquot (100 μL) of the cell-free supernatant using an Automatic Gamma Counter (1470 Wizard; Wallac, Turku, Finland). The 86Rb efflux detected in the unstimulated samples was subtracted from the values of the stimulated ones. The 86Rb release of the PMA-stimulated, DPI-free sample was taken as 100%. DPI by itself did not induce any 86Rb efflux from the cells.

Measurement of bacterial survival

Bacteria (9 × 108 cells in 900 μL H medium) were opsonized with 100 μL mixed human blood serum of at least 5 different donors for 5 minutes at 37°C. PMNs (9 × 106 in 900 μL H-medium) were then incubated with 100 μL (109/mL) opsonized bacteria for 30 minutes at 37°C. Samples (100 μL) were taken at indicated time points and lysed in 900 μL ice-cold H-medium containing 1 mg/mL saponin. In the case of S aureus, additional treatment at −80°C for 20 minutes was applied. Freezing did not impair subsequent growth of S aureus.

Bacterial growth was followed in a plate reader (Labsystems iEMS Reader MF) on the basis of changes in optical density, taking into account that in the exponential phase of bacterial growth the division time is constant. For calculations we applied the principles of real-time polymerase chain reaction (PCR); thus we characterize the samples containing unknown amount of living bacteria with an incubation time (tinc) the length of which is only dependent on the initial bacterial concentration. Specifically, lysed PMN samples were diluted in LB to decrease the initial optical density (OD) (typically a 5-fold dilution to a final volume of 200 μL per well was applied). A parallel series of 1:2 dilutions from the stock bacterial suspension (109/mL) was also prepared in LB. Then bacteria were grown in a shaking plate reader at 37°C for 5 to 8 hours, and the OD was followed continuously at 650 nm. Samples were run in 4 to 8 parallels. The data obtained in a typical experiment are shown in Fig. 1A. The time required for achieving a certain OD value (in most experiments 0.2 was chosen) was determined for each dilution and plotted as initial bacterial count versus incubation time (tinc). A straight line was fitted on the experimental points (Figure 1B), and the number of surviving bacteria was calculated with the following formula: E coli or S aureus = f × e^{−g × tinc}, where tinc indicates the incubation time required to reach the threshold OD value, f is a numerical factor, and g is the slope of the line, indicating the rate of bacterial reproduction. This calculation was then used to determine the number of viable bacteria in the lysed samples taken in the killing assay. Each experiment was calculated on the basis of its own calibration curve. The slope of the curve shown in Figure 1B allows the determination of the average division time of the investigated microorganism. Summarizing all experiments, 17.1 ± 1.91 minutes (SEM, n = 29) and 28.6 ± 1.1 minutes (SEM, n = 63) were obtained as the division times for E coli and S aureus, respectively. Thus, experiments carried out on different days showed high reproducibility.

Killing activity of neutrophils was assessed on the basis of the changes in the number of viable bacteria. Because the 30-minute incubation time with PMNs exceeded the division time of both bacteria under some experimental conditions, the final bacterial count was higher than the initial one. In Figures 1, 5, and 6, “bacterial survival” is represented as the final bacterial count expressed as percentage of the initial count.
Experiments carried out to control the semiautomated bacterial growth assay

Bacterial growth in the central or peripheral wells of the plate differed by less than 2%, and it was not influenced by the presence of nonviable bacteria or saponin-treated PMNs. The utilized concentration of saponin and dilutions of DMSO or the opsonizing serum had no effect on the growth of bacteria or saponin-treated PMNs. The utilized concentration of saponin was less than 2%, and it was not influenced by the presence of nonviable bacteria followed in time. (A) Changes in absorption at 650 nm were followed in time. (B) Initial concentration of bacteria was plotted against the obtained incubation time values (tinc). Error bars represent SD of 4 parallel samples. Fitting a straight line on the experimental data resulted in the following calibration equation:

\[ E_{coli} = 2.2200 \times e^{-0.0365t_{inc}} \times 10^{9} \, \text{cells/mL} \]

The results of 1 representative experiment out of 29 similar ones are shown. (C) Time course of the decrease in surviving bacteria followed by the semiautomated assay. (D) Time course of the decrease in surviving bacteria determined by conventional plating. Where indicated, cytochalasin B (CB) was present at a concentration of 10 μM. The results shown are representative of 4 similar experiments, and error bars represent standard deviation of 4 parallel samples.

Results

Relation of \( \text{O}_2^- \) production and membrane potential change

Neutrophil granulocytes were stimulated either with phorbol myristate acetate (PMA), an activator of protein kinase C, or with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine, fMLP (Figure 2D-F). Changes of membrane potential and \( \text{O}_2^- \) production were measured in parallel samples. \( \text{O}_2^- \) production induced by PMA (Figure 2A) was detectably inhibited by 50 nM DPI, and the inhibition was almost complete in the presence of 5 μM DPI. In contrast to \( \text{O}_2^- \) production, membrane depolarization following PMA stimulation was not influenced by 50 nM DPI, and even in the presence of 5 μM inhibitor there was only a 50% decrease in PMA-induced depolarization (Figure 2B). Data of 11 experiments are summarized in Figure 2C, where both measured parameters are expressed in percentage of the values obtained in the absence of any inhibitor. The discrepancy in the sensitivity of \( \text{O}_2^- \) production and membrane depolarization toward DPI is remarkable. In the case of fMLP stimulation, both the onset, the extent, and duration of \( \text{O}_2^- \) production and also the kinetics of membrane depolarization are different from those evoked by PMA (Figure 2D-E). Nevertheless, fMLP-initiated \( \text{O}_2^- \) generation was also inhibited at a significantly lower DPI concentration than the accompanying membrane potential change (Figure 2F). DPI did not interfere with accumulation of the potential-sensitive fluorescent dye in the cells or alter the linearity of the calibration curve obtained in solutions of different K⁺ concentrations.

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Further analysis of the data summarized in Figure 2 emphasizes the nonlinear relation between the rate of \( \text{O}_2^- \) production and the change in the membrane potential induced thereby. As shown in Figure 3, less than 5% of the maximal \( \text{O}_2^- \)-generating capacity is sufficient to induce 50% of the maximal depolarization. On the other hand, near-maximal depolarization is attained at 20% of the maximal \( \text{O}_2^- \)-producing capacity, and further increase of the rate of \( \text{O}_2^- \) production does not initiate significant changes in the membrane potential. The rather stable membrane potential prevailing under conditions when the rate of \( \text{O}_2^- \) production increases from 20% to 100% maximal capacity indicates that in this phase electron transport via the NADPH oxidase is fully compensated by movement of another ion(s).

Relation of \( \text{O}_2^- \) production and K⁺ release from granulocytes

In previous investigations H⁺ and K⁺ release were suggested as cations potentially compensating for the electron efflux via the oxidase. In the last decade ample information has been gained on the H⁺ release from neutrophils.
conductance of phagocytic cells under resting and activated conditions, whereas the importance of K⁺ movements has been emphasized recently. To gain further insight into the relation of O₂⁻ and K⁺ release, we measured the effect of DPI upon PMA-induced efflux of ⁸⁶Rb, the tracer generally used for assessing K⁺ movements. As shown in Figure 4A, at a concentration of 50 nM, DPI had no measurable effect on ⁸⁶Rb release, and even in the presence of 5 μM DPI it decreased only to 38%. Relating ⁸⁶Rb efflux to O₂⁻ production measured in parallel revealed that less than 5% of O₂⁻ production is accompanied by the release of 38% of the mobilized K⁺ and that only 30% of K⁺ movement takes place in the phase where the rate of O₂⁻ generation increases from 20% to 100% of maximal capacity. The relation is very similar to that obtained between O₂⁻ production and membrane potential changes (compare Figure 4B with Figure 3). In fact, plotting ⁸⁶Rb efflux against membrane potential changes revealed a nearly linear relationship (Figure 4C), confirming the data obtained by the fluorescent technique.

**Relation of O₂⁻ production and bacterial survival**

In the next experiments bacterial survival was measured by a new, highly effective semiautomated method using a microtiter plate reader (see “Materials and methods” and Figure 1). Intracellular ROS production occurring during bacterial phagocytosis and killing was followed in parallel by the lucigenin-based chemiluminescence method in a luminescent plate reader. DPI inhibited the chemiluminescence signal induced by either *E. coli* (Figure 5A) or *S. aureus* (Figure 5B) with similar efficiency as observed in the cases of O₂⁻ production induced by soluble stimulants. Killing of *E. coli* was only weakly dependent on O₂⁻ production. In the absence of DPI, 14.5% of the bacteria survived the 30-minute incubation with PMNs, and this value increased to 26.1% in the presence of 5 μM DPI. In contrast, survival of *S. aureus* depended significantly on the rate of O₂⁻ generation. In the presence of 5 nM DPI, when O₂⁻ production was still at 80% of the maximal capacity, bacterial survival was already increased 3-fold. Most significantly, when 5 μM DPI was present during the 30-minute incubation with PMNs, instead of decreasing, the bacterial count was doubled (Figure 5B). Other studies indicated that 5 μM DPI does not decrease the number of engulfed bacteria (W. Nauseef, personal communication, February 2004). Thus, the increase in survival must be interpreted as impairment of the killing process.

Analyzing the survival of *S. aureus* in the function of the rate of O₂⁻ generation (Figure 6) reveals a multiphase relation. Decreasing the rate of O₂⁻ production to 20% of the maximal capacity results in 90% bacterial survival. (Because bacteria multiply during the
assay, this value still indicates elimination of bacteria by neutrophils, but evidently the killing function is seriously impaired. It should be recalled that between 20% and 100% of maximal O$_2^-$-generating capacity the membrane potential is stable and only minor K$^+$ efflux takes place. Thus, impairment of bacterial killing occurs under conditions where electron flow via the assembled NADPH oxidase is completely compensated (mostly by H$^+$ ions); consequently, the driving force for other ion movements remains unchanged. We interpret the parallel increase in killing efficiency and rate of O$_2^-$ production in the presence of a stable membrane potential as an indication for the chemical effect of O$_2^-$ and the products formed thereof.

Figure 3 revealed a very sharp relation between membrane potential change and O$_2^-$ production when the latter fell below 20% of the maximal capacity. This relation is reflected in the survival of S. aureus also (Figure 6). Decreasing the O$_2^-$ generation from 20% to approximately 5% of the maximal capacity results in an increase of bacterial survival from 90% to 140%. Finally, a decrease of O$_2^-$ production by only 2% (from 5% to 3%) leads to further increase in bacterial survival (from 140% to 200%).

Effect of Zn$^{2+}$ on the membrane potential, O$_2^-$ production, and bacterial survival

To investigate further the importance of depolarization versus O$_2^-$ in the killing of S. aureus by neutrophils, we tried to uncouple O$_2^-$ generation and membrane potential change. Unfortunately, all the agents generally used for depolarizing cells, such as Na$^+$ ionophores or lipophilic cations, are toxic for bacteria. Zn$^{2+}$ ions were shown to inhibit the electrogenic H$^+$ pathway already at low concentration, and in our pilot experiments they did not impede bacterial growth. Similar to previous findings, Zn$^{2+}$ increased the membrane depolarization by 31.7% ± 7.1% and decreased the rate of O$_2^-$ generation detected upon stimulation of neutrophilic granulocytes by 30.8% ± 8.7%. The rate of O$_2^-$ production was slightly lower in the presence of 10 μM ZnSO$_4$ than in the presence of 5 nM DPI (69.2% ± 8.7% versus 80.4% ± 2.4% of the control value, n = 5). However, unlike DPI, Zn$^{2+}$ did not impair bacterial killing. In fact, bacterial survival was slightly but consistently lower than the control value: in the absence of any inhibitor 6.7% ± 6.2%, in the presence of 5 nM DPI 21.3% ± 8.3%, whereas in the presence of 10 μM ZnSO$_4$ 3.5% ± 0.53% (n = 10) S. aureus survived. Apparently, differences in the extent of membrane depolarization (ie, in the driving force for K$^+$ efflux) are able to modify the efficiency of bacterial killing independent of the rate of O$_2^-$ generation.

### Discussion

Our experiments revealed a nonlinear relation between O$_2^-$ production on one hand and membrane potential change or K$^+$ release or bacterial killing on the other hand. At very low intensity, O$_2^-$ generation is accompanied by significant depolarization and K$^+$ efflux: 38% of total K$^+$ release takes place when O$_2^-$ production proceeds at less than 5% of its maximal capacity. Because K$^+$ movement accounts only for 5% to 6% of total charge compensation, it can be calculated that in this initial phase roughly half of the electron efflux is compensated by parallel K$^+$ efflux. Our experiments indicated −60 mV as the membrane potential in resting neutrophils (Figure 2), and this value corresponds well to earlier findings. The closeness of the resting membrane potential to the calculable equilibrium potential for K$^+$ (−80 mV) indicates that under resting conditions K$^+$ conductance dominates. This conclusion is further supported by our observation that in the

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**Figure 4.** Effect of DPI on PMA-induced $^{86}$Rb efflux from human neutrophils. (A) Human neutrophils were loaded with $^{86}$Rb and pretreated with the indicated concentration of DPI for 3 minutes. $^{86}$Rb efflux was subsequently initiated with 100 nM PMA. Data are expressed as percentage of the value obtained in the absence of DPI. Error bars represent standard deviation of 4 parallel samples. (B) $^{86}$Rb release was related to superoxide production detected in the presence of the same DPI concentration. (C) $^{86}$Rb release was related to the extent of membrane depolarization obtained in the presence of the same DPI concentration.

**Figure 5.** Effect of DPI on bacterial survival and bacteria-induced ROS generation. Effect in human neutrophils stimulated with either opsonized E. coli (A) or S. aureus (B). Superoxide (ROS) production was measured by chemiluminescence and expressed as percentage of the maximal value obtained in the absence of DPI. Bacterial survival numbers represent the ratio of surviving CFUs per initial CFUs. Mean ± SEM of 5 (survival of E. coli), 3 (E. coli–induced ROS production), 10 (survival of S. aureus), and 5 (S. aureus–induced ROS production) independent experiments are presented. In the case of E. coli and S. aureus, 100% ROS production corresponds to 3.52 and 4.46 nmol O$_2^-$ per 10 minutes per 10$^6$ PMNs, respectively.

**Figure 6.** Relation of bacterial survival to superoxide (ROS) generation. Data presented in Figure 5 are replotted so that each point represents values obtained at the same DPI concentrations.
presence of 5 μM DPI, the H+ transport inhibitor Zn2+ had no detectable influence on the membrane potential (data not shown). However, the relatively high K+ conductance of the plasma membrane of neutrophilic granulocytes prevailing at rest is still limiting the rate of K+ movements and results in the abrupt change of the membrane potential upon initiation of O2- production. In our experiments (Figure 3) approximately 50% of the maximal depolarization occurs in this phase; thus, a change by 30 to 50 mV can be estimated. Elimination of bacteria is severely impeded at this low rate of O2- production, as survival of S aureus attained 200%.

At moderate rate of O2- production (between 5% and 20% of the maximal capacity) both the membrane potential change and K+ release are attenuated. Electrogenic H+ efflux pathways were shown to be potential sensitive and exhibit a threshold for opening that is, depending on the exact conditions, 20 to 40 mV more positive than the resting membrane potential.10-12 With channel-opening processes being statistical events, probably in this range of O2- generation H+-conducting pathways open gradually, and charge compensation for electron transfer is taken over more and more by H+ efflux. Bacterial killing capacity is significantly increased as indicated by the drastic fall of survival of S aureus from 200% to 95%. Assuming that the proportion of oxidase molecules to ion transporters does not basically differ between the plasma and the issuing phagosomal membrane, we related bacterial survival to the membrane potential change and to K+ efflux. Both relations resulted in a straight line (data not shown), supporting the concept that depolarization and ion movements initiated by the electrogenic oxidase may have a decisive role in the killing process. Of note, membrane potential and K+ efflux exhibited a linear relationship (Figure 5C), indicating constant K+ conductance in the measured range of membrane potential (approximately between −20 and +60 mV). This observation can be accorded with the recent report on the appearance of an outward current at −30 mV in PMA-activated neutrophils,14 although many details of these latter experiments have been criticized.22

Finally, when O2- production exceeds 20% of the maximal capacity, alteration of the membrane potential becomes minimal and the remaining K+ efflux accounts only for approximately 1% to 2% of the electron efflux. In activated eosinophils, under current-clamped conditions it was shown that the value of the membrane potential followed the equilibrium potential of H+ ions, both in the presence and in the absence of K+ in the pipette solution.20 These findings indicate that in the case of fully activated oxidase, the conductance for protons largely exceeds the conductance of any other ions. Thus, at high rate of O2- generation protons serve as dominant charge-compensating ions. At the stable membrane potential no dramatic change occurs in the driving force for any other mobile ion; thus, no serious change in the ion composition of the phagosome can be expected except for an increase of O2- itself and of the compensating H+ ions. On the other hand, survival of S aureus diminishes from 90% to 5% as O2- generation increases from 20% to 100% capacity, indicating a significant increase in the bacterial killing activity of neutrophils. Under the apparently stable electrophysiological conditions of the phagosome, an enhancement of the killing capacity developing parallel to the increase in [O2-] and [H+] must be ascribed to the chemical effect of these products (plus their derivatives).

In our experiments the variable parameter was the rate of O2- production gradually decreased by increasing doses of the inhibitor DPI. During phagocytosis, the observations detailed in the previous paragraphs may correspond to different phases following the onset of O2- production initiated by particular stimuli. We suggest that at the beginning of the process K+ efflux dominates, causing a rapid increase in [K+] inside the phagosome and allowing for the temporary alkalinization of the intraphagosomal space.14,23 As the process progresses, H+ efflux takes over and contributes to the acidification of the phagosomal milieu observed in earlier experiments.14,23 Furthermore, the experimental condition in the presence of high DPI concentration is relevant to the pathological situation when neutrophils become activated in an environment of very low oxygen tension, such as inside abscesses or in poorly perfused regions. When the availability of oxygen limits the rate of electron transfer via the NADPH oxidase, membrane depolarization and consequent ion movements may become critical in the battle with the invading pathogen.

Taken together, the presented data provide experimental support for our earlier hypothesis17 that both depolarization and secondary ion movements initiated by electron transfer via the NADPH oxidase, and the produced O2- and its derivatives as chemical compounds, participate in the killing of certain bacteria. The significance and contribution of the 2 mechanisms to the entire killing process may depend on the exact conditions, such as availability of oxygen or the species of the invading microorganism. The experiments carried out in the presence of the H+ channel inhibitor Zn2+ suggest that an increase of the membrane potential change may compensate for a decrease of O2- production. Improvement of bacterial killing by modification of ion channel activity would be a basically new concept in the therapy of infectious diseases.

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