The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H⁺ channel

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The membrane potential of cytoplasts, derived from human neutrophils, was depolarized by the activation of the superoxide-generating NADPH-dependent oxidase. The extent of the depolarization was inhibited by diphenylene iodonium and was therefore due directly to the activity of the oxidase, which must be electrogenic. The extent of the depolarization was influenced by alteration of the ΔpH across the cytoplasmic membrane, indicating that the outward translocation of H⁺ eventually compensates for superoxide generation. The depolarization of the potential is enhanced by Cd²⁺, a blocker of H⁺ currents, suggesting that the compensatory movement is via an H⁺ channel.

INTRODUCTION

Neutrophils are a major component of the body's initial cellular response against infection. They are able to migrate towards a site of infection, where they engulf, kill and digest bacteria. The plasma-membrane NADPH-dependent oxidase of human neutrophils [1] is an important contributor to the bactericidal capacity of these cells [2]. The enzyme performs the single-electron reduction of oxygen to superoxide radical (O₂^{•−}) on activation by natural and artificial stimuli. A massive change in membrane potential would ensue if the reduction of oxygen at the external surface of the membrane, by cytosolic NADPH, occurred in the absence of the movement of a compensating ion, and this might limit the activity of the oxidase. The oxidase activity is associated with a low-potential cytochrome *b* and a flavoprotein [1], and is therefore likely to comprise an electron-transport chain composed of a hydrogen acceptor, FAD, followed by a purely electron acceptor, cytochrome *b* [3]. The rapid fall in pH within the phagosome after phagocytosis of a bacterium suggests that protons, derived from the oxidation of NADPH, do translocate across the membrane [4,5], to balance the charge displacement due to O₂^{•−} formation. Therefore the two components of the oxidase might provide a path for the simultaneous translocation of the protons, they being released to the exterior on transfer of electrons from FAD to cytochrome *b*. Alternatively the protons could be released to the interior, causing the intracellular pH to fall, followed by exit via a route independent of the oxidase. The path adopted for the translocation depends upon the organization of the oxidase in the membrane and has obvious consequences for the membrane potential.

A number of probes have been used to monitor the membrane potential of neutrophils [6–9]. However, neutrophils contain a large number of internal organelles, particularly the specific and azurophilic granules, so that the use of a probe is always associated with the uncertainty as to its location. For this reason we have

used enucleated cells, cytoplasts [10], derived from human neutrophils; these are virtually free of organelles. In these right-side-out vesicles the activity of the oxidase can be initiated and maintained.

We have used oxonol V, an optical membrane-potential indicator [11], to measure the resting membrane potential and changes in this potential on induction of the activity of the oxidase using the soluble stimulus phorbol 12-myristate 13-acetate (PMA). In the present paper we report that there is a depolarization of the membrane potential associated with the activity of the oxidase and therefore the oxidase is electrogenic. We present evidence that the route for the eventual compensation for superoxide generation is an H⁺-conducting channel.

MATERIALS AND METHODS

Chemicals

Oxonol V was obtained from Molecular Probes, Eugene, OR, U.S.A., the stock solution being made up in dimethyl sulphoxide (DMSO) at a concentration of 1 mg·ml^{−1} (2.6 mM). This was diluted in the assay buffer to give a final concentration of 8 μM . The PMA was dissolved in DMSO at a concentration of 1 mg·ml^{−1}, a stock solution of 10 μM being made by dilution in DMSO. Diphenyleneiodonium sulphate (DPI) was synthesized in the laboratory as described by Collette *et al.* [12], and a stock solution of 1 mM was prepared in ethanol. Ficoll 70 and cytochalasin B were obtained from Sigma, Poole, Dorset, U.K.

Preparation of neutrophils

The neutrophils were prepared from buffy coats obtained from the Southwest Regional Blood Transfusion Centre, Southmead Hospital, Bristol, U.K. They were purified by a 1 g 1%-dextran sedimentation, centrifugation of the resulting supernatant over Ficoll-Histopaque, followed by two hypo-osmotic lyses in NaCl to remove contaminating red blood cells [13].

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleneiodonium (sulphate); DMSO, dimethyl sulphoxide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Preparation of cytoplasts

Cytoplasts were prepared from the resulting neutrophils as described by Roos *et al.* [10]. Briefly, cytoplasts were prepared on a discontinuous Ficoll 70 step gradient. The components of the gradient were 12.5, 16 and 25% (w/v) Ficoll 70, which were prepared from a stock solution of 40% (w/v) Ficoll 70 in 25 mM-Tris/HCl, pH 7.4, by dilution with 140 mM-NaCl/10 mM- K_2HPO_4 , pH 7.4. Cytochalasin B at a concentration of $5 \mu\text{g} \cdot \text{ml}^{-1}$ and 5.5 mM-glucose were present throughout the gradient. The neutrophils were loaded on to the gradient after 10 min preincubation at 37 °C in the 12.5% Ficoll 70 component. Cytoplasts were harvested from the 12.5–16% interface after centrifugation in a fixed-angle rotor for 1 h at 81 000 *g* and at 37 °C, washed twice in 150 mM-NaCl/1 mM-KCl/10 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4 (assay buffer), centrifuged at 800 *g* for 10 min and resuspended in assay buffer at approx. 5×10^8 cytoplasts $\cdot \text{ml}^{-1}$.

Determination of $O_2^{\cdot -}$ production

Superoxide generation by 1×10^7 cytoplasts was recorded continuously at 550 minus 540 nm as the DPI-sensitive reduction of horse heart cytochrome *c* (100 μM), in 1.5 ml of assay buffer at 37 °C. This reduction of cytochrome *c* was similarly inhibited by the addition of superoxide dismutase (44 $\mu\text{g} \cdot \text{ml}^{-1}$) to the assay.

Measurement of membrane potential

Cytoplasts (1.25×10^7) were pre-equilibrated for 3 min at 37 °C in 1.5 ml of assay buffer containing 8 μM -oxonol V. The uptake of the dye by the cytoplasts was monitored as a red shift in the wavelength of maximum absorbance of the dye. The changes in absorption were monitored continuously at 640 minus 600 nm, an increase corresponding to a depolarization of the membrane potential. The absorbance changes were calibrated as described by Jones *et al.* [14], with 0.5 M- K_2SO_4 additions in the presence of 2.7 μM -valinomycin.

Cell count

Cytoplast numbers were determined by using a counting chamber.

RESULTS AND DISCUSSION

The basal level of absorbance of oxonol from non-stimulated cytoplasts shown in Fig. 1(a) is equivalent to a resting membrane potential of -100 ± 6.8 mV, negative to the inside of the membrane. This agrees with that found for whole neutrophils when a similar method was used [14]. The addition of the oxidase activator PMA to cytoplasts pre-equilibrated with oxonol results in a rapid increase in dye absorbance at the recording wavelengths, indicating a depolarization of the membrane potential (Fig. 1a). Concurrently PMA induces a 120-fold increase in the generation of $O_2^{\cdot -}$ by the cytoplasts (Fig. 1b), suggesting that the oxidase activity is coupled to a depolarization of the membrane potential. The addition of PMA to cytoplasts suspended in 140 mM-NaCl/5 mM-KCl/1 mM-MgCl/0.6 mM-CaCl/5.5 mM-glucose results in similar changes in the membrane potential and the reduction of cytochrome *c*; however, the high potassium concentration precludes calibration of the oxonol (results not shown).

Fig. 1. Membrane-potential changes and $O_2^{\cdot -}$ generation by cytoplasts after addition of PMA and DPI

(a) Cytoplasts (1.25×10^7) were pre-equilibrated with oxonol V as described in the Materials and methods section. All additions were made through an injection port, and changes in absorbance (640–600 nm) were recorded continuously. The vertical bar represents the potentials obtained in the calibration using 0.5 M- K_2SO_4 additions in the presence of 2.7 μM valinomycin. The final concentrations of PMA and DPI were 33 nM and 6.7 μM respectively. (b) Superoxide generation by cytoplasts (1×10^7) monitored by the reduction of horse heart cytochrome *c* (100 μM). Both assays were performed in assay buffer (see the Materials and methods section), at 37 °C.

Inhibition of superoxide generation and the depolarization of the membrane potential by DPI

The compound DPI is an inhibitor of the NADPH oxidase of pig neutrophils [15] and rat macrophages [16]. At a final concentration of 6.7 μM it inhibited over 96% of the oxidase activity in PMA-treated cytoplasts (Fig. 1b). In cytoplasts pre-equilibrated with lower concentrations of DPI, an initial burst of enzyme activity, on adding PMA, was observed (Fig. 2a). The size of this initial burst and the final rate of $O_2^{\cdot -}$ production were DPI-concentration-dependent, as was the low level of $O_2^{\cdot -}$ generation normally observed in unstimulated cytoplasts (Fig. 2a). This initial burst of PMA-activated oxidase activity, in the presence of DPI, is probably due to the low affinity of DPI for the non-activated enzyme.

Fig. 2. Effect of the oxidase inhibitor DPI on cytoplasts

The final concentration of DPI added to the cytoplasts at zero time is shown on the right-hand side of the Figure. (a) The initial burst in superoxide generation on addition of 33 nM-PMA; (b) the depolarization of the membrane potential observed in the presence of DPI. The values shown on the extreme right are the final potentials the cytoplasts in the presence of the different concentrations of DPI. Both assays were performed as described in the Materials and methods section. Note the change of scale after 2 min.

It is probable that the oxidase undergoes a conformational change to expose a DPI-binding site as part of the process associated with initiating its activity.

The observed depolarization of the membrane potential might arise from the signal pathway which leads to the activation of the oxidase, as has been suggested by a number of authors [9,17–22]. However, this explanation is unlikely, since the addition of 6.7 μM-DPI to stimulated cytoplasts results in a repolarization of their membrane potential (Fig. 1a), and the extent of the potential change on addition of PMA is reduced by the preincubation of the cytoplasts with DPI in a concentration-dependent manner (Fig. 2b). At a DPI concentration of 26.8 μM there is virtually no change in the potential on addition

of PMA (results not shown). The effect of DPI upon the potential can be mimicked by the addition of low concentrations of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (results not shown). A potential change involved in signalling should be unaffected by the inhibition of the terminal component in the chain. The initiation of the generation of $O_2^{\cdot-}$ and the depolarization of the potentials occur coincidentally on the addition of PMA (Fig. 1). We have never observed the temporal differences in the initiation of these two processes quoted by other authors [18,22]. Therefore the change in membrane potential appears to be due directly to the enzymic production of $O_2^{\cdot-}$. A depolarization of the membrane potential is also observed on addition of PMA to oxonol-loaded cytoplasts suspended in 9% (w/v) sucrose/10 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4 (results not shown), which lacks anions and cations. Protein kinase C, the probable receptor for the phorbol esters, is located in the cytosol/membrane of cells [24], and therefore the stimulation of cytoplasts using PMA does not involve an external receptor. Thus the ion fluxes reported as components of the signalling system [25,27] do not make a significant contribution to the observed depolarization. We therefore conclude that the NADPH oxidase performs the vectorial transport of electrons and does not act directly as a proton translocator.

Investigation of the nature of the compensating ion and its means of translocation across the membrane

The membrane-potential change levels out about 1 min after stimulation. This is not due to the termination of $O_2^{\cdot-}$ generation, which continues linearly for several minutes. Calibration experiments show that the capacity of the dye to respond has not been exceeded at this time. Therefore the system must have reached a new steady state in which the flow of electrons to O_2 is exactly balanced by positive charge leaving the cytosol, negative charge entering, or a combination of both. If an outward movement of protons makes any contribution to this charge compensation, then the manipulation of the driving forces acting on them should alter the extent of the membrane-potential depolarization observed. There are two components of the force acting on protons produced by NADPH oxidation, the negative membrane potential and the concentration gradient, ΔpH , across the membrane. Net outward movement will only occur when the concentration-gradient component exceeds the unfavourable membrane-potential contribution. We varied the ΔpH across the cytoplast membrane by varying the external pH of the assay buffer. Under these conditions the ability of the cytoplasts to generate superoxide was only slightly decreased at the two extreme pH values. The result was an alteration in the extent of the potential change observed (Fig. 3). As predicted, the larger the initial proton gradient, the smaller the membrane potential response, i.e. full compensation for $O_2^{\cdot-}$ production occurs sooner. The addition of NH_4Cl to neutrophils raises their internal pH [28], and hence the potential change observed at all three external pH values is larger in its presence. Therefore the outward movement of protons is an important component in the charge compensation for the electrogenic generation of $O_2^{\cdot-}$.

If protons are not translocated by the oxidase, then how do they cross the membrane? A plasma-membrane H^+ -transporting ATPase, as found by Mollinedo *et al.* [29], would require a large amount of ATP to pump H^+

Fig. 3. Effect of the external pH on the membrane-potential change observed for cytoplasts stimulated with PMA

The external pH values of the assay buffers were 6.6 (a), 7.4 (b) and 8.3 (c). The pH of the assay buffer was adjusted to the required value by using 1 M-Tris. CdSO_4 and NH_4Cl were added at final concentrations of 0.25 mM and 20 mM respectively. The assays for the generation of superoxide and the change in the membrane potential were performed as described in the Materials and methods section.

fast enough to act as a major counter-ion. Neutrophils are dependent on glycolysis as a source of ATP because they possess few mitochondria [30], and these organelles are absent from cytoplasts (L. M. Henderson, J. B. Chappell & O. T. G. Jones, unpublished work). The production of lactate by neutrophils [31] and cytoplasts is not greatly increased on activation of the oxidase, arguing against the involvement of an ATP-driven mechanism. Cadmium (Cd^{2+}) and other heavy-metal ions have been shown, by Thomas & Meech [32], to block outward proton currents through a plasma-membrane channel in snail neurons. The addition of 0.25 mM- CdSO_4 extended the PMA-induced potential change already observed at the three external pH values used (Fig. 3). The effect of Cd^{2+} is greatest at an external pH of 8.3, where the movement of protons is otherwise most aided by the H^+ concentration gradient, and this can be overcome by the use of low concentrations of

Fig. 4. Repolarization of the membrane potential after the depolarization due to the addition of PMA

The cytoplasts were suspended in 150 mM-NaCl/1 mM-KCl/5 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4. The membrane-potential changes were measured as described in the Materials and methods section. The potential was allowed to reach its new level after the addition of 33 nM-PMA before making the additions as indicated. (a) The inhibition of the DPI (6.7 μM)-induced repolarization by 0.25 mM- Cd^{2+} . CdSO_4 and DPI were added simultaneously. (b) The repolarization as a result of the rapid increase in the external pH due to the injection of 1 M-Tris and the response in the presence of 0.25 mM- CdSO_4 . (c) As for (b), except the cytoplasts were pretreated with 6.7 μM -DPI before the addition of PMA. The potential at which the system levels out is therefore more negative than in (b).

CCCP. It therefore appears that a proton-conducting Cd^{2+} -sensitive channel could be involved.

We have investigated further the probable involvement of a proton channel in the compensation for $\text{O}_2^{\cdot -}$ generation by perturbing the established equilibrium across the membrane. In a lightly buffered medium the injection of a small volume of 1 M-Tris rapidly increases the external pH from 7.4 to 8.3. If this external pH jump is performed after the PMA-induced potential change has levelled off, it results in a repolarization of the potential (Fig. 4c). This repolarization is severely inhibited in the presence of Cd^{2+} , suggesting that the mobile species are protons. The repolarization observed on the addition of DPI is similarly inhibited by Cd^{2+} (Fig. 4a). The ability of Cd^{2+} to inhibit the initial rate of repolarization by 80% supports the probable role of a channel in this system.

The results that we have presented above suggest that the observed membrane-potential depolarization on activation of cytoplasts is due to the electrogenic nature of the NADPH oxidase. The resultant H^+ ions liberated within the cytosol would cause a very rapid fall in internal pH if they were unable to translocate across the membrane. It is most likely that their movement is via a channel in the membrane, the opening of which lags behind the activation of the oxidase. The mechanism for initiating the open of this channel is unknown at present, but could be a voltage decrease, an increase in the internal concentration of protons or a phosphorylation/conformation change. The channel might not be specific for protons alone, but could be one of the membrane cation channels through which protons can move when their concentration gradient becomes large.

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