Internal pH changes associated with the activity of NADPH oxidase of human neutrophils

Further evidence for the presence of an H⁺ conducting channel

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The internal pH (pH_i) of cytoplasts, derived from human neutrophils, falls 0.05 pH units upon activation of the superoxide-generating NADPH oxidase. The decrease in pH_i is absent in diphenylpicrylhydrazolium-treated cytoplasts and therefore it is likely to arise directly from the activity of the oxidase. The addition of amiloride, to diminish the Na⁺/H⁺ exchanger, enhanced the extent of the internal acidification but not the initial rate. However the electroneutral Na⁺/H⁺ exchanger cannot be a contributor to H⁺ efflux to compensate for charge translocated by the oxidase. In the presence of Cd ions or valinomycin, phorbol-induced acidification of the cytosol was greatly increased, suggesting an inability to translocate the cytosolic H⁺ generated by an electrogenic oxidase. In the presence of both Cd and valinomycin the cytoplasts retained 0.8 H⁺ per O₂^{•-} generated. The rate of acidification of the external medium by stimulated cytoplasts is greatly reduced in the presence of Zn and valinomycin. Our results support the view that the plasma membrane of neutrophils contains Zn²⁺- or Cd²⁺-sensitive proton-conducting channels which maintain a stable membrane potential and pH_i during the activity of the electrogenic NADPH oxidase.

INTRODUCTION

The NADPH oxidase of neutrophils is a major contributor to the ability of these cells to participate in a cellular immune response mounted by the body against an infection [1]. This plasma-membrane enzyme performs the single-electron reduction of O₂ to superoxide, using NADPH as electron donor. The major contribution of superoxide and its dismutation products to the bactericidal capacity of the body is readily demonstrated by the susceptibility to infection shown by patients with chronic granulomatous disease [2]. Neutrophils from these patients are incapable of generating O₂^{•-} as they lack a functional NADPH oxidase. In X-linked chronic granulomatous disease the product of the defective gene is a cytochrome *b* believed to be a component of the NADPH oxidase [3–6]. The formation of NADPH by the pentose phosphate pathway results from the removal of two H⁺ and two electrons from the substrates. Therefore in normal neutrophils the oxidation of cytosolic NADPH must result in the generation of one H⁺ per electron transferred to O₂. In a recent paper we have demonstrated that depolarization of the membrane (and hence a drop in potential), seen upon stimulation of the oxidase, is due directly to the activity of the oxidase [7]. As illustrated in Fig. 1, two components of the oxidase, a flavoprotein and a low-potential cytochrome *b* [8], are considered to translocate electrons from NADPH (cytosolic side) to external O₂ without the simultaneous translocation of protons generated by the oxidation of NADPH. A massive depolarization of the membrane and a rapid fall in the pH_i would be the consequence if the proton did not cross the membrane. The outward

Fig. 1. Diagrammatic representation of electrogenic superoxide-generating NADPH oxidase in the plasma membrane of human neutrophils as proposed by Henderson *et al.* [7]

The transfer of electrons from the hydrogen atom acceptor, FADH₂, to the electron acceptor, cytochrome *b*, occurs with the release of the resulting protons to the cytosolic side of the membrane. Translocation of the proton, either into the phagosome or to the exterior of the cell, occurs through a channel in the membrane sensitive to Cd and Zn. *E_m*, mid-point potential.

movement of the proton, to compensate for the electrogenic generation of O₂^{•-}, is proposed to occur through a Zn²⁺- and Cd²⁺-sensitive H⁺-conducting channel in the membrane. The delay in translocation of H⁺ that arises as a consequence of the independent transport processes

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; DPI, diphenylpicrylhydrazolium; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester.

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will give rise to a small initial fall in the pH_i of the neutrophil associated with stimulation of oxidase activity.

The pH_i of neutrophils has previously been monitored by a number of different authors using weak acid/base distribution and fluorescent indicators [9–12]. In this present paper we report that in cytoplasts, enucleated cells [13] loaded with the pH_i indicator 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF), we can detect a fall in pH_i upon activation of the oxidase. This acidification is absent in the presence of diphenyleneiodonium (DPI), an inhibitor of oxidase activity [14]. This fall in pH_i is greatly extended in the presence of Cd^{2+} , a blocker of the H^+ channel, and in the presence of valinomycin. The extent of acidification of the external medium, monitored using BCECF, seen upon initiation of oxidase activity in cytoplasts, is greatly reduced in the presence of Zn^{2+} and valinomycin in keeping with the greater fall in pH_i under the same conditions. These results strongly support the hypothesis that the oxidase is electrogenic and associated with an H^+ -conducting channel in the membrane.

MATERIALS AND METHODS

Chemicals

The fluorescent pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and non-esterified BCECF were obtained from Molecular Probes (Eugene, OR, U.S.A.). BCECF-AM was prepared as a stock solution of 1 mM by dissolving in dried dimethyl sulphoxide. A 1.67 mM stock solution of BCECF was prepared by dissolving in distilled water and adjusting the pH with NaOH. Amiloride, valinomycin, and nigericin were obtained from Sigma (Poole, Dorset, U.K.). Stock solutions of 0.1 M-amiloride, 0.4 mM-valinomycin and 1.5 mM-nigericin were prepared in dimethyl sulphoxide, ethanol and methanol respectively.

Preparation of neutrophils and cytoplasts

The neutrophils and cytoplasts were prepared from human buffy coats as previously described [7,13].

Loading of cytoplasts with BCECF-AM

Cytoplasts, at approx. 5×10^8 cells/ml, were suspended in 150 mM-NaCl/1 mM-KCl/5 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4 (Na^+ medium). The cytoplasts were incubated with 5 μ M-BCECF-AM at 37 °C for 5 min. In this period the BCECF-AM is taken up by the cytoplasts. Leakage of the indicator out of the cytoplasts is prevented by cleavage of the four acetoxymethyl groups by esterases present in the cytosol. The cytoplasts (BCECF-loaded) were pelleted (800 g for 10 min) to remove them from the remaining esterified dye, and resuspended in the above medium. Longer incubations with the esterified dye were found to cause activation of the oxidase.

Measurement of internal pH

pH_i was monitored from the difference in emitted fluorescent light at the excitation wavelengths of 490 nm and 455 nm (490–455 nm) in a dual-wavelength spectrophotometer/fluorimeter with the photomultiplier placed at 90° to the incident light. The fluorescence emission of BCECF is pH-sensitive when excited at 490 nm but pH-insensitive when excited at 455 nm [15]. This method

of monitoring fluorescence changes, by difference, eliminates the problem of light scattering caused by cell shape changes and variation in the concentration of the dye. pH_i changes (ΔpH_i) were monitored with 2×10^7 cytoplasts suspended in 1.5 ml of the appropriate medium, at 37 °C. All additions were made through an injection port and the suspension was continuously stirred. The response of the dye was calibrated using cytoplasts suspended in 150 mM-KCl/5 mM-Hepes/Tris/5.5 mM-glucose, pH 7.4 (K^+ medium) using the addition of portions of HCl in the presence of 10 μ M-nigericin [16].

Measurement of external pH changes

The fluorescent pH indicator BCECF was used to monitor changes in the external pH. The cytoplasts were suspended in 150 mM-NaCl/1 mM-KCl/1 mM-Hepes/5.5 mM-glucose/2 μ M-BCECF, pH 7.4, at 37 °C. The pH-dependent changes in the fluorescence emission of the dye were monitored as described for the measurement of pH_i . The response of the dye was calibrated, at the end of each experiment, using portions of freshly prepared NaOH. The enzyme superoxide dismutase was present at a final concn. of 33 μ g $\cdot$ ml⁻¹.

Determination of internal volume of the cytoplasts

The internal volume of portions of cytoplasts was determined using [³H]H₂O and [¹⁴C]sucrose to ascertain the excluded volume [17].

Superoxide generation

The generation of superoxide by cytoplasts was assayed as previously described [7].

Cytoplast count

Cytoplasts were counted in a counting chamber using a light microscope.

Determination of buffering capacity

The buffering capacity of the BCECF-loaded cytoplasts was determined from ΔpH_i after the addition of portions of sodium acetate in the presence of 1 mM-amiloride.

RESULTS AND DISCUSSION

The activity of the oxidase, leading to generation of superoxide anions, which is normally quiescent, is stimulated by a number of products present at a site of infection, e.g. C5A, IgG complexes, opsonized bacteria, and can also be caused by the addition of a number of artificial stimuli, e.g. phorbol esters, formyl-Met-Leu-Phe, some detergents and latex beads.

Changes in pH_i associated with the activity of the oxidase

The addition of phorbol 12-myristate 13-acetate (PMA) to cytoplasts suspended in an Na^+ medium results in a transient fall in pH_i of 0.05 pH units, as shown in Fig. 2(b). This fall in pH_i has previously been observed in neutrophils and cytoplasts [18,19]. The plasma membrane of neutrophils has been shown to contain a Na^+/H^+ exchanger which is involved in the regulation of pH_i and the volume of neutrophils [20,21]. In the presence of 1 mM-amiloride, a competitive inhibitor of the exchanger, the fall in pH_i is extended slightly (Fig. 2a). The additional fall in pH_i is due to inhibition

Fig. 2. Internal pH changes in cytoplasts

The cytoplasts were loaded with BCECF and changes in fluorescence monitored as described in the Materials and methods section. The cytoplasts were suspended in the Na^+ medium (a, b, c and e) and the K^+ medium (d) in the presence of 1 mM-amiloride (a), 6.7 μM -DPI (c). The activity of the oxidase was stimulated by the addition of 33 nM-PMA (a, b, c and d). Phenazine methasulphate (PMS) was added at a final concn. of 4.6×10^{-4} M to (e).

of the recovery in pH_i towards its starting value as seen in the absence of amiloride (Fig. 2a,b). The initial rate of acidosis is unaltered by the presence of amiloride. The pH_i of cytoplasts suspended in a K^+ medium, in which the efflux of H^+ by the exchanger is absent, falls by 0.15 pH units (Fig. 2d). The efflux path for H^+ ions must itself be electrogenic if it is to compensate for the generation of $\text{O}_2^{\cdot-}$ by the oxidase. The Na^+/H^+ exchanger performs the electroneutral antiport of one H^+ ion for one Na^+ ion. This therefore precludes the exchanger from being a major route for the translocation of H^+ accompanying the activity of the oxidase. The fall in pH_i on adding PMA is prevented by the addition of DPI (Fig. 2c). It has been shown that DPI has a high affinity for only the active state of the enzyme, however the

sensitivity of this assay is insufficient to detect the initial burst in activity of the enzyme seen using the cytochrome *c* assay [7]. *N*-Ethylmaleimide, an inhibitor of $\text{O}_2^{\cdot-}$ generation and the metabolic inhibitor deoxyglucose, which prevents the supply of substrate to the enzyme, have previously been shown to prevent the fall in pH_i of neutrophils [18]. The addition to cytoplasts of phenazine methosulphate, an electron acceptor, promotes the generation of acid by the oxidation of NADPH, causing a fall in pH_i (Fig. 2e) [18]. Therefore it is possible that the fall in pH_i on addition of PMA to cytoplasts arises as a direct consequence of NADPH oxidation due to the activity of the oxidase.

According to Fig. 1 one H^+ is generated per $\text{O}_2^{\cdot-}$ formed in the phagosome or externally. Taking the obtained value for buffering capacity of 120 mM per pH unit and an internal volume of the cytoplasts per assay of 0.49 μl , the fall in pH_i exhibited by the cytoplasts upon addition of PMA, is equivalent to the generation of 2.94 nmol of H^+ internally. Under the same conditions these cytoplasts generated 50 nmol of $\text{O}_2^{\cdot-} \text{ min}^{-1}$ (not shown). Therefore the amount of H^+ measured falls dramatically short of the expected value. This suggests that the acid produced is due to the oxidation of NADPH, as illustrated in Fig. 1, followed by a rapid efflux of H^+ to compensate for the generation of $\text{O}_2^{\cdot-}$.

Inhibition of H^+ translocation by valinomycin and Cd

As shown in Fig. 1 the oxidase is unable to perform simultaneous translocation of H^+ and an electron across the membrane. A transient fall in pH_i would be predicted from such a mechanism for the oxidase. However a massive depolarization of the membrane would occur if charge compensation for the generation of $\text{O}_2^{\cdot-}$ was not rapid. The addition of valinomycin to cytoplasts (a K^+ uniporter [22]), suspended in an Na^+ medium, allows outward movement of K^+ down its concentration gradient to compensate for $\text{O}_2^{\cdot-}$ generation. Under these conditions the fall in pH_i is greatly extended (Fig. 3a). The retention of H^+ in the cytosol of cytoplasts is likely to be a direct consequence of the fact that their movement across the membrane is no longer required for compensation of $\text{O}_2^{\cdot-}$ generation. For this accumulation of H^+ in the cytosol to occur, H^+ must have been released to the cytosolic side of the membrane, during the normal course of NADPH oxidation by the enzyme. This supports our view [7] that the oxidase is electrogenic, as diagrammatically represented in Fig. 1. In the absence of valinomycin, the diminished acidification of the cytosol results from rapid translocation of H^+ ions as they are generated.

We have previously described a Cd^{2+} -sensitive H^+ -conducting channel in the plasma membrane of cytoplasts through which H^+ , from oxidation of NADPH, are considered to translocate the plasma membrane [7]. It therefore follows that the extent of acidification of the pH_i should also be increased by blockage of the translocation of H^+ . As shown in Fig. 3(a), the recorded fall in pH_i is much greater in the presence of Cd^{2+} , and also Zn^{2+} (not shown). This is strong supporting evidence for a $\text{Cd}^{2+}/\text{Zn}^{2+}$ -sensitive H^+ -conducting channel in the membrane of these cells, having a direct role in the movement of H^+ in compensation for generation of $\text{O}_2^{\cdot-}$. From the model for the activity of the enzyme it would be predicted that the inhibition of movement of H^+ by Cd^{2+} should result in a massive depolarization of the

Fig. 3. Changes in the pH_i of cytoplasts (a) and the generation of superoxide by cytoplasts (b) in the presence of valinomycin and Cd

Both assays were performed as described in the Materials and methods section on cytoplasts loaded with BCECF. The cytoplasts were suspended in the Na⁺ medium, at 37 °C, in the presence of 1 mM-amiloride. Valinomycin (Val) and CdSO₄ were added at final concns. of 2.7 μM and 0.25 mM respectively. Activity of the oxidase was stimulated by the addition of 33 nM-PMA.

membrane and inhibition of the oxidase. However under these conditions there is only a slight inhibition of the generation of superoxide (Fig. 3b). Under these conditions it would be predicted that the movement of another ion must occur to act as a compensator (most probably K⁺). The greatest fall in the value of pH_i is seen in the presence of valinomycin and Cd²⁺ (Fig. 3a). Therefore even in the presence of valinomycin some conduction through the H⁺ channel occurs. This fall in pH_i is equivalent to 26 nmol of H⁺. The cytoplasts, under the same conditions, generated 33 nmol of superoxide, Fig. 3(b), and therefore retain 0.8 H⁺ per O₂⁻ generated. The ability of cytoplasts to generate superoxide is strongly inhibited in the presence of both valinomycin and Cd²⁺.

Changes in pH of the external medium caused by activity of the oxidase

The above enhancement in the fall of pH_i on PMA stimulation of oxidase activity seen, in the presence of Cd²⁺, valinomycin and Cd²⁺ plus valinomycin, is suggested to arise from the inhibition of or deficiency in the translocation of H⁺ across the plasma membrane. Under normal conditions the efflux of H⁺ from the cytoplasts, in response to O₂⁻ generation, should result in a fall in pH of the medium. However cytoplasts suspended in a lightly buffered medium show very little increase in the rate of acidification of the external medium on addition of PMA (L. M. Henderson, J. B. Chappell & O. T. G. Jones, unpublished work). However, spontaneous dismutation of the superoxide anion

results in the removal of one H⁺ from the external medium per O₂⁻ generated. It is therefore probable that the enhancement in the rate of acidification of the medium on addition of a stimulus to cytoplasts is smaller than would be predicted due to the rapid spontaneous dismutation of the O₂⁻ generated.

Fig. 4. Changes in external pH caused by PMA stimulation of oxidase activity in cytoplasts

The assays were performed as described in the Materials and methods section using cytoplasts suspended in a lightly buffered Na⁺ medium containing 2 μM-BCECF. Activity of the oxidase was stimulated by addition of 33 nM-PMA. Valinomycin (Val) and ZnSO₄ were added at final concns. of 2.7 μM and 1 mM respectively. The presence of superoxide dismutase ensures that one H⁺ is removed from the external medium for every O₂⁻ generated. Any deficiency in H⁺ translocation will result in a reduced acidification of the external medium.

Superoxide dismutase catalyses the dismutation of O₂⁻, as outlined in the equation above. In the presence of the enzyme one H⁺ is removed from the external medium per O₂⁻ generated. Under these conditions any disparity between the generation of O₂⁻ and the translocation of H⁺ would lead to reduced acidification or perhaps even alkalization of the external medium. Such an alkaline shift in external pH is observed and is greatest when the oxidase is stimulated in the presence of valinomycin and Zn²⁺ (Fig. 4), as expected from the enhanced fall in pH_i under these conditions. Therefore the retention of H⁺ in the cytosol of cytoplasts correlates with the inhibition of their translocation.

As shown in the equation above, dismutation normally consumes one H⁺ per O₂⁻, and the pH of the external medium should be unaffected. The slight increase in the rate of efflux of H⁺ upon the addition of PMA (Fig. 4) is equivalent to 1/5 H⁺ per O₂⁻ generated and probably arises from the CO₂ generated by the increased activity of the hexose monophosphate pathway necessary to supply NADPH for the enzyme. This increase in the rate of change of external pH is not observed in DPI-treated cytoplasts.

The observed fall in pH_i upon addition of stimuli to cytoplasts, arises directly from activity of the oxidase. Retention of H⁺ in the cytosol upon stimulation of the oxidase in the presence of valinomycin and Cd²⁺ is directly mirrored by a reduced rate of pH_o change. These results strongly support the proposed hypothesis for the independent translocation of electrons, by the electrogenic oxidase, and protons, by a channel across the plasma membrane of the cell.

Activity of the NADPH oxidase of neutrophils is stimulated during the phagocytosis of a bacterium. The pH within the resulting phagocytic vacuole has been shown [23,24] to rise slightly in the first 2 min following

addition of bacteria to the neutrophils, before falling below the initial value. The results we have presented in this paper, showing a fall in the pH_i together with a rapid spontaneous dismutation of O_2^- , suggest that this elevation in phagosomal pH could arise from the delay in translocation of H^+ across the phagosome membrane relative to generation of O_2^- by the electrogenic oxidase.

Conduction of H^+ by the mitochondrial respiratory chain occurs through proteins that form integral components of three of the complexes of the inner mitochondrial membrane [25,26]. Therefore the possibility that the Cd^{2+} -sensitive H^+ channel of the plasma membrane of neutrophils is a component of NADPH oxidase cannot be eliminated by our results. Such an arrangement would allow for simultaneous regulation of both O_2^- generation and H^+ conduction.

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