Inhibition of the neutrophil NADPH oxidase and associated H⁺ channel by diethyl pyrocarbonate (DEPC), a histidine-modifying agent: evidence for at least two target sites

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Diethyl pyrocarbonate (DEPC), a histidine-modifying reagent, has been utilized to demonstrate the importance of histidine residues in the functioning of proteins. In previous studies of the NADPH oxidase, histidine residues have been determined to be important in the ability of gp91^{phox} to function as an H⁺ pathway and in the binding of haem and FAD. We have investigated the ability of DEPC to inhibit H⁺ flux and superoxide generation by human neutrophils. Proton flux through the NADPH oxidase-associated H⁺ channel was inhibited by DEPC only if applied simultaneously with an activator of the channel. This suggested that the site modified by DEPC is not accessible in the closed channel. Superoxide generation by the NADPH oxidase was also inhibited by DEPC when applied after or simultaneously with the activator. Translocation of the NADPH oxidase cytosolic components, p67^{phox} and p47^{phox}, to the membrane was un-

affected by DEPC. In a cell-free system, DEPC-treated membranes failed to support superoxide generation or the reduction of Iodonitrotetrazolium Violet and showed a loss of the characteristic cytochrome *b*₅₅₈ spectrum. Superoxide generation by DEPC-treated cytosol was inhibited slightly. Therefore it can be concluded that there are two sites within the NADPH oxidase that interact with DEPC, one in the H⁺ pathway, only accessible in the activated oxidase, and a second accessible prior to activation of the NADPH oxidase. The latter non-proton pathway DEPC site is located within the membrane components of the NADPH oxidase and is associated with the binding of haem in the enzyme complex.

Key words: proton channel, superoxide, therapeutic.

INTRODUCTION

The NADPH oxidase is an enzyme complex, located in the plasma membrane of phagocytes, which generates superoxide as part of the bactericidal activity of the immune system. The enzyme consists of a flavocytochrome *b*₅₅₈, formed by two integral membrane proteins (gp91^{phox} and p22^{phox}), and at least two cytosolic proteins, p47^{phox} and p67^{phox}, which upon activation translocate to the cytochrome in the membrane [1]. The flavocytochrome contains two non-identical haem moieties and an FAD cofactor [2,3]. The characteristics of the Raman spectra and low-temperature absorption spectra obtained from neutrophil membranes are consistent with histidine residues acting as the fifth and sixth co-ordinating positions of the Fe²⁺ of the haem moieties [4–6]. The haems have been proposed to be located either within gp91^{phox} alone [7] or, alternatively, one of the pair may be carried between gp91^{phox} and p22^{phox} [8,9]. A chronic granulomatous disease (CGD) patient with the mutation His-338 → Tyr in gp91^{phox} has been described as lacking an FAD spectrum [10]. Therefore histidines may play an important role in the binding of cofactors in the NADPH oxidase complex.

The activity of the NADPH oxidase has been demonstrated to be associated with an efflux of H⁺ ions through a Zn²⁺- and Cd²⁺-inhibited H⁺ channel [11,12]. It has been shown previously that the N-terminal 230 amino acids of gp91^{phox} are capable of forming a functional H⁺ channel [13] and that mutation of His-111, -115 and -119 to Leu or of His-115 alone to either Leu or Lys

renders the N-terminal region of gp91^{phox} unable to conduct H⁺ ions [14]. We therefore proposed that His-115 is important in the H⁺ efflux through the NADPH oxidase-associated H⁺ channel. Therefore histidine residues have been suggested to perform at least two separate roles necessary for the functioning of the NADPH oxidase enzyme complex [4–6,14].

Diethyl pyrocarbonate (DEPC) is a known histidine-modifying reagent that has been utilized to demonstrate the importance of histidine residues in the functioning of a number of different proteins. DEPC modifies the imidazole ring of histidines to form the non-functional *N*-carbethoxyimidazole [15]. As histidines have been identified as important to the H⁺ flux through the H⁺ channel, and have been proposed to co-ordinate the haem moieties, we investigated the effect of DEPC on both the flux H⁺ ions and the generation of superoxide by the NADPH oxidase. In this paper we report that DEPC inhibited both H⁺ flux and superoxide generation, but not the assembly of the NADPH oxidase, through interaction with at least two sites within the NADPH oxidase.

MATERIALS AND METHODS

Materials

2',7'-Bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.), and a stock solution of 1 mM was

Abbreviations used: AA, arachidonic acid, sodium salt; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DEPC, diethyl pyrocarbonate; DPI, diphenylene-iodonium chloride; INT, Iodonitrotetrazolium Violet; SOD, superoxide dismutase; pH_o, external pH; pH_i, internal pH.

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prepared in DMSO dried by freeze-thawing. Diphenyleneiodonium chloride (DPI) was obtained from Calbiochem-Novabiochem U.K. (Nottingham, U.K.), and a stock solution of 1 mM was prepared in DMSO. DEPC, arachidonic acid, sodium salt (AA), PMA, carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), valinomycin and superoxide dismutase (SOD) were all supplied by Sigma (Poole, Dorset, UK). Owing to its instability in aqueous solutions, DEPC was diluted to the appropriate concentration in 150 mM NaCl/1 mM KCl/5 mM Hepes/5 mM glucose at pH 6 (DEPC buffer) immediately before use. A 1 mM stock solution of AA was prepared in 50% ethanol, degassed with nitrogen and stored under nitrogen at -20°C . Stock solutions of the following were prepared in the appropriate solvents: 16 μM PMA in DMSO, 10 mM CCCP in ethanol, 400 μM valinomycin in ethanol and 10 mg/ml SOD in water.

Preparation of neutrophils and cytoplasts

Human neutrophils were prepared from buffy coat residue (Blood Transfusion Service, Southmead Hospital, Bristol, U.K.) as described previously [11]. The cytoplasts were prepared from the resulting neutrophils as described previously [11,16]. The cytoplasts were suspended at 1×10^8 cytoplasts/ml in Na^+ buffer (150 mM NaCl/1 mM KCl/5 mM Hepes/5.5 mM glucose, pH 7.3, with the pH adjusted with Tris).

Preparation of membrane and cytosol fractions for the cell-free system

Membrane and cytosol fractions for the cell-free system were prepared from neutrophils based on the method described by Pilloud et al. [17]. Human neutrophils were resuspended in PBS (137 mM NaCl/2.7 mM KCl/8.1 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) at 1×10^8 cells $\cdot\text{ml}^{-1}$, and disrupted by sonication on ice (4×15 s). The unbroken cells, granules and nuclei were removed by centrifugation (10000 *g* for 10 min at 4°C). The membrane and cytosolic fractions were separated by centrifugation at 130000 *g* for 40 min at 4°C and the membrane pellet resuspended in PBS by homogenization. The cytosolic component was further purified by passage through a Sephadex G-50 gel-filtration column [18].

The membrane fraction from activated neutrophils was prepared from neutrophils that had been suspended in Na^+ buffer at 37°C and activated with PMA (50 nM) for 5 min [19] prior to disruption by sonication and isolation as described above.

Determination of superoxide generation

The generation of superoxide by whole cells was performed in Na^+ buffer, at 37°C , using 5×10^5 neutrophils $\cdot\text{ml}^{-1}$ or 5×10^6 cytoplasts $\cdot\text{ml}^{-1}$, as described previously [11]. The cell-free assay for superoxide generation contained 300 μg of cytosolic fraction, 10 μg of membrane fraction, 100 μM AA, 2.5 mM Mg^{2+} and 20 μM guanosine 5'-[γ -thio]triphosphate to give a final volume of 300 μl with PBS, which was incubated for 5 min at 22°C , prior to assay. The generation of superoxide was monitored continuously as the DPI-sensitive reduction of 100 μM horse heart cytochrome *c* (in PBS with 2.5 mM Mg^{2+}) following the addition of an NADPH-regenerating system (10 mM NADP $^+$, 60 mM glucose 6-phosphate and 20 μg of glucose-6-phosphate dehydrogenase) to the assay cuvette.

DEPC-treated whole cells, membrane and cytosolic fractions

Neutrophils (1×10^7 cells $\cdot\text{ml}^{-1}$), cytoplasts (1×10^8 cells $\cdot\text{ml}^{-1}$) or membrane and cytosolic fractions for the cell-free system (0.5 mg

of protein $\cdot\text{ml}^{-1}$) were suspended in DEPC buffer and incubated with freshly diluted DEPC for 10 min at room temperature (20°C). DEPC was subsequently removed from both whole cells and the membrane fraction by centrifugation as described above. A DEPC-free cytosolic fraction was obtained after passage through a Sephadex G-50 gel-filtration column. Control cells and membrane and cytosolic fractions were treated in an identical manner but in the absence of DEPC.

For the DEPC inhibition of active oxidase, cytoplasts were suspended at 1×10^7 cells $\cdot\text{ml}^{-1}$ and neutrophils at 1×10^6 cells $\cdot\text{ml}^{-1}$ in DEPC buffer, at 37°C , in the presence of 100 nM PMA. The DEPC was rapidly removed by centrifugation prior to assay.

Measurement of intracellular pH

Cytoplasts were loaded with the pH-sensitive fluorescent indicator BCECF-AM by incubation with 5 μM BCECF-AM in Na^+ buffer for 10 min at 37°C . An inward pH gradient (i.e. the external pH, pH_o , is less than the internal pH, pH_i , so that H^+ ions flow inwards) was imposed on cytoplasts suspended in a Na^+ buffer at 37°C by the addition of valinomycin (2.7 μM), to hyperpolarize the membrane potential, and 10 mM Hepes ($\text{pH}_o = 6.7$). An outward pH gradient ($\text{pH}_o > \text{pH}_i$, so H^+ ions flow outwards) was imposed by suspending cells in 150 mM KCl/5 mM Hepes/5 mM glucose, pH 7.3 (K^+ buffer, with pH adjusted by Tris), at 37°C , following the addition of valinomycin to depolarize the membrane potential and 10 mM Tris ($\text{pH}_o = 8.6$). The opening of the proton channel, following the addition of AA (10 μM), was detected as the flux of H^+ ions, monitored as the change in fluorescence of BCECF, at an excitation wavelength of 490–455 nm as described previously [9,20]. The response of BCECF to changes in pH is such that an increase in pH_i causes a decrease in fluorescence, and a decrease in pH_i results in an increase in fluorescence. The protonophore CCCP equilibrates protons across membranes and was utilized to demonstrate the maximal change in BCECF fluorescence/ pH_i that could be achieved in each assay [20].

The response of BCECF was calibrated using additions of HCl to cytoplasts suspended in K^+ buffer, in the presence of 10 μM nigericin as described previously [12,21].

Iodonitrotetrazolium Violet (INT) reduction by the cell-free system

INT was prepared in PBS to give a stock solution of 322 μM and diluted to a final concentration of 53 μM in the assay. The cell-free system with composition as described above was incubated for 5 min at 22°C prior to assay for INT reduction. The SOD (40 $\mu\text{g} \cdot\text{ml}^{-1}$)-insensitive reduction of INT was recorded at 500 nm, following the addition of an NADPH-regenerating system. The reaction was halted by the addition of 10 μM DPI [22].

Absorbance spectroscopy

Difference spectra (reduced-minus-oxidized spectra, at 400–600 nm) were obtained using a Beckman DU 640 spectrophotometer from control and DEPC-treated neutrophil membrane fractions. The cytochrome b_{558} content was quantified using a molar absorption coefficient, $\epsilon_{558-540}$, of $21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [23].

Western blotting

The translocation of the two cytosolic oxidase components p47^{phox} and p67^{phox} to the membrane fraction upon activation of

the NADPH oxidase was examined by Western blot using peptide-specific polyclonal antibodies to p47^{phox}, p67^{phox} and p22^{phox} [24]. The level of p22^{phox} in the membrane was monitored as a control. The membrane fractions were isolated from unactivated neutrophils, neutrophils treated with 50 nM PMA and neutrophils pre-treated with DEPC and 50 nM PMA, as described above. The membranes (10 µg per track) were solubilized in sample buffer [20% (v/v) glycerol/4% (w/v) SDS/62.5 mM Tris/HCl, pH 6.8/10% (v/v) mercaptoethanol/4.5% (v/v) saturated Bromophenol Blue] and separated by electrophoresis on a discontinuous SDS/polyacrylamide gel (4% stacking gel, pH 6.8; 12% separating gel, pH 8.8) using 0.3% (w/v) Tris/1.44% (w/v) glycine/0.1% (w/v) SDS as electrophoresis buffer. The separated proteins in the polyacrylamide gel were transferred, by electrophoresis, to nitrocellulose membrane using cold 0.303% (w/v) Tris/1.44% (w/v) glycine/20% (v/v) methanol as transfer buffer. The membrane was rotated for 30 min in 137 mM NaCl/2.7 mM KCl/3.21 mM Na₂HPO₄/1.47 mM KH₂PO₄/0.01% (v/v) Tween 20/1% (w/v) non-fat dried milk (blocking solution) prior to probing with anti-oxidase-component polyclonal antibodies for 1 h in blocking solution. The presence of the primary antibody was detected following the addition of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1 h), using the Western-blotting ECL detection kit (Amersham) and photographic film [25].

RESULTS

Three histidine residues of gp91^{phox}, His-111, -115 and -119, have been shown, by mutagenesis, to be functionally important for the movement of protons through the NADPH oxidase-associated H⁺ channel [14]. This suggested that modification of these residues would result in inhibition of the H⁺ channel. However, to demonstrate that the H⁺ channel is a potential therapeutic target, it is necessary to demonstrate that residues within the H⁺ channel are accessible for modification by externally added reagents. We therefore investigated the ability of DEPC to inhibit proton flux through gp91^{phox} and the flow of electrons through the NADPH oxidase.

Inhibition of proton flux by DEPC

In a Na⁺ buffer, the decrease in pHi observed following the addition of AA was slower and smaller in cytoplasts pre-treated with 500 µM or 1 mM DEPC (Figures 1b and 1c) prior to assay, compared with non-DEPC-treated cytoplasts (Figure 1a), suggestive of a reduction of the influx of H⁺ ions in DEPC-treated cells. However in a K⁺ buffer, the efflux of H⁺ ions was observed to be faster and the overall response larger in cells treated with either concentration of DEPC (Figures 1e and 1f) compared with control untreated cytoplasts (Figure 1d). No inhibition of this H⁺ efflux was observed, even at higher concentrations of DEPC (results not shown). That Zn²⁺ ions inhibited this observed H⁺ efflux (results not shown) demonstrates that the NADPH oxidase-associated H⁺ channel carries the proton efflux. These conflicting observations (Figure 1) would at first seem to indicate that DEPC inhibited the influx of protons through the H⁺ channel, but had no effect on or even enhances the efflux of protons from the cells. However, the assay for transmembrane proton movement relies on the establishment of a pH gradient across the plasma membrane by rapidly altering the pH of the external solution [20]. The initial pHi of the cytoplasts treated with 1 mM DEPC (Figures 1c and 1f) is much more acidic than that observed in control cytoplasts (Figures 1a and 1d). Cytoplasts

Figure 1 AA-activated H⁺ channel in cytoplasts pre-treated with DEPC

The flux of H⁺ ions through the NADPH oxidase-associated H⁺ channel was determined in cytoplasts pre-treated with DEPC for 10 min and loaded with the pH-sensitive fluorescent probe BCECF, as described in the Materials and methods section. The flux of H⁺ ions was monitored in cytoplasts treated in the absence of DEPC (a, d) or pre-treated with either 500 µM (b, e) or 1 mM (c, f) DEPC. Following the addition of 10 µM AA, an influx of H⁺ ions was recorded for cytoplasts suspended in a Na⁺ buffer, pH_o = 6.7 (a–c), or an efflux from those suspended in a K⁺ buffer, pH_o = 8.3 (d–f), both in the presence of 2.7 µM valinomycin. The protonophore CCCP was added at a final concentration of 66 µM as indicated. All measurements were performed at 37 °C in a continuously stirred cuvette. The data are representative of those obtained from three preparations of neutrophil-derived cytoplasts.

treated with DEPC had an acidic pHi compared with the controls, and were therefore exposed to a smaller pH gradient across their membranes following the addition of Hepes (pH_o = 6.7, Figures 1a–1c), and a larger gradient following the addition of Tris (pH_o = 8.6, Figures 1d–1f). Therefore the apparent inhibition of H⁺ influx (Figures 1b and 1c) by DEPC observed above is due to a reduction in the pH gradient driving H⁺ movement and is not the result of a direct interaction with the proton channel.

Inhibition of proton flux in activated NADPH oxidase

Amino acid residues believed to line the pore of the K⁺ channel have recently been reported to be exposed for chemical modification, and hence inhibition of the channel, only when the channel is in the open or active state [26]. The failure, described

Figure 2 DEPC inhibition of H⁺ efflux in activated NADPH oxidase

Cytoplasts, suspended in a K⁺ buffer, incubated with either 250 μ M DEPC (a) or 250 μ M DEPC + 50 nM PMA (b) to open the H⁺ channel. The efflux of H⁺ ions was recorded as described in the Materials and methods section. CCCP was added at a final concentration of 66 μ M where indicated. The data are representative of those obtained from three preparations of neutrophil-derived cytoplasts.

above, to observe inhibition of the H⁺ channel by DEPC may be accounted for by the histidines and/or other target residues being buried and inaccessible in the closed channel. The H⁺ channel has been reported to be opened by the addition of either arachidonate [20] or PMA [27,28]. To investigate the ability of DEPC to inhibit the channel in the open state, cytoplasts were treated simultaneously with DEPC and PMA. As activation of superoxide generation by PMA has previously been reported to result in an initial decrease in pH_i (by 0.1 pH unit) [12,29–31] and a lower pH_i was observed in cytoplasts treated with DEPC, we examined the effect of DEPC upon the AA-stimulated efflux of H⁺ ions only. This would minimize false positives arising from an acidic starting pH_i (Figure 1) as it favours rather than inhibits H⁺ efflux.

The addition of AA to cytoplasts treated with 250 μ M DEPC + PMA failed to initiate an efflux of H⁺ ions (Figure 2b), which was observed in cytoplasts treated with 250 μ M DEPC alone (Figure 2a). That this arises from an inhibition of the H⁺ pathway and not from differences in the pH gradient or the response of BCECF is demonstrated by observation of the full and expected pH_i change upon addition of the protonophore CCCP (Figure 2b). Therefore DEPC inhibits the NADPH oxidase-associated H⁺ channel but only when added in the presence of an activator of the channel. The residue(s) whose modification results in the inhibition of H⁺ efflux are exposed to the DEPC only in the open channel.

Reduction of superoxide generation in DEPC-treated cytoplasts

PMA not only activates the H⁺ channel but is also a good activator of superoxide generation by the NADPH oxidase. Therefore we examined the ability of DEPC to inhibit superoxide generation. The high rate of superoxide generation observed in Figure 3(a) confirms that the pre-treatment of cytoplasts with 50 nM PMA, $pH_o = 6.0$, utilized above (Figure 2), does activate the NADPH oxidase. The simultaneous treatment of cytoplasts

Figure 3 Inhibition of superoxide generation by DEPC

Cytoplasts, suspended in Na⁺ buffer, were incubated simultaneously with 50 nM PMA and either buffer (a) or 200 μ M DEPC (b), or pre-treated with either buffer (c) or 200 μ M DEPC (d) prior to the addition of 50 nM PMA. The generation of superoxide was monitored at 37 °C as the DPI (10 μ M)-sensitive reduction of cytochrome c (550–540 nm), as described in the Materials and methods section. The data are representative of at least five determinations.

with PMA and DEPC resulted in an inhibition of superoxide generation (Figures 3a and 3b). The addition of 26 mM sodium acetate to cytoplasts reduces pH_i by 0.37–0.4 pH units [14], and acid loading of cells in the presence of nigericin has been shown to reduce pH_i to as low as 6.3 [30]. The rate of superoxide generation activated by 50 nM PMA was unaffected by either sodium acetate- or nigericin-induced acidosis of pH_i (results not shown). Therefore the fall in pH_i , following DEPC treatment and PMA activation, does not by itself account for the inhibition of superoxide generation observed (Figure 3).

Figure 4 pH dependence of superoxide inhibition by DEPC

Cytoplasts were resuspended in DEPC buffer at pH 6.0, 7.0, 8.0 and 9.0 and pre-treated with either 400 μ M DEPC (solid line) or buffer (dotted line) for 10 min prior to assay for PMA (50 nM)-stimulated superoxide generation. The maximum rate of DPI-sensitive reduction of cytochrome *c* was plotted versus the DEPC buffer pH. The values plotted are the means of those obtained from two separate preparations of cytoplasts.

The stimulation by PMA of superoxide generation by the NADPH oxidase was inhibited in cytoplasts pre-treated with DEPC (Figures 3c and 3d) prior to the addition of PMA. Under these conditions DEPC failed to inhibit the proton channel (Figure 2a). The maximum rate of superoxide generation following the addition of PMA to control cells, $pH_o = 6$ (18.32 nmol $\cdot$ min⁻¹), is comparable with that for cells suspended in Na⁺ buffer, pH 7.4 (18.58 nmol $\cdot$ min⁻¹). Therefore pre-exposure of the cells to buffer at pH 6 alone does not inhibit the ability of PMA to activate superoxide generation. The ability of DEPC to inhibit superoxide generation both prior to the addition of PMA (non-active oxidase) and when added simultaneously with PMA (active oxidase), but to only inhibit the H⁺ channel when added simultaneously with PMA (active oxidase), indicates that there are at least two types of site within the NADPH oxidase that interact with DEPC. One type of site results in inhibition of the H⁺ channel and is accessible to DEPC only in the open channel. Another type of site results in the inhibition of superoxide generation and is accessible to DEPC in the non-activated enzyme complex. Inhibition of superoxide in the activated oxidase could arise from interaction of DEPC with either or both types of site.

The pH_o dependence of DEPC's interaction with NADPH oxidase

DEPC has been shown to chemically modify a number of amino acid residues, His, Cys, Arg and Tyr [15]. To determine whether the DEPC inhibition of unactivated NADPH oxidase showed pH dependence, cytoplasts were pre-treated with 400 μ M DEPC while suspended in buffer at pH 9.0, 8.0, 7.0 and 6.0. As shown in Figure 4, DEPC pre-treatment inhibited PMA-stimulated superoxide generation in cytoplasts in a pH-dependent manner. Maximum inhibition occurs in cells pre-treated with DEPC in buffer at pH 6.0 and 7.0 (pH_o). The rate of superoxide production observed following addition of PMA increased with increasing pH of the pre-treatment buffer, until at pH 9.0 DEPC failed to inhibit superoxide generation. Control cells pre-treated by exposure to buffer of pH 6.0–9.0 in the absence of DEPC showed

Figure 5 Assembly of NADPH oxidase following DEPC pre-treatment

The translocation of the cytosolic oxidase components p47^{phox} and p67^{phox} to the membrane was monitored in the membrane fraction isolated from whole cells treated with buffer control (lanes i), 50 nM PMA (lanes ii) and 500 μ M DEPC + 50 nM PMA (lanes iii) using specific anti-peptide antibodies. The membrane fraction was isolated, separated by 12% discontinuous SDS/PAGE and transferred to nitrocellulose as described in the Materials and methods section. The Western blots were probed with anti-p22^{phox} (a), anti-p67^{phox} (b) and anti-p47^{phox} (c). The data are representative of three separate Western blots.

no inhibition of the subsequent PMA-stimulated superoxide generation (Figure 4).

The effect of DEPC on the assembly of NADPH oxidase

The generation of superoxide requires translocation of the cytosolic oxidase components p47^{phox} and p67^{phox} to the cytochrome *b₅₅₈* in the membrane. Flux of H⁺ ions through the H⁺ channel does not require the assembly of the active oxidase complex [9]. The inhibition of superoxide generation in Figures 3(c) and 3(d) could result from either a direct interaction of DEPC with one or more components of the NADPH oxidase or alternatively with protein(s) involved in the activation pathway between PMA and the NADPH oxidase e.g. protein kinase C. To investigate the possibility that DEPC inhibits superoxide via inhibition of the assembly of the oxidase, the ability of p47^{phox} and p67^{phox} to translocate to the membrane was examined. No or little p67^{phox} or p47^{phox} was associated with the membrane fraction isolated from unactivated cells (Figures 5b and 5c, lanes i). An increase in the level of both p47^{phox} and p67^{phox} (Figures 5b and 5c, lanes ii) was observed in membranes isolated from neutrophils treated with 50 nM PMA. The levels of p47^{phox} and p67^{phox} associated with the membranes isolated from cells treated with DEPC before PMA (Figures 5b and 5c, lanes iii) was comparable with that observed in membranes isolated from neutrophils treated with PMA alone (Figures 5b and 5c, lanes ii). The level of p22^{phox} at the membrane, in keeping with it being an integral membrane protein, was similar in both unactivated and PMA-stimulated (with or without DEPC) membranes (Figure 5a). Therefore, translocation of the cytosolic oxidase components p47^{phox} and p67^{phox} to the membrane upon activation is unaltered by DEPC.

DEPC inhibition of the cell-free system

DEPC has been shown to inhibit PMA-stimulated superoxide production while having no obvious inhibitory effect upon the assembly of the oxidase. The activity of the oxidase can be stimulated in a cell-free system consisting of membrane and cytosolic fractions. To investigate whether the second type of interaction site with DEPC is within the NADPH oxidase, the

Figure 6 DEPC inhibition of membrane and cytosolic fractions of a cell-free system

The cell-free system was prepared as described in the Materials and methods section, from control neutrophils (a) and neutrophils pre-treated with 250 μ M DEPC (b). The generation of superoxide by the cell-free system was monitored as the DPI-sensitive reduction of cytochrome *c* following the addition of a NADPH-regenerating system (NR). (c) Rate of superoxide production by DEPC-pre-treated cytosol + untreated membranes (dashed line) or untreated cytosol + DEPC-pre-treated membranes (solid line) were plotted versus DEPC concentration. The data points are the means and the bars represent the maximum and minimum values obtained from three or more cell-free preparations for each concentration of DEPC.

ability of DEPC pre-treatment to inhibit superoxide generation in the cell-free system was examined.

The cell-free system prepared from human neutrophils that had been pre-treated with 250 μ M DEPC prior to fractionation and isolation had a much reduced level of NADPH-dependent superoxide generation (Figure 6b) when compared with a cell-free system prepared from untreated neutrophils (Figure 6a). Therefore, DEPC can modify one or more of the components of the NADPH oxidase, which results in inhibition of superoxide generation by the assembled NADPH oxidase.

To determine whether the DEPC inhibition of superoxide generation in the cell-free system was due to modification of the membrane, the cytosol or both fractions, the membrane and cytosolic fractions were treated individually with increasing

concentrations DEPC. The DEPC-treated membrane fraction was assayed for ability to generate superoxide in combination with untreated cytosol, and vice versa. As can be seen in Figure 6(c), the DEPC-pre-treated membrane fraction was unable to support superoxide generation when combined with control cytosol, in a manner dependent upon the DEPC concentration. At a concentration of 100 μ M DEPC the function of the membrane fraction in the cell-free system was inhibited by 80 %, but the cytosol was inhibited by 15 %. The membrane fraction was inhibited 90 % by 200 μ M, and the cytosol by 25 %. The control membrane and cytosolic fractions, treated in the absence of DEPC, showed no reduction in superoxide generation (results not shown). We conclude that the major site of interaction with DEPC that results in inhibition of superoxide generation by the

Figure 7 DEPC inhibition of the SOD-insensitive reduction of INT by the cell-free system

The reduction of INT (diaphorase activity) by the cell-free system was monitored (500 nm) in the presence of 50 ng · ml⁻¹ SOD, following the addition of the NADPH-regenerating system (NR). (a) Control untreated membrane fraction + control cytosol. (b) Membrane fraction pre-treated with 250 μM DEPC + control cytosol. (c) Maximum rate of INT reduction in DEPC-pre-treated membrane fraction (solid line) and maximum rate of cytochrome *c* reduction (dotted line) versus DEPC concentration. The values shown are means obtained from three or more assays.

cell-free system is contained in the membrane fraction. Therefore, either or both of the NADPH oxidase transmembrane components, gp91^{phox} and p22^{phox}, could be modified by DEPC.

The effect of DEPC on the INT reduction of NADPH oxidase

INT is a colourless dye that changes to violet upon reduction. The reduction of INT by the NADPH oxidase is reported to be due to a direct transfer of electrons from NADPH oxidase to the dye [22], and not through interaction with the superoxide, as is the case with cytochrome *c*, or in the reaction of hydrogen peroxide with Dihydrorhodamine 123 [32]. Hence the reduction of INT by the NADPH oxidase is insensitive to SOD but is still inhibited by DPI. INT reduction by the NADPH oxidase only occurs in the cell-free system and not in whole cells. It has

therefore been proposed that the transfer of electrons from NADPH to INT occurs on the inside of the neutrophil membrane [22]. The increase in INT reduction under anaerobic conditions has been proposed to be due to competition for electrons between INT and oxygen, although not necessarily at the same site [22]. As DEPC inhibited superoxide generation by the cell-free system we examined its ability to inhibit INT reduction in order to investigate further the location of the possible DEPC interaction site.

The SOD-insensitive reduction of INT catalysed by a cell-free system comprising DEPC-pre-treated membranes and control cytosol was reduced (Figure 7b) when compared with that of control membranes (Figure 7a). The concentration-dependence of DEPC inhibition of INT reduction (Figure 7c) and of the cell-free system (results not shown) were similar to that seen for inhibition of superoxide generation (cytochrome *c*) in DEPC-

Figure 8 Reduced-minus-oxidized cytochrome b_{558} difference spectra from control and 250 μM DEPC-pre-treated membranes

The dithionite reduced-minus-oxidized absorption spectra (400–600 nm) were recorded from buffer-treated (a) and 250 μM DEPC-pre-treated (b) membrane fractions as described in the Materials and methods section. (c) Cytochrome b_{558} content (pmol·mg of protein⁻¹; 558–540 nm) was plotted versus concentration of DEPC. The scans are representative of data obtained from three neutrophil membrane preparations.

pre-treated whole cells (Figure 3d). DEPC inhibition of INT reduction could result from either an inhibition of NADPH oxidation, or the movement of electrons through the oxidase.

DEPC and the haem group of cytochrome b_{558}

The membrane components of the NADPH oxidase, gp91^{phox} and p22^{phox}, comprise cytochrome b_{558} , which contains the two haem groups [3]. As neutrophils are highly differentiated and possess little or no endoplasmic reticulum and mitochondria, they contain very few cytochromes [33]. The presence of the haem of cytochrome b_{558} can be detected by obtaining a reduced-minus-oxidized difference spectrum of neutrophil membranes. The difference spectrum (400–600 nm) from the control untreated membrane fraction from the cell-free system contains peaks at 426 nm and 558 nm (α -peak) and a much smaller one at 527 nm (Figure 8a) [34], indicative of the presence of cytochrome b_{558} . These peaks were not observed in the membrane fraction, which had been pre-treated with 250 μM DEPC (Figure 8b). The plot of α -peak height ($A_{558} - A_{540}$) versus DEPC concentration (Figure 8c) illustrates that the extent of the loss of cytochrome b_{558} spectrum is concentration-dependent. The concentrations of DEPC required to inhibit the cell-free system (Figure 6c) are comparable with those that result in the loss of the cytochrome b_{558} spectrum. We therefore suggest that the loss of the haem groups is the non-H⁺-channel-type of site in the NADPH oxidase whose modification by DEPC results in the inhibition of superoxide generation.

From the experiments outlined above it can be concluded that DEPC inhibits H⁺ flux through the NADPH oxidase-associated H⁺ channel and that this site is only accessible to DEPC in the open channel. DEPC inhibits the generation of superoxide by whole-cell and cell-free systems but not the translocation of the cytosolic components of the NADPH oxidase to the membrane. As the inhibition of superoxide generation by DEPC is independent of the presence of the oxidase activator, PMA, there are therefore at least two types of DEPC-interactive site within the NADPH oxidase.

DISCUSSION

In this article we have reported that DEPC only inhibited the flux of H⁺ ions through the AA-activated, NADPH oxidase-associated H⁺ channel when added simultaneously with an activator of the channel. This suggests that this type of DEPC-interaction site is buried/inaccessible and becomes exposed upon opening of the channel. As gp91^{phox} has been proposed to form the H⁺ channel [9], it can be concluded that, upon activation of the NADPH oxidase, gp91^{phox} must undergo a change in conformation which permits the flux of H⁺ ions through the protein. DEPC has been reported previously to inhibit the influx of H⁺ ions, but not their efflux or the activity of NADPH oxidase, measured as electron current, in eosinophils [35]. In human neutrophil-derived cytoplasts we observed an apparent reduction in H⁺ influx following DEPC treatment, but this was found to be the consequence of a decrease in pH_i and hence a severe reduction in the proton-motive force driving H⁺ ions inward. The disparity in observed effect of DEPC between these two cell types requires further investigation.

DEPC also inhibited the ability of the NADPH oxidase to generate superoxide in whole cells and in a cell-free system. Unlike the H⁺ channel, superoxide generation was inhibited by DEPC whether it was added prior to or simultaneously with PMA. The absence of the characteristic cytochrome b_{558} haem spectrum from DEPC-treated neutrophil membranes suggests that the inhibition of superoxide may result from a loss or alteration in the environment of one or both haems. The loss of the reduced-minus-oxidized difference spectrum obtained from

horseradish peroxidase has been reported following treatment of the enzyme with 60 mM DEPC [36]. The loss of the difference spectrum of cytochrome *b*₅₅₈ from neutrophil membranes following treatment with iodonium biphenyl has also been reported previously [37]; iodonium biphenyl inhibited superoxide generation by binding directly to, and altering, the structure of the porphyrin ring of the haem groups on cytochrome *b*₅₅₈ [37]. The current data do not distinguish between the loss of spectrum resulting from either an alteration in the haem structure or a loss of the haem moiety from the protein.

The reduction of INT by the NADPH oxidase has been reported recently to occur as a result of the passage of electrons from the haems of cytochrome *b*₅₅₈ directly to INT [38]. The observed loss of or alteration to the haem(s) of the oxidase reported here therefore also explains the ability of DEPC to inhibit INT reduction.

The H⁺ flux through the NADPH oxidase-associated H⁺ channel is only inhibited in the presence of an activator, whereas superoxide generation is inhibited in the presence and absence of the activator. Therefore it can be concluded that DEPC inhibits the oxidase through modification of at least two types of site within the enzyme complex. The amino acid(s) modified by DEPC resulting in loss of the haem spectrum differ from the residue(s) required to conduct H⁺ ions across the membrane. The inhibition of superoxide by DEPC when both inactivated and when added simultaneously with the activator suggests that this site is continuously exposed. However, the activity of the NADPH oxidase is tightly coupled to the efflux through the H⁺ channel such that Zn²⁺ inhibition of the channel inhibits the generation of superoxide [39]. The possibility that, upon activation, the previously exposed site (superoxide-inhibition site) is buried/made inaccessible to DEPC and that the subsequent inhibition of the oxidase arises due to the inhibition of the H⁺ channel cannot be ruled out by the current data.

The application of site-directed mutagenesis has provided additional supporting evidence for the importance of histidine in the biological functioning of proteins. We have previously shown an absence of the AA-activated H⁺ flux in Chinese hamster ovary cells expressing N-terminal gp91^{phox} in which His-115 was mutated to either leucine or lysine and in which His-111, -115 and -119 were all mutated to leucine [14]. Therefore these histidine residues are important in the conduction of H⁺ ions through the channel. The combination of this previous mutation data with the ability of DEPC to inhibit H⁺ efflux could be taken to suggest that one or more of these histidine residues forms the site modified by DEPC, resulting in inhibition of the H⁺ channel. However, conformation of this will require further investigation. The inhibition of the H⁺ channel suggests that there are functionally important amino acids that are accessible to externally added reagents and which could therefore provide targets for potential therapeutic drugs.

The characteristics of the Raman spectra and low-temperature absorption spectra obtained from neutrophil membranes are consistent with histidine residues acting as the fifth and sixth co-ordinating positions of the Fe²⁺ of the haem. Yeast iron reductase, FRE1, contains two non-identical haems and shares sequence homology with gp91^{phox}. The mutagenesis of one of four histidine residues of FRE1 resulted in the total loss of the haem spectrum and associated loss of enzyme activity [40]. Therefore it was proposed that histidines at amino acid positions 294, 308, 364 and 378 act as the axial co-ordinating ligands for the two haem moieties of FRE1. The loss of the characteristic haem spectrum in DEPC-treated neutrophil membranes may result from modification of one or more of the axial histidine residues. The identification of the amino acid(s) associated with

the haem that can be modified by DEPC requires further investigation.

Inhibition by micromolar or millimolar concentrations of Zn²⁺ or Cd²⁺ ions is one of the characteristic features of H⁺ conductances in both phagocytic-like and non-phagocytic cells [11,12,41–46]. It has been demonstrated that Zn²⁺ inhibition of neutrophil H⁺ conductance shows strong pH dependence in the pH range 5.0–8.0. Therefore it was concluded that histidine residue(s) form the external Zn²⁺ inhibitory binding site in the neutrophil H⁺ channel [47].

Here we have demonstrated the inhibition of both superoxide generation and the NADPH oxidase-associated H⁺ channel by the histidine-modifying agent DEPC, and that there are at least two types of interaction site within the membrane components of the NADPH oxidase. The previously reported roles for histidine residues in both H⁺ efflux and as co-axial residues for the haems suggest candidate sites for modification by DEPC, which require further investigation. Compounds targeted to either or both sites could have therapeutic benefits.

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