The NADPH-oxidase-associated H^+ channel is opened by arachidonate

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The H^+ channel associated with the generation of $O_2^{\cdot-}$ by NADPH oxidase and the oxidase itself must both be activated in response to stimuli (e.g. phorbol esters, chemotactic peptides, certain fatty acids). We have investigated the effects of membrane potential, an imposed pH gradient and a combination of the two (the protonmotive force) on the H^+ conductivity of the cytoplasmic membrane. H^+ conductivity was observed only in the presence of arachidonate and not in its absence. In the presence of arachidonate, H^+ movement was determined by the protonmotive force. The effect of arachidonate was probably on a channel, since this fatty acid did not significantly increase the H^+ permeability of artificial phospholipid membranes. It appears, therefore, that arachidonate is required both for the activation of $O_2^{\cdot-}$ production and the associated H^+ -channel-mediated efflux.

INTRODUCTION

NADPH oxidase, which is located in the plasma membrane of neutrophils, is a major contributor to the bactericidal capacity of these cells. It catalyses the single-electron reduction of oxygen to superoxide ($O_2^{\cdot-}$) using electrons donated by NADPH. The activity of this enzyme is normally low, but it can be activated by the addition of a number of physiological and artificial stimuli, e.g. opsonized bacteria, complement factors, phorbol esters and arachidonic acid [1,2]. It has been shown previously that a sustained depolarization of the membrane potential is associated with the stimulation of $O_2^{\cdot-}$ generation. We have shown that this depolarization is due to the electrogenic activity of the oxidase and that an efflux of H^+ ions through a Zn^{2+} - and Cd^{2+} -inhibitable channel provides the necessary charge compensation [3,4].

If H^+ ions were capable of distributing freely across the plasma membrane of non-stimulated neutrophils, in accordance with the membrane potential (-60 mV), then the internal pH (pH_i) would be 1 pH unit lower than that of the surrounding medium (pH_o). However, the pH_i has been reported by us [4] and other authors [5–7] to be only 0.1–0.2 pH units lower than pH_o . Therefore, as with most eukaryotic cells [8], the plasma membrane of non-stimulated neutrophils is relatively impermeable to H^+ ions, i.e. the H^+ channel is closed. It follows that the stimulation of $O_2^{\cdot-}$ generation must be associated with an opening of the H^+ channel and an associated increase in the H^+ permeability of the plasma membrane, i.e. the channel must be gated. The depolarization of the membrane potential associated with the initiation of NADPH oxidase activity suggests that the efflux of H^+ ions initially lags behind the generation of $O_2^{\cdot-}$ [3]. It is therefore possible that the H^+ channel is voltage-gated and that the depolarization is required to initiate H^+ efflux. We and other authors have previously shown that both neutrophils and cytoplasts exhibit a slight fall in pH_i following the activation of NADPH oxidase [4–7]. Therefore it is also possible that the H^+ channel is gated by ΔpH or by a combination of both membrane potential and ΔpH , i.e. the protonmotive force.

The nature of this control process has been investigated. It appears that voltage gating, variation of pH or a combination of the two are not important in controlling the H^+ channel, but that arachidonate is the vital factor.

MATERIALS AND METHODS

Chemicals

Oxonol V and 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxy-fluorescein acetoxymethyl ester (BCECF-AM) were obtained from Molecular Probes (Eugene, OR, U.S.A.), and stock solutions of 2.6 mM and 1 mM respectively were prepared in dimethyl sulphoxide. Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) and arachidonate (Na^+ salt) were obtained from Sigma (Poole, Dorset, U.K.). Stock solutions of 400 μM , 25 mM and 1 mM were prepared in ethanol (100%, 100% and 50% respectively). The pH of the CCCP solution was adjusted to 7.4.

Preparation of neutrophils and cytoplasts

Neutrophils and cytoplasts were prepared from human buffy coats, obtained from the South West Regional Blood Transfusion Centre (Southmead Hospital, Bristol, U.K.), as previously described [3,9]. The cytoplasts were routinely resuspended in 150 mM-NaCl/1 mM-KCl/1 mM-Hepes/5.5 mM-glucose, pH 7.3 (Na^+ medium).

Measurement and calibration of membrane potential and pH_i

The membrane potential of cytoplasts [$(1-2) \times 10^7$] was measured using the optical probe, Oxonol V, and a dual-wavelength spectrophotometer/fluorimeter. The cytoplasts were pre-equilibrated for 3 min at 37 °C in Na^+ medium containing 2 μM -Oxonol V. The changes in absorbance (640–600 nm) were monitored continuously in a stirred cuvette at 37 °C. The changes in absorbance were calibrated by addition of 0.5 M- K_2SO_4 in the presence of 2.7 μM -valinomycin [3].

Cytoplasts (5×10^8 /ml) were loaded with BCECF by incubating them in Na^+ medium containing 5 μM -BCECF-AM for 5 min at 37 °C. The cytoplasts were pelleted (800 g for 10 min) and resuspended in Na^+ medium. Internal pH changes were monitored continuously as the difference in fluorescence emitted using 490 and 455 nm excitation wavelengths (490–455 nm). The assays were performed using $(1-2) \times 10^7$ cytoplasts at 37 °C in a continuously stirred cuvette. The response of the indicator was calibrated as previously described using additions of HCl in the presence of 10 μM -nigericin [4].

The changes in membrane potential and pH_i were monitored

Abbreviations used: pH_i and pH_o , internal and external pH respectively; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; DPI, diphenyleneiodonium; ψ , membrane potential; Δp , protonmotive force.

Fig. 1. Changes in the H^+ permeability of the cytoplasmic membrane induced by changes in the ΔpH or $\Delta \psi$, and following the addition of arachidonic acid

The internal pH of a suspension of cytoplasts was monitored as described in the Materials and methods section. An efflux or influx of H^+ ions would be expected to be reflected in changes in the value of pH_i . (a) The external pH of the Na^+ medium was lowered rapidly from 7.3 to 6.6 by the addition of 10 mM-Hepes. A second addition of 10 mM-Hepes lowered the pH_o further to 6.3. This provided a large pH gradient favouring the influx of H^+ ions. Arachidonate was added at a final concentration of 8 μM . (b) The addition of 2.7 μM -valinomycin to cytoplasts suspended in a Na^+ medium facilitated the efflux of K^+ ions and hyperpolarizes the membrane potential. As in (a), the pH_o fell rapidly to 6.6 following the addition of 10 mM-Hepes. The efflux of K^+ ions should act as a counter-ion for the influx of H^+ ions. Arachidonate was added at a final concentration of 8 μM . (c) The membrane potential of cytoplasts suspended in a K^+ medium was depolarized only following the addition of 2.7 μM -

separately but on the same cytoplast preparation. All additions were made directly into the cuvette through an injection port.

Acid loading of the cytoplasts

The pH_i of cytoplasts suspended in Na^+ medium was lowered by the addition of 10 μM -nigericin as described by Grinstein *et al.* [10]. The acidification was stopped by the addition of 0.1 mg of BSA/ml. The cytoplasts were sedimented rapidly in an Eppendorf centrifuge and resuspended in 150 mM-KCl/1 mM-Hepes/5.5 mM-glucose/pH 7.3 (K^+ medium).

Preparation of liposomes

Phospholipids were prepared from hen egg yolks according to the method of Bangham *et al.* [11]. The liposomes were prepared by resuspending the phospholipids in K^+ medium containing 5 μM -BCECF. The suspension was sonicated using a Ultrasonics sonicator (4×15 s) and centrifuged at 100 000 g for 30 min. The liposomes were separated from the unincorporated BCECF by passing them down a Sephadex G-25 molecular exclusion column.

The changes in the pH_i of the liposomes were monitored as described above for the cytoplasts.

RESULTS AND DISCUSSION

Cytoplasts lack the nucleus, granules and internal organelles of their parent neutrophils, but have been shown previously to retain their functional characteristics, including a fully competent stimutable NADPH oxidase activity [3,8]. As cytoplasts are a single-membrane, single-compartment system, they have obvious advantages over neutrophils for use with optical probes.

Gating of the H^+ channel

As described previously, the stimulation of $O_2^{\cdot -}$ generation must be associated with an increase in the efflux of H^+ ions through a channel. The possible role of membrane potential ($\Delta \psi$), pH gradient (ΔpH) or the protonmotive force ($\Delta p = \Delta \psi + 60 \Delta pH$) in the gating of the channel was investigated where $O_2^{\cdot -}$ generation was suppressed by the addition of the diphenyleneiodonium (DPI) [12].

Fig. 1(a) shows the effect on pH_i (initially pH 7.2) of a sudden decrease of pH_o from 7.3 to 6.6 induced by the addition of 10 mM-Hepes. Even the further addition of 10 mM-Hepes, which decreased pH_o to 6.3, did not cause a major change in pH_i . At this point it was calculated that the Δp , composed of a pH gradient of 1.0 and a membrane potential of -60 mV (negative inside), would assume a value of -120 mV, tending to cause internal acidification. This observation implies either that the membrane is under these conditions impermeant to H^+ or that permeability of H^+ is limited by movement of a counter-ion. That the latter is not the case is shown by the experiment depicted in Fig. 1(b), in which 2.7 μM -valinomycin was added. Under these conditions $\Delta \psi = -80$ mV and $\Delta pH = 0.1$ (acid inside), giving a Δp of approx. -70 mV. So, despite the ready permeation of K^+ [13] and a favourable Δp , no significant internal acidification occurred. This was still the case when Δp was increased to approx. -110 mV by addition of 10 mM-Hepes. These experiments clearly indicate that the membrane is impermeant to H^+ .

In both the presence and the absence of valinomycin (Figs. 1a and 1b) the addition of 8 μM -arachidonate caused a rapid internal

valinomycin. The addition of 10 mM-Tris raised the pH_o from 7.3 to 8.3. This provided a large outward pH gradient and a path for electroneutral efflux of H^+ ions in the absence of a substantial membrane potential. Arachidonate was added at a final concentration of 8 μM .

Fig. 2. The value of Δp dictates the direction of the arachidonate-induced H^+ movement

The pH_i of cytoplasts is slightly lower than pH_o , favouring an efflux of H^+ ions. (a) The addition of valinomycin ($2.7 \mu M$) to cytoplasts suspended in a high- K^+ medium resulted in a depolarization of the membrane potential. An efflux of H^+ followed the addition of $8 \mu M$ -arachidonate. (b) However, in a Na^+ medium the membrane potential was hyperpolarized by addition of $2.7 \mu M$ -valinomycin. The addition of $8 \mu M$ -arachidonate induced an influx of H^+ .

Fig. 3. Proton efflux from acid-loaded cytoplasts

The internal pH_i of the cytoplasts was lowered by acid loading as described in the Materials and methods section. The addition of $2.7 \mu M$ -valinomycin resulted in a depolarization of the membrane potential. Significant efflux of H^+ occurred only following the addition of $8 \mu M$ -arachidonate.

acidification, as indicated by the negative value of Δp . Thus when valinomycin was added to cytoplasts suspended in an alkaline K^+ medium (pH_o 8.3, pH_i 7.2, $\Delta\psi$ approx. 0 mV, Δp +66 mV) there was a rapid internal alkalization only on addition of arachidonate (Fig. 1c).

That the membrane potential alone does not allow H^+ permeability was shown by the following experiment (Fig. 2). In a KCl medium (pH_o 7.3, pH_i 7.2, $\Delta\psi$ approx. 0 mV after the addition of $2.7 \mu M$ -valinomycin) no pH_i change occurred until $8 \mu M$ -arachidonate was added. Then there was a very rapid internal acidification (probably representing an internal accumulation of arachidonic acid) followed by a slow and small H^+ efflux. Acidification of the external medium lead to rapid internal

Fig. 4. Ion selectivity of arachidonate-induced permeability

The membrane potential of a cell responds to changes in the concentration gradient of ions to which it is significantly permeable. The membrane potential of cytoplasts suspended in a Na^+ medium was monitored as described in the Materials and methods section. As in Fig. 2(a) the pH_o rapidly fell following the addition of 10 mM-Hepes. The additions of valinomycin ($2.7 \mu M$) and arachidonic acid ($8 \mu M$) were made where indicated. The external concentration of K^+ ions was raised by additions of K_2SO_4 to give the final concentration at the places indicated.

acidification. In a NaCl medium (pH_o 7.3, pH_i 7.2, $\Delta\psi$ -80 mV after the addition of $2.7 \mu M$ -valinomycin), no change in pH_i occurred until arachidonate was added.

The experiments shown in Figs. 1(a), 1(b) and 1(c) establish that H^+ conduction occurs in the presence of arachidonate. Neither a membrane potential nor a pH gradient, nor a combination of the two, was adequate. Δp controls only the direction of flow, and it was possible that the absolute value of pH_i acts as a trigger. That this is not the case is shown by the experiment depicted in Fig. 3. The pH_i of cytoplasts was decreased to approx. 6.8 as described in the Materials and methods section, and they were then exposed to valinomycin in a KCl medium [Δp 0.4 (acid inside), $\Delta\psi$ approx. 0 mV, Δp +24 mV]. Outward efflux of H^+ occurred only on addition of arachidonate.

Ion selectivity of arachidonate-induced membrane permeability

The membrane potential is determined by the activity ratios of various ions in the system for which there is a conduction pathway, the magnitude of the potential being given by the Goldman equation [14]. If the permeability for any given ion is very much greater than that for any other, then this ion is the major determinant of the membrane potential. Fig. 4(a) illustrates this with regard to valinomycin, H^+ and K^+ ions. The NaCl medium in which the Oxonol-treated cytoplasts were suspended contained 1 mM-KCl. The addition of valinomycin led to a rapid fall in the membrane potential to -100 mV. Variations in pH_o had no significant effect on the potential, but there was a rapid Nernstian response to K^+ .

In a separate experiment (Fig. 4b) membrane potential was monitored in the same medium but in the presence of arachidonate. In this case changes in potential were induced by changes in pH_o , but not changes in K^+ . From this experiment it may be concluded that arachidonate induces H^+ permeability, but not K^+ permeability, or (from other experiments; results not shown) Na^+ or Cl^- permeability.

This effect of arachidonate in inducing H^+ permeability could be a specific effect on a channel or through arachidonate acting as a protonophore (i.e. like an uncoupling effect).

Fig. 5. K^+ - H^+ exchange in protein-free liposomes

The liposomes incorporated BCECF and high $[K^+]$ as described in the Materials and methods section. The changes in the liposome pH_i were recorded as for the BCECF-loaded cytoplasts. The uncoupler, CCCP (66 μM), valinomycin (2.7 μM) and arachidonic acid (8 μM) were added where indicated.

K^+ - H^+ exchange in liposomes

The ion selectivity of the response to arachidonate and the changes in pH_i reported above are no different from those which would be predicted if an uncoupler were to be substituted for arachidonate. Liposomes are a protein-free system in which the ion selectivities of ionophores have been previously investigated [13]. Fig. 5(a) depicts an experiment in which the effects of adding both valinomycin and an uncoupling agent on the pH_i of BCECF-loaded liposomes is shown. The addition of CCCP produced little effect, but the subsequent addition of valinomycin caused a marked acidification, because K^+ efflux is compensated by H^+ entry. Replacing CCCP by arachidonate (Fig. 5b) did not produce the same effect, and only a small acidification occurred. The subsequent addition of CCCP led to a full-blown response. From this experiment it may be deduced that arachidonate does not act as a protonophore in liposomes, and it may be inferred that this is also the case in cytoplasts. If this inference is correct then arachidonate must activate a H^+ channel.

Conclusions

We have previously presented evidence that $O_2^{\cdot -}$ generation in neutrophils involves an electrogenic transfer of an electron to O_2 from NADPH and a charge-compensating transfer of H^+ through a putative H^+ channel [3,4]. It follows that activation of $O_2^{\cdot -}$ generation by any means (phorbol esters, chemotactic peptides etc.) must also involve activation of the H^+ channel.

We have previously shown that inhibitors of phospholipase A_2 are good inhibitors of the phorbol 12-myristate 13-acetate-stimulated generation of $O_2^{\cdot -}$, and that activity was restored following the addition of arachidonate [15]. These observations suggest that phospholipase and arachidonate have a role in the activation of NADPH oxidase. Evidence is presented here that arachidonate is an activator of the H^+ channel, as follows. (1) Arachidonate allows H^+ movement across the cytoplasmic membrane in either direction in accordance with the existing thermodynamic gradient, the protonmotive force. (2) In the presence of arachidonate, the membrane potential of cytoplasts responds to ΔpH , but not to concentration gradients of other ions, e.g. K^+ , Na^+ or Cl^- . (3) Arachidonate, at least at the concentrations used, did not induce H^+ permeability in liposomes.

It would appear that a possible explanation for these findings is that arachidonate is an activator of the H^+ channel. In our hands arachidonate does not lead to detectable phosphorylation of any protein component [16], which would appear to rule out the possibility of arachidonate activation of protein kinase C, or indeed of any other protein kinase [17].

The stimulation of $O_2^{\cdot -}$ generation by a range of activators has been reported to be associated with an increase in the level of free arachidonate [18]. It is therefore possible that the above ability of arachidonate to gate the H^+ channel may have some physiological significance. However, at present it is not possible to rule out the possibility that its effects on the H^+ channel are mediated by a metabolite of arachidonate.

The ability of a number of other fatty acids to induce H^+ movement in cytoplasts was investigated. In a preliminary study the unsaturated fatty acids oleic, linolenic and linoleic acid could substitute for arachidonate, but were not as effective (results not shown). Interestingly, these unsaturated fatty acids can also stimulate NADPH oxidase [19]. The ability of the unsaturated fatty acids to activate both the flow of electrons to O_2 and the efflux of H^+ ions could be taken to suggest that both pathways are activated in a single event, and therefore that the H^+ channel is a component of the NADPH oxidase complex. However, as arachidonate is probably a mobile stimulus, the activation of the two pathways may consist of two separate but co-ordinated events. The question of the involvement of the H^+ channel in the oxidase complex remains to be resolved. The exact mechanism by which arachidonate stimulates both NADPH oxidase and the H^+ channel is unknown.

In this paper we have demonstrated that exogenous arachidonate can open the H^+ channel of intact cytoplasts, in the absence of superoxide generation. The role of internally generated arachidonate in the activation of both the channel and the oxidase requires further investigation. Using patch-clamp techniques it has been shown previously that arachidonate, some of its metabolites and other fatty acids can activate K^+ -selective channels in rat atrial cells, smooth muscle cells and hippocampal pyramidal neurons [20–22]. Therefore arachidonate may have a wider role in gating specific channels.

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