The arachidonate-activatable, NADPH oxidase-associated H⁺ channel is contained within the multi-membrane-spanning N-terminal region of gp91-*phox*

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The generation of superoxide by the NADPH oxidase of neutrophils is accompanied by the efflux of H⁺ ions through a H⁺ channel. gp91-*phox*, a protein component of the oxidase, has been shown previously to function as a H⁺ channel [Henderson, Banting and Chappell (1995) J. Biol. Chem. **270**, 5909–5916]. We have constructed a CHO cell line (CHO-N) that expresses an N-terminal fragment of gp91-*phox* containing the predicted multiple

transmembrane domains of the protein. These cells exhibit H⁺ fluxes in response to an imposed proton motive force and in the presence of arachidonate (to open the channel). The H⁺ fluxes were indistinguishable from those observed in cells expressing full-length gp91-*phox*. Therefore the N-terminal 230 amino acids of gp91-*phox* contain all that is required to function as the NADPH oxidase-associated H⁺ channel.

INTRODUCTION

Superoxide is released by neutrophils during phagocytosis of immune complexes and other bodies [1,2]. The phagocyte oxidase generates superoxide by an electrogenic process involving the liberation of protons, from the oxidation of NADPH, on the cytosolic surface of the plasma membrane, while oxygen is reduced to superoxide at the exterior. The associated efflux of H⁺ ions, through a selective channel, compensates for this charge separation and prevents what would otherwise be a substantial fall in intracellular pH (pH_i) [3,4]. As inhibitors of the H⁺ channel are inhibitors of the generation of superoxide [5], the activity of the channel is essential to maintain oxidase activity and hence the phagocyte's bactericidal properties. It has been shown previously that the channel is normally closed, but that it can be opened, *in vitro*, following the addition of arachidonate [6]. The ability of arachidonate to stimulate superoxide generation suggests the involvement of a mechanism for coordinating the initiation of electron flow through the oxidase with the efflux of H⁺ ions.

An inherited failure to generate superoxide renders individuals with chronic granulomatous disease (CGD) highly susceptible to infection [7]. Analysis of genetic lesions found in these patients facilitated the identification of the protein components of the phagocyte oxidase (*phox*), including gp91-*phox*, the product of the X-linked CGD gene. CGD cell lines from which gp91-*phox* is absent have been shown previously to be deficient in H⁺ ion flux [8]. The suggestion that gp91-*phox* functions as the H⁺ channel was further supported by the reported presence of an arachidonate-activatable H⁺ flux in Chinese hamster ovary (CHO) cell lines transfected with and expressing gp91-*phox*. This H⁺ flux was not present in control cell lines that did not express gp91-*phox*.

The hydropathy plot for gp91-*phox* suggests the existence of multiple (four to six) transmembrane domains at the N-terminus of the protein and a large hydrophilic C-terminal domain which,

as it contains the predicted FAD and NADPH binding sites, is assumed to be on the cytosolic side of the plasma membrane. As ion channels and transporters must, by definition, cross the membrane, we have investigated the ability of the N-terminal fragment, containing the predicted transmembrane domains of gp91-*phox*, to function as a H⁺ channel. We report that CHO cell lines transfected with and expressing this N-terminal fragment of gp91-*phox* exhibit an arachidonate-activatable H⁺ flux, further supporting a role for gp91-*phox* as the NADPH oxidase-associated H⁺ channel.

MATERIALS AND METHODS

Materials

2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.), and a stock solution of 1 mM was prepared in DMSO and dried by freeze-thawing. Valinomycin (0.4 mM in ethanol), carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) (8 mM and 0.8 mM in ethanol), arachidonate (Na⁺ salt) (1 mM in 50% ethanol) and nigericin (1.5 mM in methanol) were obtained from Sigma (Poole, Dorset, U.K.). The pH of the CCCP solution was adjusted to 7.4 with NaOH. The compositions of the salt solutions were as follows: 150 mM KCl, 5 mM Hepes/Tris and 5.5 mM glucose, pH 7.3 (K⁺ medium); and 150 mM NaCl, 1 mM KCl, 5 mM Hepes/Tris and 5.5 mM glucose, pH 7.3 (Na⁺ medium).

Generation of the N-terminal fragment of gp91-*phox*

The N-terminal fragment of gp91-*phox* (amino acids 1–230) was amplified by PCR using the synthetic oligonucleotides CTCA-GCTAAGCTTGCCACCATGGGGAAC and AGCTGACCG-CGGGCCACGTACAATTCG, and inserted as a *Hind*III/

Abbreviations used: BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; CHO cells, Chinese hamster ovary cells; CHO-N cells, CHO cells transfected with the N-terminal fragment of gp91-*phox*; CHO-91 cells, CHO cells transfected with full-length gp91-*phox*; CGD, chronic granulomatous disease; HA epitope, haemagglutinin epitope (YPYDVPDYAG); Δp, proton motive force; pH_i, intracellular pH.

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SacII fragment into the plasmid pCMUIV [9] to generate pCMUIVgp91N. pCMUIV contains a sequence encoding three tandem copies of the haemagglutinin (HA) epitope (YPYDVPDYAG). gp91NHA was excised as a *HindIII/BamHI* fragment from pCMUIVgp91NHA and inserted downstream from the metallothionein promoter in the multiple cloning site of the plasmid pMEP4 (Invitrogen).

Construction and maintenance of CHO cell lines

Stable CHO cell lines expressing the N-terminal fragment of gp91-*phox* (CHO-N) were established following transfection (electroporation) and selection (hygromycin B) procedures, as described previously [8,10]. CHO cell lines were maintained in culture in Ham's F-12 nutrient mixture (Gibco) with L-glutamine supplemented with 10% (v/v) fetal calf serum, 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco). The medium was replaced every 2–3 days and the cells were passaged once a week following trypsinization [8]. Under the control of the metallothionein promoter, the expression of the N-terminal fragment of gp91-*phox* was induced by exposure of the cells to 10 µM Cd²⁺ for 24 h, as described previously [8,10].

Immunocytochemical staining and imaging of CHO cell lines

CHO-N cells were fixed with paraformaldehyde (4%) and permeabilized (0.02% Triton X-100) prior to detection of the HA-tagged N-terminal fragment of gp91-*phox* with a monoclonal anti-HA antibody. The cells were imaged on a Bio-Rad MRC confocal scanning optical microscope following detection of bound primary antibody with an FITC-conjugated rabbit anti-mouse Ig (Sigma) secondary antibody [8].

Measurement of pH_i

The CHO cell lines were removed from the tissue-culture flasks by trypsinization and washed twice by centrifugation in PBS (800 g for 10 min). The cells were loaded with the pH_i indicator BCECF by incubating them for 5 min at 37 °C in Na⁺ medium containing 5 µM BCECF-AM. The cells were pelleted (800 g for 10 min) and resuspended in the appropriate assay medium.

Changes in pH_i were monitored continuously as the difference in fluorescence emitted using alternating excitation at 490 nm and 455 nm (490 minus 455 nm). The assays were performed at 37 °C in a continuously stirred cuvette. All additions were made directly into the cuvette through an injection port. An inward proton motive force ($\Delta p = \Delta \psi + (RT/F) \cdot \ln([H^+]_o/[H^+]_i)$), where R is the gas constant, T is temperature, F is the Faraday constant and $[H^+]_o$ and $[H^+]_i$ are the external and internal H⁺ concentrations respectively} was imposed on the cells in Na⁺ medium by the addition of valinomycin (2.7 µM) and Hepes (external pH 6.7). An outward-acting Δp was imposed on cells in K⁺ medium in the presence of valinomycin (2.7 µM) and Tris (external pH 8.2). Opening of the channel was measured following the addition of 8–12 µM arachidonate, as described previously [6]. The response of the indicator was calibrated as described previously using additions of HCl in the presence of 10 µM nigericin [4,11].

RESULTS AND DISCUSSION

From amino acid sequence similarities, it has been suggested that the membrane-spanning protein gp91-*phox* contains the binding sites for the oxidase substrate NADPH and the cofactor FAD. We have shown previously that it also functions as the oxidase-associated H⁺ channel [8]. A hydropathy plot of the gp91-*phox*

protein sequence (Figure 1a) indicated, as suggested previously by others [12], that the predicted transmembrane domains are located within the N-terminal 230 amino acids. The DNA sequence corresponding to the N-terminal fragment (blocked in Figure 1b) comprising residues 1–230 of gp91-*phox* was amplified by PCR and inserted in-frame with the nucleotide sequences encoding three copies of the HA epitope, downstream of the inducible metallothionein promoter, into the plasmid pMEP4. This generated a plasmid (pMEP4gp91NHA) encoding the putative transmembrane domains of gp91-*phox* with a C-terminal HA epitope tag to allow immunodetection. pMEP4gp91NHA was used to generate stable transfected CHO cells (CHO-N) as described in the Materials and methods section. The advantage of the vector pMEP4 is that it allows the regulated expression of inserted cDNAs by virtue of the fact that expression is driven by the CdCl₂-inducible human metallothionein IIa promoter. The N-terminal fragment of gp91-*phox* was expressed upon treatment of CHO-N cells with Cd²⁺, as demonstrated by the positive immunostaining visualized with an anti-HA antibody in conjunction with a confocal fluorescence microscope (Figure 2). This immunostaining was not present in CHO-N cells not treated with Cd²⁺ (Figure 2d) or in CHO cells (results not shown). As the confocal microscope excites and collects fluorescence from only within the plane of focus of the objective lens, the annular pattern of immunostaining suggests that the protein is located in or near the plasma membrane of the cells.

Arachidonate-activatable H⁺ fluxes

The pH indicator BCECF responds to a fall in pH with a decrease in fluorescence (490–455 nm), and to a rise in pH with an increase in fluorescence. In a high-K⁺ medium, on the addition of valinomycin (depolarization) and Tris (elevation of external pH) the cells are exposed to a Δp that will drive an efflux of H⁺ ions (alkalinization of pH_i), whereas in a high-Na⁺ medium the addition of Hepes (decrease in external pH) and valinomycin (hyperpolarization) generates a Δp that is capable of driving an influx of H⁺ ions. However, the predicted flux of H⁺ ions can only occur in the presence of a H⁺ conduction pathway, i.e. a functioning channel protein or a protonophore.

We have previously described an arachidonate-induced H⁺ flux in CHO cells transfected and induced to express full-length gp91-*phox* (CHO-91), and have proposed that this is due to the ability of gp91-*phox* to function as a H⁺ channel [8]. To determine if the transmembrane domains of gp91-*phox* alone can function as the H⁺ channel, we exposed CHO-N cells to Δp that favoured either the efflux (Figure 3a) or the influx (Figure 3e) of H⁺ ions, and observed the changes in pH_i following the addition of arachidonate. Under conditions of alkalinization in K⁺ medium (Figure 3a) and of acidosis in Na⁺ medium (Figure 3e), large and rapid changes in pH_i were observed following the addition of arachidonate to CHO-N cells induced to express the N-terminal fragment gp91-*phox*. We failed to detect such arachidonate-induced H⁺ fluxes in CHO-N cells that had not been induced to express the N-terminal fragment of gp91-*phox* (Figures 3b and 3f). These arachidonate-induced H⁺ fluxes were very similar to those observed in CHO-91 cells expressing full-length gp91-*phox* (Figure 3c), as reported previously [8]. This observed H⁺ flux was not due to the fact that induction of expression of the protein requires the cells to be exposed to Cd²⁺ (Figures 3g and 3h). In untransfected CHO cells only the pH_i change due to the addition of arachidonate was seen both in cells treated with and without 10 µM Cd²⁺, despite the imposition of a Δp . As the slight change in pH_i upon addition of arachidonate was observed in protein-free liposomes, Figures 3(g) and 3(h) indicate the absence of a H⁺

Figure 1 N-terminal fragment of gp91-*phox*

(a) The hydropathy plot of gp91-*phox* suggests the presence of between four and six transmembrane domains and a large hydrophilic C-terminal domain [12]. The predicted transmembrane domains are indicated by bars, and the proposed binding sites for FAD and NADPH are shown. (b) Diagrammatic representation of gp91-*phox*, showing the region containing the predicted transmembrane domains in (a) that was amplified by PCR (solid block).

Figure 2 Expression of the N-terminal fragment of gp91-*phox* in CHO cells

CHO-N cells were immunostained and imaged as described in the Materials and methods section. (a) CHO-N cells induced to express the N-terminal fragment ($\times 40$ objective); (b) enlarged 4-fold following acquisition; (c) enlarged 8.5-fold following acquisition; (d) non-induced CHO-N cells ($\times 40$ objective). The diameter of the cells in each case is 15–20 μm .

conduction pathway in CHO cells. The addition of the protonophore CCCP provided a H⁺ conduction pathway and resulted in the expected pH_i changes (Figures 3g and 3h). As the CHO-N cells induced to express the N-terminal fragment of gp91-*phox* exhibited arachidonate-activatable H⁺ conductance, we conclude that these N-terminal 230 amino acids, which include the predicted transmembrane domains of gp91-*phox*, contain all that is required for a fully functional H⁺ channel. Under the experimental conditions described here, the H⁺ flux through the N-terminal fragment of gp91-*phox* was

indistinguishable from that observed previously in CHO-91 cells expressing full-length gp91-*phox*.

Conclusions

We have previously presented evidence that gp91-*phox* functions as the NADPH oxidase-associated, arachidonate-activatable H⁺ channel, and that it does so independently of the other subunits of the oxidase. In the present paper we have demonstrated that all that is required for a functional H⁺ channel is within the N-

Figure 3 Arachidonate-induced pH_i changes in CHO-N, CHO-91 and CHO cells

The arachidonate-induced changes in pH_i were monitored in CHO-N cells (**a, b, e and f**), in CHO-91 cells (**c and d**) and in control untransfected CHO cells (**g and h**) that had either been treated for 24 h with $10 \mu\text{M}$ Cd^{2+} (**a, c, e and g**) or left untreated (**b, d, f and h**). Cells suspended in K^+ medium (**a–d**) were exposed to a Δp favouring the efflux of ions, whereas in Na^+ medium (**e–h**) the Δp favours the influx of H^+ ions. Tris (5 mM), Hepes (5 mM), valinomycin (Val; $2.7 \mu\text{M}$), arachidonate (AA; $8 \mu\text{M}$; $4 \mu\text{M}$ in **b**) and CCCP ($53 \mu\text{M}$) were added as indicated.

terminal 230 amino acids of gp91-*phox*, which contains the predicted transmembrane domains. Our conclusion that the N-terminal fragment of gp91-*phox* functions as the oxidase-associated H^+ channel does not conflict with the previously suggested role of gp91-*phox* as the flavocytochrome of the oxidase [13]. The predicted binding sites for FAD and the enzyme substrate NADPH are contained within the cytosolic C-terminal hydrophilic domain [13], which was excluded from our N-terminal fragment construct. The location of the haem moiety in the complex remains uncertain, although the involvement of the α -subunit of cytochrome *b* (p22-*phox*) has been suggested [8,14]. Moreover, it could be suggested that close physical association of the pathways for the efflux of electrons to oxygen (superoxide generation) and for the efflux of H^+ ions (charge compensation) would greatly facilitate the regulation and co-ordination of the pathways and hence the activation and functioning of the NADPH oxidase.

A recent paper by Doussiere et al. [15] reported a shift in the co-ordination of the haem moiety of the oxidase from a low-spin hexaco-ordinated state to a high-spin pentaco-ordinated state upon the addition of arachidonate. This shift in the haem co-ordination may reflect the change in the oxidase that is necessary to convert it from an inactive into an active enzyme, capable of binding oxygen. This demonstration of an arachidonate-triggered conformational change in the cytochrome may also reflect the process(es) involved in the arachidonate gating of the H^+ channel.

Arachidonate and other unsaturated fatty acids are reported to increase the fluidity of membranes. It is at present not possible to determine whether these effects of arachidonate are due to a direct interaction with the protein or to an indirect effect of an increase in membrane fluidity facilitating a conformational change.

Electrophysiological studies, by a number of groups, have demonstrated the presence of H^+ conductances in snail neurons [16,17], muscle cells [18], oocytes [19], epithelial cells [20,21], phagocytes and phagocyte cell lines [22–24]. The high selectivity reported for these H^+ conductances may be a consequence of the presence of specific amino acids within and/or at the mouth of the pore. Alternatively, it may be the result of a narrow pore diameter that restricts the entry of large ions, as has been suggested previously for the ion-conducting peptides [25]. Since, with the exception of the phagocytes, the proteins responsible for these conduction pathways are unknown, a comparison of amino acid sequences cannot be made. A number of proteins translocate H^+ ions across membranes as a consequence of their function, e.g. viral coat proteins, NADH:NADPH transhydrogenase and cytochrome oxidase. Comparison of the amino acid sequences of these proteins with that of gp91-*phox* may help to identify the functionally important amino acids.

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