Perspective

Proton Conduction through gp91[phox]

LYDIA M HENDERSON[1] and ROBERT W. MEECH[2]

[1]Departments of Biochemistry and [2]Physiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, United Kingdom

Introduction

Previous Perspectives in the *Journal of General Physiology* have considered (a) the different advantages of two models of ion channel permeation and (b) whether ryanodine receptor adaptation might arise from flash photolysis. Each argument, over the choice of model or influence of technique, is an example of a classic scientific dispute. Here we turn to another hoary old problem—the identification of a "prime mover."

The specific problem we are dealing with here concerns the structure and function of the voltage-gated proton channel in mammalian neutrophils. We have presented evidence recently that a component of the NADPH oxidase, gp91[phox], functions as a proton channel and as such plays an essential role in neutrophil action (Henderson and Meech, 1999). The proposal has proved controversial because the evidence we provided was necessarily circumstantial.

Each time this kind of controversy arises standard pieces of circumstantial evidence are assembled. The following is a list of the kinds of evidence that would generally be considered relevant:

(a) Deletion: destruction of gp91[phox] abolishes function.

(b) Addition: recovery of function when gp91[phox] is restored.

(c) Modification: altered gp91[phox] structure leads to altered function.

(d) Location: gp91[phox] is located at a site appropriate to its proposed function.

(e) Quantity: gp91[phox] is present in sufficient quantity to perform the function.

(f) Conditions: gp91[phox] is active under conditions appropriate to its proposed function.

The key question is whether gp91[phox] is a "prime mover," i.e., a proton channel, or whether it simply acts as some kind of cofactor. It has been suggested that gp91[phox] is not the proton channel itself but the modulator of a proton channel formed by a different, as yet unidentified protein (DeCoursey et al., 2001). The experimental basis for this suggestion comes from *Xenopus* oocytes where high levels of heterologous expression of many different membrane proteins induce the appearance of an endogenous chloride current (Shimbo et al., 1995; Tzounopoulos et al., 1995). The question is an important one because the proton channel poses an interesting problem in membrane permeation; if gp91[phox] is the channel, further analysis of proton permeation becomes possible.

Before we start to evaluate the evidence we have tried to summarize the key findings in this area over the past 20 yr. So new readers start here!

Background

(1) In 1980, one of us (Robert Meech) found that depolarized neurons of the land snail, *Helix aspersa*, were highly proton permeable. In fact, depolarization increased the proton permeability so much that the intracellular pH (pH$_i$) was set by the trans-membrane potential. Subsequent experiments in Roger Thomas's laboratory showed that injection of protons at depolarized membrane potentials produced a large outward current which declined in an exponential fashion once the injection had stopped (Thomas and Meech, 1982; Meech and Thomas, 1987). The acidified cell cytoplasm returned to its resting pH with a similar time course; both pH$_i$ recovery and outward current were blocked reversibly by 2 mM Cd^{2+}. After proton injection at the resting potential, pH$_i$ remained acidified and there was no obvious outward current. Thus, the current appeared when protons moved through a voltage-gated pathway. It always seemed simpler to think of this pathway as a Cd^{2+}-sensitive "channel."

(2) Further characterization of this voltage-gated proton channel took place in Lou Byerly's laboratory in 1982 and 1983. Byerly and Hagiwara (1982) had identified a "residual current" present in the perfused neurons of the pond snail, *Lymnaea stagnalis*, when the major ions, both internal and external, had been replaced by large impermeable substitutes. Armed with the knowledge that snail neurons contained a voltage-gated proton pathway, our key experiment was to show that "residual" tail current reversed at E$_H$, the H$^+$ equilibrium potential (Byerly et al., 1984). The protocol used to control pH$_i$ in the perfused neurons was that developed by Byerly and Moody and later published by them (Byerly and Moody, 1986); variants on this protocol have been used in all subsequent experiments on voltage-gated proton channels.

(3) The perfused neuron experiments not only established that the "residual" conductance was proton selec-

tive, they also showed that its voltage dependence was affected by both internal and external pH. Acidification of pH_i shifted activation toward less positive potentials; acidification of the bathing medium shifted activation in the opposite direction. As a result, activation seemed to occur when the main driving force for protons was outwards. It was as if the channel's primary role was to promote recovery from acidification. Protons could move inwards through the channel—during tail current experiments for example—but these movements were short lived. A second important finding was that Cd^{2+} inhibited the channel in two ways; it not only shifted activation toward positive membrane potentials but also reduced the maximum available conductance. Cd^{2+} increased the time to half steady-state current, but this effect was reduced or absent at positive membrane potentials.

(4) Patch clamp experiments by Byerly and Suen (1989) established that proton channels were not distributed evenly over the cell surface, that the proton current had a Q_{10} of 2.1 and that extracellular divalent cations blocked the proton current in the sequence: $Cu^{2+} = Zn^{2+} > Ni^{2+} > Cd^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} = Ca^{2+} = Ba^{2+}$. Mahaut-Smith (1989) showed that in land snails Zn^{2+} block at 45 mV had the form of a monomolecular reaction with a K_d of 1.48×10^{-5}M; 1 mM Zn^{2+} produced 100% block.

(5) Byerly and Suen (1989) examined the current fluctuations at different membrane potentials and found that activation of the proton conductance produced no significant increase in the current variance. They estimated the unitary proton current to be <0.004 pA with a driving force of $\sim$100 mV. Thus, the unitary conductance was <0.04 pS.

(6) In human neutrophils, a Cd^{2+}-sensitive proton–permeable pathway becomes evident once the enzyme NADPH oxidase is made active. NADPH oxidase is found in the plasma and granular membranes of the white blood cells (basophils, neutrophils, macrophages, and eosinophils) that surround and kill invading infectious microorganisms. The enzyme uses electrons derived from the oxidation of NADPH to reduce O_2 to the reactive radical, superoxide $O_2{}^-$. It is not continuously active but is rapidly (0.5–2 min) activated by a range of compounds and physiological stimuli. Activation results in a rapid depolarization of the plasma membrane to a level that depends on the transmembrane pH gradient; the larger the pH gradient the smaller the depolarization. Activation can be inhibited by diphenelyene iodonium (DPI) (Henderson et al., 1987).

(7) Activation of the NADPH oxidase leads to the synthesis of $O_2{}^-$ and an associated efflux of electrons recorded as an electric current by Schrenzel et al. (1998). The electron efflux is sufficient to depolarize the cell membrane by several volts and the remaining protons are sufficient to acidify the cell cytoplasm by many pH units. Henderson (1988) proposed that a proton channel associated with the NADPH oxidase would account for the observed high proton permeability in activated neutrophils. Their proposal was that the channel provided a path for proton movement to compensate for both the intracellular acidosis and the charge difference set up by the efflux of electrons. A reduced compensatory movement of protons explained the increased acidosis of pH_i in the presence of the K^+ ionophore valinomycin, or 1 mM Cd^{2+}.

(8) A number of compounds activate the NADPH oxidase. These include PMA, phorbol ester, and unsaturated fatty acids such as arachidonic and oleic acids. If there is an electrochemical gradient for protons, concentrations of arachidonic acid that activate the NADPH oxidase initiate a rapid change in pH_i when studied in cell suspensions. Henderson and Chappell (1992) concluded that arachidonate induces a selective increase in the permeability of the membrane to protons and suggested that it acted on the NADPH oxidase associated proton channel.

(9) The NADPH oxidase is a multisubunit enzyme (phagocyte oxidase), consisting of two membrane proteins, gp91^{phox} and p22^{phox}, and two cytosolic proteins, p47^{phox} and p67^{phox}. Upon activation, the two cytostolic subunits move to the membrane and bind to the membrane subunits. The gene for gp91^{phox} is X-linked and a mutation in this or any of the genes encoding one of the other subunits results in chronic granulomatous disease (CGD). Patients with CGD lack a functional NADPH oxidase and are highly susceptible to infection.

(10) In cell lines established from CGD patients, the absence of an arachidonic acid–activated proton channel correlates with the absence of gp91^{phox} rather than the absence of p47^{phox} or p67^{phox} (Henderson et al., 1995). Furthermore, suppressing the expression of p22^{phox} in differentiating HL60 cells had little effect on the arachidonic acid–activated proton flux associated with superoxide production. Consequently, gp91^{phox} seemed likely to be the protein that forms the proton channel.

(11) Soon after the description of voltage-gated proton currents in *Helix* and *Lymnaea* neurons, Barish and Baud (1984) identified similar currents in *Ambystoma* oocytes. Proton currents were also found in rat alveolar epithelial cells (DeCoursey, 1991), microglial cells (Morihata et al., 2000), and a range of myeloid or myeloid derived cells, such as human neutrophils (DeCoursey and Cherny, 1993) and human granulocytes (Demaurex et al., 1993). In addition, the selective proton permeability of the influenza virus M_2 channel was established (Chizhmakov et al., 1996; Shimbo et al., 1996)

(12) Arachidonic acid increases the amplitude of the voltage-gated proton current in human neutrophils (DeCoursey and Cherny, 1993). This important finding indicated that the proton channel associated with the NADPH oxidase might correspond to the voltage-gated proton channel. This idea is supported by whole-cell

clamp experiments on gp91^{phox}-transfected Chinese hamster ovary (CHO) cells. CHO cells, studied in suspension, show no evidence of having an arachidonate-activated proton pathway until they are transfected with cDNA encoding for gp91^{phox} (Henderson et al., 1995). What is more, studied under whole cell patch-clamp, these transfected cells generate large outward proton currents even during short (150–750 ms) depolarizing command pulses (Henderson and Meech, 1999). These currents, absent in control cells, have all the classical characteristics of the proton channels described by Byerly et al. (1984): their tail currents reverse at E_H and the reversal potential shifts 56 mV for 1 U change in pH$_o$. They are also rapidly and reversibly inhibited by 200 μM Zn^{2+} which produces 60% inhibition during activation at 100 mV (see section 3 above). In addition, their amplitude is reversibly increased by arachidonic acid.

(13) The actual membrane topology of gp91^{phox} is currently unknown. The hydropathy plot suggests that it consists of either four or six transmembrane domains, followed by a large hydrophobic domain, which contains the predicted binding sites for the NADPH oxidase cofactor FAD and the substrate NADPH. CHO cells expressing only the putative transmembrane domain (amino acids 1–230) of gp91^{phox} (CHO-NT) exhibit all the properties of a voltage-gated proton pathway (Henderson et al., 1997; Henderson and Meech, 1999). The third transmembrane domain of gp91^{phox} contains four histidine residues, of which 111, 115, and 119 are predicted to align one above another in an α-helix. If the histidine at position 115 is mutated to either leucine or lysine, the expressed protein appears at the cell membrane, but neither voltage-gated proton currents nor an arachidonic activated proton flux can be elicited. However, some function is retained if the histidine is mutated to aspartic acid (Henderson, 1998; Henderson and Meech, 1999). Aspartic acid and histidine each have a similar pK$_a$, suggesting that proton permeation may require the amino acid side chain at position 115 to alternate between accepting and donating protons at physiological pH.

(14) In recent years it has become evident that the human genome contains more than one gene encoding a gp91^{phox}-like protein. At present, four homologues have been identified, each with a similar molecular weight to gp91^{phox}. They have been given the names Nox1 (from NADPH Oxidase), Nox2 (the renamed gp91^{phox}), Nox3, and Nox4 (Lambeth et al., 2000). In addition, there is a higher molecular weight homologue, Nox5, with an NH$_2$-terminal extension containing three EF hands (Banfi et al., 2001) and two Duox proteins, expressed in the thyroid, which consist of a fusion of NH$_2$-terminal peroxidase and COOH-terminal gp91^{phox} domains (Dupuy et al., 1999). Splice variants of Nox1 (Nox1S) (Banfi et al., 2000) and Nox5

(Banfi et al., 2001) have been reported. Voltage-dependent proton currents have been recorded from HEK-293 cells transfected with either Nox1S (Banfi et al., 2000) or Nox5 (Banfi et al., 2001). However, untransfected HEK-293 cells express low levels of Nox4 (Shiose et al., 2001) that could account for the proton currents observed in these wild type cells.

(15) The predicted membrane topology for the different Nox homologues is of four to six transmembrane domains with a large cytosolic domain at the COOH-terminal, much as described for gp91^{phox} (Nox2). The histidines in position 115 and 119 in Nox2 are conserved in all the human homologues, in keeping with the proposed mechanism for proton permeation.

Is gp91^{phox} (Nox2) a Voltage-gated Proton Channel? A Review of the Evidence

The question presented for discussion contains a number of tangled threads so that it would be as well to tease apart and identify the different components, as we understand them. First, is gp91^{phox} a voltage-gated proton channel? Second, is such a channel required for superoxide generation in neutrophils? Third, is gp91^{phox} the voltage-gated proton channel in neutrophils?

We have attempted to answer the first question by showing that CHO cells expressing gp91^{phox} have large time and voltage-dependent proton currents (Henderson and Meech, 1999). We cannot exclude the possibility that gp91^{phox} expression simply up-regulates a channel already present in the wild-type CHO cells (DeCoursey et al., 2001) but we feel that some observations are difficult to reconcile with a hypothesis that requires a specific protein–protein interaction of this kind. A key finding is that histidine 115 is essential for function. This supreme specificity is very different to the situation in *Xenopus* oocytes where the expression of a wide variety of cellular and synthetic proteins, such as phospholemman and the NB protein of influenza B virus, can activate an endogenous chloride conductance (Shimbo et al., 1995).

As to whether a voltage-gated proton channel is essential for superoxide generation in neutrophils, this appears to be well established so there is little need for further discussion here. The final question is more difficult to answer. There is little direct evidence but much indirect inference based on neutrophil surrogates. Our discussion reviews the evidence from a number of different cell types.

(a) Deletion: Does Destruction of gp91^{phox} Abolish Function?

Finding voltage-gated proton currents in the absence of membrane-bound gp91^{phox} would exclude gp91^{phox} from consideration as the only voltage-gated proton channel in neutrophils. We know of no unequivocal evidence of this kind from neutrophils but exon 3 of the gene en-
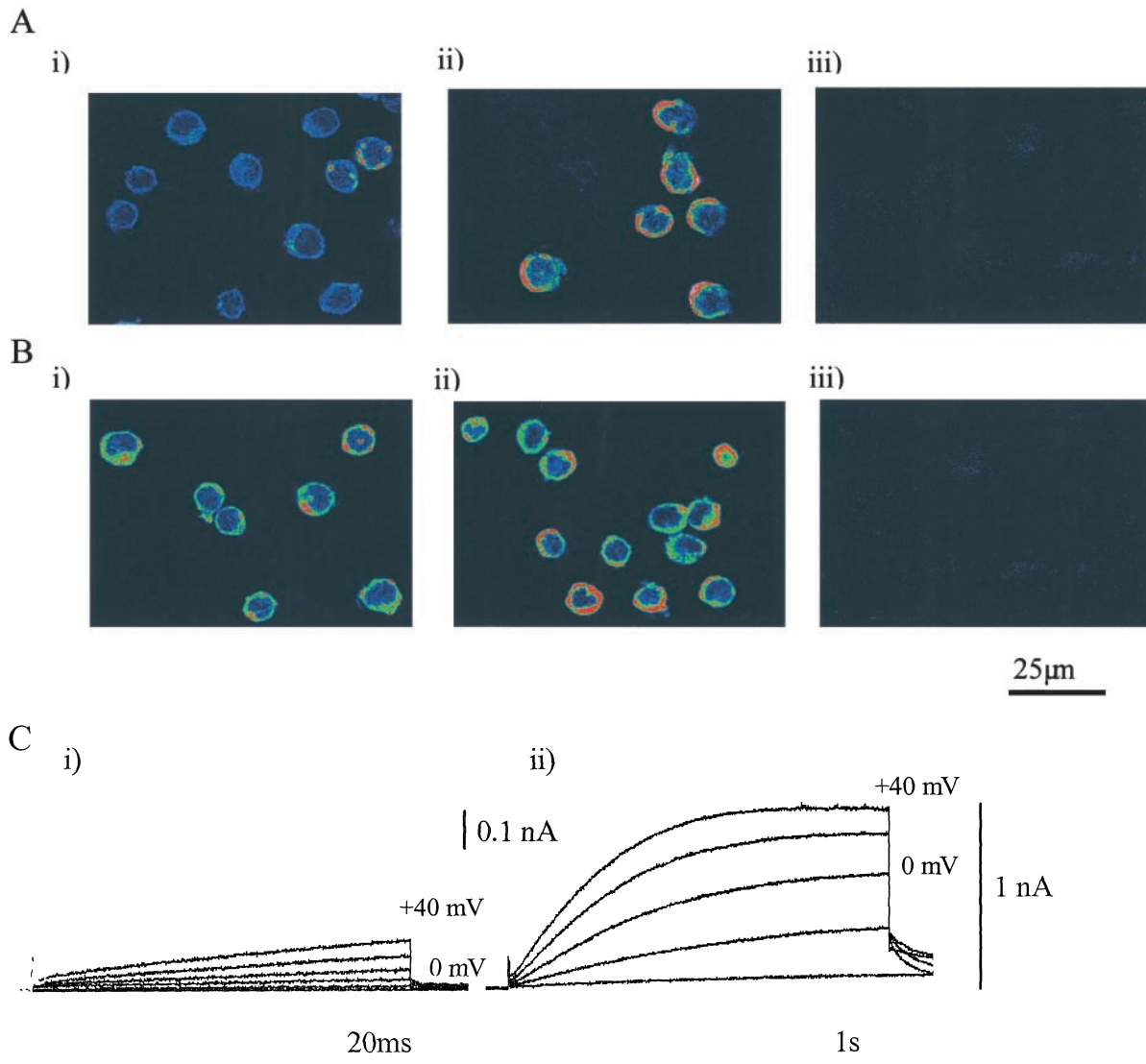
FIGURE 1. Expression of NADPH Oxidase (Nox) protein in PLB and PLB$_{KO}$ cells. (A) Nox protein immunodetected in undifferentiated (i) and differentiated (ii) PLB-298 cells after incubation with an anti–COOH-terminal gp91^{phox} polyclonal antibody and FITC-labeled anti–rabbit antibody. The polyclonal anti-gp91^{phox} was raised to the COOH-terminal 14-amino acid peptide coupled to keyhole limpet hemocyanin. It was verified by Western blot against detergent-solubilized membrane proteins from human neutrophils (Henderson et al., 1995). The images, which show an optical slice close to the cell center, were obtained using a BioRad MRC 600 inverted confocal microscope. A pseudocolor scale in which the highest intensity of emitted fluorescence is denoted by red through orange, yellow and green to blue, low fluorescence, indicates the presence of a Nox protein. Differentiated PLB-985 cells treated in the absence of the anti-gp91^{phox} antibody are shown in iii. (B) Immuno-detection of Nox protein in PLB$_{KO}$ cells using the same technique and antibody as described for A. As before, undifferentiated (i), differentiated (ii), and control cells in the absence of anti-gp91^{phox} antibody (iii) are shown. (C) Whole-cell currents recorded from a single differentiated PLB-985 cell under conditions designed to maximize the amplitude of H$^+$ current (see Henderson and Meech, 1999). The patch pipette–filling solution, which was adjusted to pH 6.5, contained 119 mM TMA hydroxide, to block potassium currents, a small quantity of calcium buffer (3.7 mM EGTA, 0.74 mM CaCl$_2$) and ~120 mM MES pH buffer. The bathing solution contained 110 mM TMA methane-sulphonate, 2 mM Ca(OH)$_2$, 2 mM Mg(OH)$_2$, 5 mM glucose, and 100 mM EPPS buffer adjusted to pH 8.0. Low amplitude prepulses in the linear current–voltage range (−60 to −100 mV) were scaled by PCLAMP 6 software (Axon Instruments, Inc.) and used for online subtraction of linear capacitive and leakage currents (Armstrong and Bezanilla, 1974). The superimposed currents shown were recorded in response to a standard stepped-voltage protocol; commands were in the range -40 to 40 mV (20-mV increments). The command duration was 100 ms (i) or 5 s (ii). The immunostaining of PLB-985 and PLB$_{KO}$ cells and recording of whole cell currents are the work of X. Wen Hu. Mary C. Dinauer provided the PLB$_{KO}$ cells. EPPS is N-[2-hydroxyethyl]-piperazine-N'-[3-propane-sulphonic acid]; TMA, tetramethylammonium.

coding gp91^{phox} has been knocked out in a PLB-985 cell line. The resulting PLB$_{KO}$ cells appear not to express gp91^{phox}, but nevertheless exhibit large outward proton currents (DeCoursey et al., 2001). In Fig. 1 C we reproduce typical proton currents recorded from the PLB$_{KO}$ cells given to us by Dr. Mary C. Dinauer. The figure also

shows that both PLB-985 and PLB_{KO} cells immunostained with an antibody to gp91[phox] have high levels of stain in the region of the cell membrane. As might be expected, differentiation of PLB-985 cells results in an increase in the expression of this gp91[phox]-like protein (Fig. 1 A) together with an increase in PMA-activated superoxide generation (not depicted). However, undifferentiated PLB_{KO} cells also show a high level of expression of a gp91[phox]-like protein, and once again expression increases after differentiation (Fig. 1 B). The COOH-terminal regions of other Nox proteins have such a high degree of sequence homology that we would expect the anti-gp91[phox] antibody would detect any of them. We have confirmed the presence of gp91[phox]-like protein in PLB_{KO} cells with a second anti-gp91[phox] antibody supplied by M.C. Dagher (Grenoble, France).

The presence of a gp91[phox]-like protein in the gp91[phox] knockout cell line may be a consequence of an enhanced expression of other Nox homologues. Compensation of this kind is seen in osteoclasts isolated from the gp91[phox] knockout mouse; these osteoclasts express Nox4 at a higher level than osteoclasts from wild-type mice (Yang et al., 2001). Compensatory expression of one or more of the homologues of gp91[phox] in the PLB_{KO} cells would account for the presence of the voltage-dependent proton current reported by DeCoursey et al., (2001). The identity of the gp91[phox] homologue expressed in these cells is currently under investigation.

(b) Addition: Does Function Recover if gp91[phox] Is Restored?

Once again we know of no direct evidence of this kind. However, gp91[phox] has been inserted into both CHO cells (Henderson and Meech, 1999; see section 12 above) and Green monkey kidney (COS-7) cells (Maturana et al., 2001), and time- and voltage-dependent currents were recorded in each case. Now the question arises: do the characteristics of the expressed gp91[phox] channels correspond to those of the voltage-gated proton conductance in neutrophils?

A puzzling aspect of the voltage-gated proton channels recorded from many mammalian cells is that their kinetics are so slow. Fig. 1 C shows whole cell currents from a single differentiated PLB-985 cell on two different time scales. In Fig. 1 C, ii, the currents take several seconds to reach a steady-state, even at 40 mV, and at -20 mV the currents fail to reach a steady-state after 5 s. However, although they take many seconds to reach steady-state, the proton currents do develop without a delay (see Figure C i). The currents recorded from gp91[phox]-expressing CHO cells are similar. In most of our studies on CHO cells we have used short command pulses to demonstrate the early stages of the development of the proton current and to avoid changes in the proton gradient with time. As a practical consideration, these slow kinetics mean that a true steady-state activation curve is hard to achieve and the position of the "activation curve" on the voltage axis will depend on the duration of the test pulse. As always, it is essential to compare like with like.

Whether CHO cells have detectable endogenous proton currents is in dispute but it is generally accepted that they are absent from COS-7 cells (Maturana et al., 2001; Morgan et al., 2002). However, the large volume of these cells (cell diameter as much as 50 μm) makes it hard (perhaps impossible) to control pH_i satisfactorily using a standard patch pipette with a 1 μm tip. Cell cytoplasm contains highly effective mobile and fixed pH buffers that dictate pH_i. Byerly and Moody (1986) found that it was not possible to control pH_i in dialyzed snail neurons (100 μm diameter) if the pipette diameter was less than one third of the diameter of the cell, even if the dialysis solution contained 120 mM pH buffer. The molecular size of the buffer, the volume of the cell and the diameter of the pipette tip govern the time required for full dialysis. In CHO cells (diameter 25 μm), it takes at least 10 min for a fluorescent dye (with a comparable molecular weight to pH buffer) to perfuse from a 3-μm pipette tip throughout the entire cell (Henderson and Meech, 1999). Even so pH_i dropped to pH 6.8 only after 20 min dialysis with a pH 5.5 pipette solution. Problems associated with controlling pH_i when recording voltage-gated proton currents have also been reported by Morihata et al. (2000) in experiments on microglial cells.

Characterization of the voltage-dependent currents that Maturana et al. (2001) recorded from their gp91[phox]-transfected COS-7 cells remains uncertain because the presumed proton tail currents do not reverse at E_H as calculated from the pH of the pipette solution. However, our experience with CHO cells would lead us to suggest that pH_i was in reality close to normal and not the pH of the pipette solution. Whether or not the time-dependent currents observed were proton currents they stand in contrast to the findings of Morgan et al. (2002), who were unable to see comparable currents even after 24 min dialysis. The COS-7 cells used were transfected with all four of the main subunits of NADPH oxidase (although direct evidence for gp91[phox] in the cell membrane is absent). The cells were smaller than normal COS-7 cells ($\sim$15–25 instead of 20–50 μm) and they regularly developed a high leakage current before any proton current became evident. We can confirm that even in CHO cells this is an ongoing problem that we attribute to cell swelling. In the smaller CHO cells, proton currents may be evident because pH_i becomes acidified before irreversible damage is done to the cell membrane and the cell contents. In conclusion: COS-7 cells transfected with gp91[phox] may generate time-dependent currents but there appears to be considerable variability in the response.

(c) Modification: Does Altered gp91^{phox} Structure Lead To Altered Function?

"Deletion" and "addition" experiments of the kind considered above do not exclude the possibility that the structure concerned is an intermediate agent rather than a "prime mover." More direct evidence is obtained by modifying the gp91^{phox} product by mutagenesis (Henderson and Meech, 1999). Conserve the positive charge at position 115 (i.e., by changing the histidine residue to lysine), and the expressed product fails to conduct protons. But change the positive charge at position 115 to negative (i.e., by changing the histidine to aspartic acid), and the expressed product retains the capacity to conduct protons, at least partially. Such observations are difficult to reconcile with a hypothesis that requires a specific protein–protein interaction between gp91^{phox} and a second proton channel–forming protein. However, if gp91^{phox} is the proton channel these findings fall into place with a simpler, more general hypothesis, that the histidine residue, with its capacity to undergo cycles of protonation and deprotonation, is the key element of the proton conduction pathway.

This proposal finds support not only in work on the influenza virus M_2 proton channel, where histidine 37 is essential for both ion selectivity and activation (Wang et al., 1995), but also in experiments on the S4 region of the *Shaker* K$^+$ channel (Starace and Bezanilla, 2001). S4 is thought to act as a voltage sensor because every third amino acid residue has a positive charge (arginine or lysine). When the arginine in positions 365, 368, and 371 is changed to histidine the resulting channels are capable of conducting protons down their electrochemical gradient. Starace and Bezanilla conclude that because the three histidine residues are accessible to the cytosol and to the external environment they are able to conduct protons across the cell membrane.

(d) Location: Is gp91^{phox} Located at a Site Appropriate to its Proposed Function?

A variety of cell fractionation experiments show that there are large quantities of gp91^{phox} in the neutrophil cell membrane.

(e) Quantity: Is gp91^{phox} Present in Neutrophils in Sufficient Quantity?

If we were to measure the amount of gp91^{phox} present in neutrophils and show that the concentration was insufficient to generate the measured proton conductance the discussion would be over. Instead we can show by calculation that there is sufficient gp91^{phox} present in the neutrophil surface membrane to account for the proton currents observed. The concentration of cytochrome b_{558} present in human neutrophils corresponds to $\sim 1.66 \times 10^6$ molecules of gp91^{phox} (Henderson, 1988). Of this, $\sim 25\%$ of the gp91^{phox} molecules are in the surface membrane (Clark et al., 1987). If we take the *Shaker* K$^+$ channel as our model and assume that there are four subunits of gp91^{phox}/proton channel, we end up with $\sim 1 \times 10^5$ channels/neutrophil. If each proton channel had a unitary conductance of no more than 0.04 pS (Byerly and Suen, 1989), the neutrophil would have a total proton conductance of no more than 4.2 nS. This value, which is ~ 20 times higher than that actually found, suggests that there would be sufficient gp91^{phox} even if the individual proton channels had an unusually low open probability or if we have overestimated the unitary conductance.

(f) Conditions: Is gp91^{phox} Active under Appropriate Conditions?

Direct activation of neutrophils by agents such as arachidonic acid leads to production of superoxide, depolarization of the cell membrane and acidification of the cell contents. In gp91^{phox}-expressing CHO cells, cell acidification and arachidonic acid both shift the voltage dependence of the proton conductance toward the resting potential. Thus, the characteristics of the proton conductance are exactly those required to fulfill its function.

Indirect activation by PMA is thought to be via a cytosolic phospholipase A_2 (cPLA$_2$) that releases arachidonic acid. Experiments using a PLB-985 cell line deficient in cPLA$_2$ have established that the enzyme is essential for the PMA activation of proton efflux (Lowenthal and Levy, 1999). This is presumably because arachidonic acid shifts the voltage dependence of the proton conductance toward the resting potential. The generation of superoxide by PMA is also dependent on the expression of cPLA$_2$ (Dana et al., 1998). It follows that PMA should shift the activation curve of the proton conductance in PLB-985 cells but not that in a cPLA$_2$-deficient cell line.

Future

At one time gp91^{phox} expression was believed to be restricted to the phagocytes and myeloid-derived cells. Now, however, homologues of gp91^{phox} have been found in the genomes of a wide range of organisms such as *Drosophila, Dictystelium, C. elegans,* Arabidopsis, tomato, rice, and some bacteria. Function has yet to be assigned to gp91^{phox}-like proteins in these species, although superoxide generation is assumed. Histidines 115 and 119 are conserved in each of the gp91^{phox} homologues so far identified, and so they too may function as proton channels. That homologues have been shown to be expressed in such a wide range of tissues and species forces us to re-evaluate the assumption that cell lines and tissues are devoid of gp91^{phox} unless shown otherwise. It may be safer to assume that gp91^{phox} has a widespread distribution and that it performs an essential function as a proton permeable pathway in all cells.

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