*Perspective*

# Voltage-gated Proton "Channels": a Spectator's Viewpoint

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A battle is raging over the molecular identity of the voltage-gated, $H^+$-selective conductance that has been recorded in activated and/or depolarized phagocytes. Having stepped out of this arena, we feel uncommitted to any particular viewpoint and hope to be able to provide below an unbiased opinion of what we feel has been established unambiguously, and what requires additional confirmation before conclusive statements can be issued.

The innate immune system is the first line of defense against invading microorganisms. This immune response is performed by specialized cells, macrophages, neutrophils, eosinophils, and dendritic cells, that recognize, ingest, and destroy pathogenic organisms. These professional phagocytes have developed an arsenal of microbicidal strategies aimed at ridding the organism of potential infectious agents. After engulfment, the foreign organism is trapped in a specialized vesicle, called the phagosome, which acquires microbicidal ability as a result of a series of coordinated fission and fusion events with other endomembrane compartments, culminating in the formation of the phagolysosome. These maturation events include the incorporation of vacuolar proton pumps and other integral proteins into the phagosomal membrane, as well as the delivery of microbicidal and lytic enzymes into the vacuolar lumen.

Generation of reactive oxygen species (ROS) in the phagosomal lumen is one of the key strategies used by phagocytes to kill ingested organisms. Superoxide is initially generated in phagosomes and at the cell surface by the two-electron reduction of molecular oxygen, a reaction catalyzed by the NADPH oxidase complex. The resulting superoxide anion can then be converted to more reactive radical molecules. Hydrogen peroxide is produced by dismutation and is itself a substrate of myeloperoxidase, which generates hypochlorous acid (HOCl) (Hampton et al., 1998). Superoxide can also generate the extremely reactive hydroxyl radicals via the Haber-Weiss reaction. The importance of the NADPH oxidase to the immune response is highlighted by the manifestation of chronic granulomatous disease (CGD). Patients with this genetic disorder lack a functional NADPH oxidase and, as a result, suffer from chronic susceptibility to infection by various bacterial strains, which can be lethal.

The NADPH oxidase is composed of two transmembrane proteins, $p22^{phox}$ and $gp91^{phox}$, that jointly form the cytochrome $b_{558}$; three cytosolic factors, $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$; plus the small molecular weight protein Rac (for reviews see Curnutte, 1992; Chanock et al., 1994; Babior, 1999). Because electrons from cytosolic NADPH are translocated across the bilayer to luminal (extracellular) oxygen, the activation of the oxidase generates a sizable current across the phagosomal membrane, which can be measured by whole-cell patch clamping (Schrenzel et al., 1998). This electron current triggers the depolarization of the phagosomal (or plasma) membrane and is accompanied by an acidification of the cytoplasm, particularly in the vicinity of the oxidase, due to the release of 2 protons during the oxidation of NADPH. Cytoplasmic acidification is further compounded by acid generated by the hexose monophosphate shunt, which is activated in order to replenish NADPH (Borregaard et al., 1984). To prevent excessive acidification and to neutralize the net charges displaced by the oxidase, the cells have devised compensatory mechanisms. Chief amongst these is an $H^+$-selective conductance that, when activated, can translocate cytosolic $H^+$ (equivalents) into the phagosomal lumen, contributing to relieve the cytosolic acid burden and providing a counterion to neutralize the flux of electrons. The $H^+$-conductive pathway is voltage sensitive and displays marked outward rectification. In addition to depolarization, the conductance can also be activated by phorbol esters and by arachidonic acid, which increase the maximal $H^+$ current and shift the I/V relationship to more negative values. A similar voltage-gated $H^+$ conductance was first described in snail neurons (Thomas and Meech, 1982) and then reported also in axolotl oocytes (Barish and Baud, 1984). In mammalian cells, an analogous $H^+$-selective pathway was initially identified in alveolar epithelial cells (DeCoursey, 1991) and later characterized in white blood cells (DeCoursey and Cherny, 1993; Demaurex et al., 1993). Though often referred to as a channel, the actual mode of ion translocation through the $H^+$-conductive pathway remains undefined. The properties of the $H^+$ conductance have been extensively reviewed (e.g., Eder and DeCoursey, 2001) and

will not be detailed here, except where relevant to the issue of its molecular identification.

The activities of the H$^+$ "channel" and the NADPH oxidase are intimately linked. The H$^+$ current is thought to be triggered by the depolarization caused by transport of electrons across the membrane, and the conductance is also regulated by the proton gradient generated by activation of the oxidase. Moreover, the same phorbol esters and arachidonic acid that stimulate the H$^+$ current are also effective activators of the NADPH oxidase (Nanda and Grinstein, 1991; Henderson and Chappell, 1992; Kapus et al., 1994; Kaldi et al., 1996).

There is a general consensus in the field regarding the existence of the H$^+$ conductance in phagocytes, its basic physiological features and its susceptibility to products of phospholipase A2 (Henderson et al., 1989; Susztak et al., 1997; Lowenthal and Levy, 1999). However, that is where the agreement ends. The molecular identity of the conductance is being hotly disputed and even the number of distinct entities involved is the subject of debate. The objective of this paper is to review, as impartially as possible, the available evidence and to draw tentative conclusions regarding the nature and number of conductive pathways. To this end, we have assembled a set of criteria that would need to be fulfilled for a candidate molecule to be identified as the mediator of the conductance. These criteria, which are listed below, will then be applied systematically and critically to the existing observations. The criteria, which individually may be suggestive yet not definitive, are listed in order of increasing stringency.

(a) A quantitative correlation should exist between the abundance of the candidate molecule and the magnitude of the conductance. The correlation can be established among various cell types and/or during the course of differentiation.

(b) The candidate molecule should be the target of pharmacological agents that affect the conductance. The drugs may exert their effect directly or indirectly, but the putative "channel" molecule must be ultimately affected.

(c) Expression of the candidate molecule should induce the appearance of the conductance. It should be borne in mind that, if the "channel" is a multisubunit complex, expression of a single subunit may fail to elicit the conductance, unless all others are endogenously expressed. Conversely, it is important to consider that the expressed molecule may be a modulator of the channel, as opposed to an essential subunit thereof. Therefore, fulfillment of this criterion provides suggestive but not conclusive evidence.

(d) The conductance should be absent in cells lacking the candidate molecule. As above, the channel could be a multi-subunit structure, in which case this criterion may yield only a partial identification of its components.

(e) Proper assembly of all the essential components in pure lipid bilayers should reconstitute the conductance in vitro. While most convincing, this criterion has not yet been applied to the study of H$^+$ channels and will not be considered hereafter.

*Is gp91$^{phox}$ the Elusive H$^+$ "Channel"?*

The first attempt to identify the molecular entity underlying the H$^+$ conductance was made by Henderson et al. (1987). Based on their coexistence and simultaneous activation, these authors speculated that one or more components of the NADPH oxidase may be the conductive unit. This suggested dual role of the oxidase in electron and proton transfer would be ideally suited to coordinate spatially and temporally the generation of protons and their disposal. Subsequently, Henderson et al. (1995) more specifically attributed the H$^+$ current to the gp91$^{phox}$ subunit of the NADPH oxidase. Existing evidence that supports this notion is reviewed next in the frame of the criteria stipulated above: (a) The H$^+$ conductance increases during the differentiation of HL-60 cells into neutrophils, in parallel with the appearance of the components of the NADPH oxidase (Henderson et al., 1995; Qu et al., 1994). (b) The proton current is activated by the same agonists that stimulate the NADPH oxidase (Henderson and Chappell, 1992; Henderson et al., 1995; Banfi et al., 1999, 2001). Moreover, inhibition of the H$^+$ conductance by Zn$^{2+}$ also affects the activity of the oxidase (Henderson et al., 1995). The histidine-specific reagent diethyl pyrocarbonate (DEPC) similarly inhibited both the oxidase and the conductance (Mankelow and Henderson, 2001). (d) The expression of gp91$^{phox}$ conferred H$^+$-conductive properties to cells normally devoid of an endogenous H$^+$ current (Henderson et al., 1995, 1997). Additionally, mutation of conserved histidines in the third transmembrane segment of gp91$^{phox}$, which had been implicated in the binding of hememoieties and are potential targets of DEPC, altered the activation properties of the conductance and rendered it less sensitive to DEPC (Henderson, 1998; Maturana et al., 2001).

Together, the data from Henderson's laboratory make a strong case in favor of gp91$^{phox}$ as the direct mediator of the H$^+$ flux. However, data from other groups are inconsistent with this notion and the relative weight of the following counter-arguments should be considered according to the hierarchical criteria.

(a) The correlation between the appearance of the oxidase and the conductance during differentiation is not perfect. Moreover, an H$^+$ conductance that is similar, if not identical, to the one of phagocytes has been reported in cell types that express no detectable levels of gp91$^{phox}$ (see above). (b) There are also considerable differences in the susceptibilities of the oxidase and con-

ductance to pharmacological inhibitors. A variety of studies have demonstrated that the oxidase and the conductance can be inhibited independently (Bianchini et al., 1994; Schrenzel et al., 1998; DeCoursey et al., 2000; Cherny et al., 2001). (c) Dinauer and colleagues recently undertook the mammoth task of reconstituting the phagocyte NADPH oxidase in nonphagocytic cells by heterologous transfection of all the required subunits, including gp91$^{phox}$ (Price et al., 2002). The transfected COS-7 cells acquired the ability to generate superoxide in response to agonists. Remarkably, when such cells were tested electrophysiologically by DeCoursey's group, they failed to exhibit the anticipated H$^+$ conductance (Morgan et al., 2002). It is conceivable that, unlike CHO cells, COS-7 cells express an inhibitor of the conductance, but this possibility appears unlikely. (d) Cells from CGD patients lacking detectable amounts of gp91$^{phox}$ have a readily measurable H$^+$ conductance (Nanda et al., 1994). Moreover, myeloid cells in which gp91$^{phox}$ was knocked out by gene targeting similarly displayed the outward-rectifying H$^+$ conductance. The activation properties of the conductance in cells lacking gp91$^{phox}$ are somewhat altered, which suggests a modulatory effect, rather than an essential constitutive role of this protein (Nanda et al., 1993; DeCoursey et al., 2001). This is discussed in more detail below.

Clearly, most of the latter findings are incompatible with the concept that gp91$^{phox}$ mediates the H$^+$ flux. How, then, can Henderson's data and other observations linking the channel with the oxidase be explained? Parallel expression of the two systems is not a strong index of causal association and could be accounted for by coordinated expression of sets of genes activated jointly during differentiation. The concomitant inhibition of the two systems by metals or by DEPC could be attributed to the poor specificity of these reagents. However, the appearance of the conductance in CHO cells upon transfection of gp91$^{phox}$ is more difficult to explain. It is conceivable that in CHO cells gp91$^{phox}$ acts as a regulator or functions as a subunit of an inactive or poorly active endogenous channel that is absent in COS-7 cells. In accordance with this interpretation, Cherny et al. (1997) reported the presence of a proton channel in untransfected CHO cells. It is noteworthy that the properties of the channel reconstituted in CHO cells by expression of gp91$^{phox}$ differ significantly from those seen in bona fide phagocytes: the current activates faster, is larger, and less Zn$^{2+}$ sensitive. This raises the possibility that a different type of conductance was elicited by heterologous expression of gp91$^{phox}$.

*One Conductance or Two?*

Is it conceivable that the confusion arose because not one, but two different H$^+$ conductive pathways exist in leukocytes? This possibility has been proposed by Banfi et al. (1999), who detected two types of H$^+$ currents in eosinophils, one associated with activation of the NADPH oxidase and another that was present in CGD cells and is therefore independent of a functional oxidase. These conductances differed in their threshold of activation, in their rates of activation and inactivation, and in their sensitivity to Zn$^{2+}$. Most striking, the channel associated with the oxidase was able to conduct H$^+$ inwardly, in sharp contrast with the outward rectifying properties of the conventional channel (Banfi et al., 1999).

In search for the identity of this novel H$^+$ conductance, the groups of Krause and Demaurex probed for the existence of homologues of gp91$^{phox}$ and undertook their functional characterization. They succeeded in cloning a related protein expressed as two splice variants: NOX1L and S (originally designated as NOH-1L and S). The latter, shorter variant lacks the last two transmembrane domains of the longer form (Banfi et al., 2000).

*Is NOX1S An H$^+$ Conductance?*

Krause and Demaurex and their coworkers proceeded to express NOX1S heterologously and to analyze the transfectants by electrophysiological means. In cells with low endogenous conductance, expression of NOX1S unmasked large H$^+$ currents with properties resembling those of unstimulated leukocytes (Banfi et al., 2000). As in the case of gp91$^{phox}$, there is currently insufficient evidence to judge whether NOX1S is itself the conductive moiety, a subunit thereof, or a modulator of a distinct conductive entity.

To complicate matters further, NOX1 may not be the only oxidase homologue linked to the conductance. There are now five members of the NOX family of oxidase-related proteins (for review see Lambeth et al., 2000) and all have the potential to induce or modulate H$^+$ currents, though only NOX1S, NOX2 (i.e., gp91$^{phox}$), and NOX5 have been tested to date (Banfi et al., 2001).

*Where Do We Stand?*

Can conclusions be reached at present regarding the identity of the conductive pathway(s)? An essential first step is to establish whether more than one type of conductive entities exist in phagocytes. While the data of Banfi et al. (1999) are suggestive of two separate pathways, the results are equally consistent with a single entity that undergoes kinetic alterations when a modulator is activated. Which of these hypotheses is tenable at present? The English philosopher William Ockham stated that "entities should not be multiplied needlessly; the simplest of two competing theories is to be preferred." This principle, known as Ockham's razor,

would favor the choice of a single, modulated channel, at least until the existence of a second channel is verified. Indeed, channels that change their electrophysiological profile upon posttranslational modification or association with ancillary molecules have been amply documented.

Let us assume, therefore, that a single type of $H^+$ channel exists. It is our view that definitive statements cannot be made at present regarding its molecular identity based on the available evidence. However, some interim conclusions can be reached. First, the weight of the existing evidence seems to rule out gp91$^{phox}$ as an essential component of the conductive pathway. This applies not only to nonmyeloid tissues, but also to phagocytes where gp91$^{phox}$ is naturally expressed. Second, despite the reported correlations, it is premature to conclude whether other members of the NOX family are *sine qua non* components of the conductive pathway. The existing data can be explained equally well by postulating the existence of an independent channel that is modulated by expression and/or activation of NOX family members, including gp91$^{phox}$.

In summary, it is our point of view that only one type of $H^+$ conductance needs to be postulated for the time being and that its molecular identity cannot yet be assigned. It appears clear that gp91$^{phox}$ is not the conductive entity, but it is equally obvious that the oxidase component plays a modulatory role on the conductance. Because of their homology with gp91$^{phox}$, we are inclined to believe that other members of the NOX family will similarly prove to be modulators, rather than essential components of the conductive pathway, but confirmation of this "hunch" will have to await further experimentation. We feel that this is at present the most conservative interpretation of the existing data, i.e. a close shave with Ockham's razor. Ultimately, application of criterion (e), in vitro reconstitution of the conductance, may be the sole way to unambiguously identify the putative $H^+$ channel.

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