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TOPICAL REVIEW

Physiological roles of voltage-gated proton channels in leukocytes

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Voltage-gated proton channels are designed to extrude large quantities of cytosolic acid in response to depolarising voltages. The discovery of the *Hvcn1* gene and the generation of mice lacking the channel molecule have confirmed several postulated functions of proton channels in leukocytes. In neutrophils and macrophages, proton channels are required for high-level production of superoxide anions by the phagocytic NADPH oxidase, a bactericidal enzyme essential for host defence against infections. In B lymphocytes, proton channels are required for low-level production of superoxide that boosts the production of antibodies. Proton channels sustain the activity of immune cells in several ways. By extruding excess cytosolic acid, proton channels prevent deleterious acidification of the cytosol and at the same time deliver protons required for chemical conversion of the superoxide secreted by membrane oxidases. By moving positive charges across membranes, proton channels limit the depolarisation of the plasma membrane, promoting the electrogenic activity of NADPH oxidases and the entry of calcium ions into cells. Acid extrusion by proton channels is not restricted to leukocytes but also mediates the intracellular alkalinisation required for the activation of spermatozoids. Proton channels are therefore multitalented channels that control male fertility as well as our innate and adaptive immunity.

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Voltage-gated proton channels have fascinated physiologists for three decades before the discovery of the channel molecule in 2006. Since the initial report of proton currents in snail neurons by Thomas & Meech (1982), proton currents have been recorded in almost all cell types, the most recent being human sperm (Lishko *et al.* 2010). After a longstanding controversy regarding their molecular identity, the unique nature of proton channels was revealed in 2006 when the *Hvcn1* gene was discovered by two independent groups (Ramsey *et al.* 2006; Sasaki *et al.* 2006). The human *Hvcn1* gene encodes a 273 amino acid protein that bears an astonishing resemblance to the voltage-sensing domains of other ion channels, and hence the alternative names VSOP (for voltage-sensing only protein) and Hv1 (for human voltage-gated proton channel 1) of the channel protein (Ramsey *et al.* 2006; Sasaki *et al.* 2006). Proton channels differ in a fundamental aspect from other members of the channel family as they lack the S5–S6 segments that form the pore of other channels. Structure–function studies revealed that VSOP/Hv1 channels are dimers, with each monomer containing a separate conduction pathway and a voltage sensor (Koch *et al.* 2008; Lee *et al.* 2008; Tombola *et al.* 2008). The monomers in the dimer do not function independently, but gate cooperatively

Nicolas Demaurex (right) and **Antoun El Chemaly** (left) work in the department of Cell Physiology and Metabolism at the University of Geneva and collaborate to study the role of ion channels in phagocytic white blood cells. Their background is in physiology and cell biophysics, respectively. Using mouse genetics, ion imaging, and electrophysiology, they have determined that VSOP/Hv1 proton channels sustain neutrophils migration and bacterial killing.

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(Gonzalez *et al.* 2010; Musset *et al.* 2010*a*; Tombola *et al.* 2010). The two monomers are closely apposed, enabling Zn^{2+} and other divalent cations to bind simultaneously to two externally accessible histidine residues from each monomer (Ramsey *et al.* 2006; Sasaki *et al.* 2006; Musset *et al.* 2010*b*) and to prevent the movement of the two monomers during channel opening. Only one isoform of the gene was detected in all tested species so far, and mice bearing a targeted deletion of the *Hvcn1* gene have been generated by three independent laboratories. These VSOP/Hv1-deficient mice provide a valuable animal model to study the physiological role of proton channels.

The properties of voltage-gated proton channels were firmly established long before the discovery of the channel protein, due in large part to the dedicated work of Thomas DeCoursey (Decoursey, 2003). Proton currents are activated by depolarising voltages in a pH-sensitive manner and inhibited by extracellular metal ions such as Zn^{2+}, the prototypical inhibitor of proton channels. The slowly developing outward currents strongly resemble delayed rectifier potassium currents, but are carried exclusively by hydrogen ions since proton channels are perfectly selective for protons. The transmembrane pH gradient sets the voltage dependence of proton channels such that, under most conditions, the channels activate ~20 mV above the equilibrium potential for H^+ ions and only catalyse acid extrusion. One notable exception is the so-called 'enhanced gating mode' observed in phagocytic white blood cells and osteoclasts activated with phorbol esters. The enhanced gating reflects protein kinase C dependent phosphorylation (Morgan *et al.* 2007) of a threonine residue on the N-terminus of the channel (Musset *et al.* 2010*a*). Currents across phosphorylated proton channels are larger, activate faster and at ~40 mV lower voltages, thereby enabling the bidirectional flux of protons (Banfi *et al.* 1999; DeCoursey *et al.* 2000; Petheo *et al.* 2003). Since the membrane potential and trans-membrane pH gradient almost invariably favour proton efflux however, phosphorylated proton channels in fact extrude protons more efficiently. The major physiological consequence of the enhanced gating is to boost the activity of proton channels during activation of the phagocyte NADPH oxidase, an enzyme that assembles at the plasma or phagosomal membrane upon phosphorylation (see below).

The physiological role of voltage-gated proton channels has been much less controversial than their molecular identity. In fact, a role for proton channels was proposed even before proton currents were detected in phagocytes. Proton channels alter both their chemical and electrical environment as they transport acid equivalents and move positive charges across membranes. Proton channels are highly efficient acid transporters, a single proton channel being able to transport up to 10^4 ions per second under physiological conditions (Decoursey, 2003). Since the physiological concentration of H^+ ions is low, around 40 nM (pH 7.4) and 100 nM (pH 7.0) in the intracellular and extracellular fluids, respectively, the flux of protons across voltage-gated proton channels can cause very rapid pH variations both inside and outside cells. Extreme pH variations are predicted to occur in small, membrane-enclosed compartments such as the phagocytic vacuole.

Due to their high capacity to extrude cytosolic acid, proton channels were immediately proposed to regulate the pH of the cytosol [pH_i] and of extracellular fluids [pH_o]. Several studies reported that proton channels contribute to the maintenance of the intracellular pH, based on the effect of Zn^{2+}, the best inhibitor of proton channels identified so far. Zn^{2+} prevents the pH_i recovery from physiological acidifications or experimentally induced acid loads in a whole range of cell types (reviewed in DeCoursey, 2010), including neutrophils (Nanda *et al.* 1992; Demaurex *et al.* 1996), mast cells (Kuno *et al.* 1997), basophils (Musset *et al.* 2008) and alveolar epithelial cells (Murphy *et al.* 2005). Zn^{2+} also inhibits acid secretion by human airway epithelia (Fischer *et al.* 2002), consistent with a role for proton channels in the acidification of the airway surface liquid (Schwarzer *et al.* 2004).

By extruding positively charged protons from cells, proton channels alter the membrane potential and act as repolarising devices. Like delayed-rectifying potassium channels, proton channels activate slowly upon depolarisation, but their ability to repolarise the plasma membrane is limited because proton channels can only repolarise cells to the equilibrium potential for H^+ ions. Under physiological pH conditions ($pH_{i/o}$ 7.1/7.4), proton channel activation will thus only repolarise the membrane potential to −10 mV. In cells exposed to severe acid loads, however, proton channels can repolarise cells to values close to the resting membrane potential (−60 mV at $pH_{i/o}$ 6.4/7.4). Because of this dual regulation by voltage and acidic pH, proton channels were proposed to stabilise the membrane potential of human cardiac fibroblasts during ischaemia (El Chemaly *et al.* 2006), a condition associated with massive acidification of the cytosol (Yan & Kleber, 1992).

The physiological role of proton channels is determined in large part by the voltage and pH-dependent gating of these unique channels. Proton channels are designed to extrude cytosolic acid and their optimal activation requires a sustained depolarisation combined with a massive cellular acidification. Both conditions are seldom met and only occur in very specific situations. One such situation is the so-called 'respiratory burst' that occurs during the activation of phagocytic white blood cells such as neutrophils and eosinophils. These cells are equipped with a powerful enzyme that produces large amounts of reactive oxygen species (ROS) essential for

antimicrobial defence, the NADPH oxidase. The NADPH oxidase moves electrons from cytosolic NADPH across the membrane to reduce extracellular or phagosomal oxygen. The oxidase is electrogenic (Henderson *et al.* 1987) and generates electron currents that can be measured with the patch-clamp technique (Schrenzel *et al.* 1998). The electron currents are voltage dependent and their amplitude decreases linearly at positive voltages (DeCoursey *et al.* 2003; Petheo & Demaurex, 2005). To prevent self-inhibition by membrane depolarisation, the translocation of electrons must therefore be compensated by the flux of counter-ions.

Voltage-gated proton channels were proposed very early to provide the compensating charge for the oxidase, for two reasons. First, the NADPH oxidase provides optimal conditions to activate proton channels as the enzyme depolarises the plasma membrane and generates large quantities of cytosolic acid. Second, proton channels are perfect devices to sustain the activity of the oxidase. Proton channels can extrude the acid generated in the cytosol, compensate the charge translocated across the plasma membrane, and deliver the extracellular protons needed for the conversion of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and then to hypochlorous acid (HOCl) (Fig. 1). It was this intricate relationship between proton channels and the NADPH oxidase of phagocytes that led to disputed claims that the oxidase itself is a proton channel (Henderson *et al.* 1987; Banfi *et al.* 1999; DeCoursey *et al.* 2001; Maturana *et al.* 2001, 2002; DeCoursey *et al.* 2002; Morgan *et al.* 2002; Demaurex & Petheo, 2005). Oxidase activity goes *pari passu* with proton channel activity, and cells that have the highest

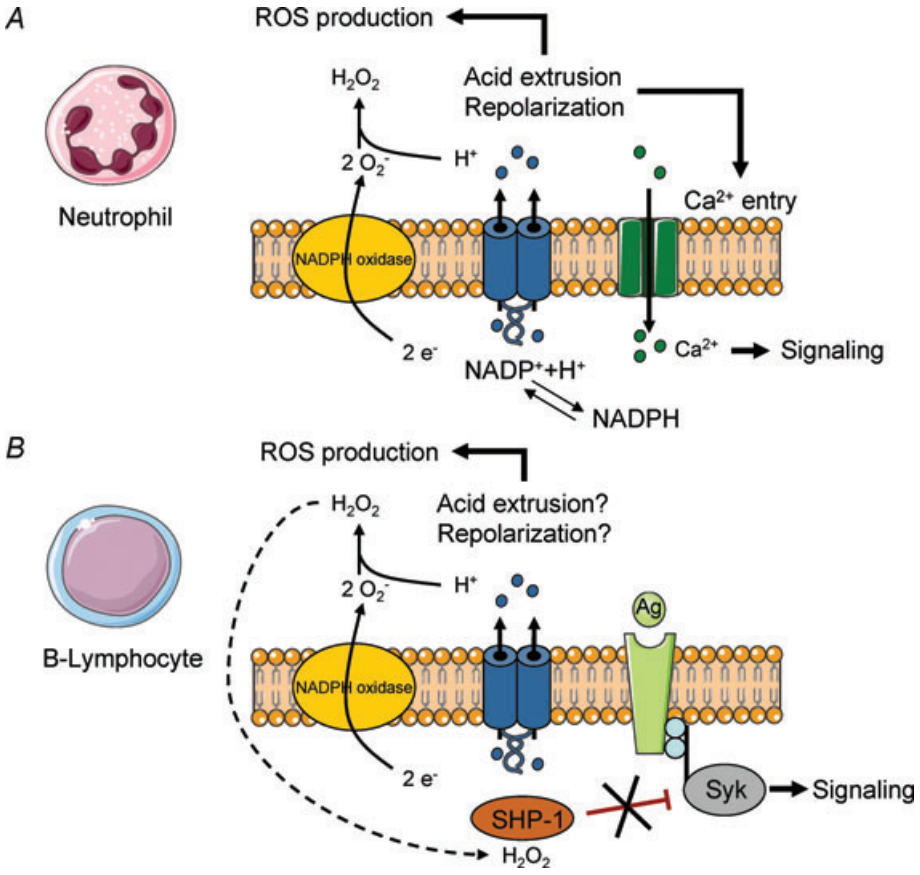

Figure 1. Functional roles of voltage-gated proton channels of immune cells
Both neutrophils and B lymphocytes possess a superoxide-generating enzyme, the NADPH oxidase that assembles at the plasma membrane upon phosphorylation. *A*, neutrophils produce high levels of superoxide radicals (O_2^-) that help kill bacteria. The large flux of electrons across the oxidase (yellow) depolarises the plasma membrane, whereas the protons released by the oxidation and regeneration of NADPH acidify the cytosol, two conditions that, together with phosphorylation, activate voltage-gated proton channels (blue). Proton channels extrude the cytosolic acid, repolarise the plasma membrane, and deliver extracellular protons used to convert superoxide to hydrogen peroxide (H_2O_2). Acid extrusion and membrane repolarisation sustain the activity of the oxidase and enhance the entry of calcium across membrane channels to boost cell signalling. *B*, in activated B lymphocytes, VSOP/Hv1channels also sustain the production of superoxide, but the H_2O_2 generated diffuses inside cells to oxidise and inactivate the tyrosine phosphatase SHP-1, promoting the phosphorylation of the antigen-bound B cell antigen receptor to boost cell signalling.

levels of oxidase activity such as neutrophils, eosinophils, macrophages, and microglia also have the highest density of proton currents (Eder & DeCoursey, 2001; Morgan & DeCoursey, 2003). In all of these phagocytic cells, Zn^{2+} inhibits both proton currents and superoxide production, strongly suggesting that proton channels sustain the activity of the NADPH oxidase (Henderson *et al.* 1988; Demaurex *et al.* 1996; DeCoursey *et al.* 2003; Rada *et al.* 2004; El Chemaly *et al.* 2010; Schilling & Eder, 2010; reviewed in DeCoursey, 2010).

Although the best established role for proton channels is to sustain superoxide production in phagocytes, concurrent depolarisation and cytosolic acidification also occur in non-phagocytic cells. In osteoclasts, the cells that reabsorb bone, depolarisation and acidification are triggered by calcitonin and elevated extracellular Ca^{2+}, two conditions that prevent osteoclasts from reabsorbing bone. Osteoclasts express proton channels (Nordstrom *et al.* 1995) that were shown to protect these cells from depolarisation and acidosis (Mori *et al.* 2003). Proton channels could thereby maintain the bone reabsorbing activity of osteoclasts. In human skeletal muscle, proton channels are activated during action potentials and were proposed to eliminate excess cytosolic acid during prolonged exercise (Bernheim *et al.* 1993).

The cloning of the *Hvcn1* gene and the generation of viable VSOP/Hv1-deficient mice now enable investigators to confirm the functional role of voltage-gated proton channels. As mentioned above, only one isoform of the *Hvcn1* gene has been detected in any animal species tested so far. Accordingly, all attempts to record proton currents in phagocytes from VSOP/Hv1-deficient mice yielded absolutely flat traces, even after activation of cells with phorbol 12-myristate 13-acetate (PMA) (Morgan *et al.* 2009; Okochi *et al.* 2009; Ramsey *et al.* 2009; El Chemaly *et al.* 2010). This confirms that VSOP/Hv1 is the only proton channel protein expressed in phagocytes and definitively rules out the possibility that the oxidase is a proton channel. Ablation of the *Hvcn1* gene dramatically reduced PMA-activated ROS production in mouse neutrophils and macrophages (Okochi *et al.* 2009; Ramsey *et al.* 2009; El Chemaly *et al.* 2010), yet these cells express a functional oxidase, as demonstrated by direct recordings of electron currents (Morgan *et al.* 2009; El Chemaly *et al.* 2010). These data clearly indicate that proton channels sustain oxidase activity and are required for high-level production of superoxide by phagocytes.

As could be anticipated, the impaired ROS production of VSOP/Hv1 deficient neutrophils was associated with an increased cytosolic acidification (Morgan *et al.* 2009; El Chemaly *et al.* 2010) and with an increased depolarisation. VSOP/Hv1 deficient neutrophils were nearly one pH unit more acidic than wild-type neutrophils when stimulated with PMA (El Chemaly *et al.* 2010), and 0.4 pH unit more acidic after ingestion of opsonised zymosan particles (Morgan *et al.* 2009). These data confirm that VSOP/Hv1 proton channels extrude the excess cytosolic acid that neutrophils generate during the respiratory burst. VSOP/Hv1 deficient neutrophils were also significantly more depolarised than control cells, confirming that VSOP/Hv1 proton channels provide most of the compensating charge required to balance the transport of electrons by the NADPH oxidase. Thus, VSOP/Hv1 proton channels prevent both the acidification and the depolarisation generated by the activity of the NADPH oxidase. The oxidase is inhibited at depolarised voltages (DeCoursey *et al.* 2003; Petheo & Demaurex, 2005) and at acidic pH (Morgan *et al.* 2005), but whether the reduced superoxide production observed in neutrophils exposed to Zn^{2+} or lacking the *Hvcn1* gene is due to detrimental acidification or depolarisation is impossible to tell, since proton channel ablation has both chemical and electrical effects.

The increased depolarisation observed in VSOP/Hv1-deficient neutrophils caused an important secondary defect by reducing the driving force for the entry of cations into cells. In neutrophils, the influx of Ca^{2+} into cells occurs predominantly through store-operated Ca^{2+} entry (SOCE). As predicted from the increased depolarisation, SOCE was dramatically impaired in VSOP/Hv1 deficient neutrophils and the calcium signals evoked by chemoattractants were reduced by 85% (El Chemaly *et al.* 2010). This calcium signalling defect was due to the increased depolarisation, as normal calcium signals could be recovered by preventing the depolarisation with the ionophore gramidicin. Ca^{2+} signals control several functions of neutrophils. The loss of calcium influx is thus likely to abort Ca^{2+}-dependent mechanisms such as the depolymerisation of the cortical actin cytoskeleton, which is required for neutrophil migration and efficient maturation of phagosomes. Accordingly, VSOP/Hv1 deficient neutrophils failed to migrate effectively upon stimulation with chemoattractants and exhibited thicker actin rings around ingested particles (El Chemaly *et al.* 2010). The combination of calcium signalling defects, impaired superoxide production, and altered cytosolic pH can all account for the defective bacterial killing observed in neutrophils from VSOP/Hv1 deficient mice (Ramsey *et al.* 2009).

Despite the multiple cellular defects observed in their innate immune cells, VSOP/Hv1-deficient mice did not suffer from recurrent bacterial infections. The preserved immunity can be explained by the residual neutrophil activity in mice lacking proton channels. Neutrophils from VSOP/Hv1 deficient mice were still able to migrate, ingest foreign particles and to secrete superoxide, although at much lower rates than control neutrophils. In humans, chronic granulomatous disease (CGD) requires complete loss of oxidase activity, as illustrated by carriers of the

X-linked CGD gene who are usually asymptomatic despite a 50% reduction in neutrophil superoxide production.

In CGD mouse models, the absence of superoxide production is associated with hyper-inflammation due to the abnormal termination of inflammation (Hultqvist *et al.* 2004; Schappi *et al.* 2008). We did not observe any sign of hyper-inflammation in VSOP/Hv1 deficient mice upon intradermal injection of fungal cell wall products into the ear dorsum (unpublished observation), a procedure that causes severe sterile inflammation in NOX2-deficient mice (Schappi *et al.* 2008). This suggests that VSOP/Hv1-deficient mice produce sufficient amounts of superoxide to degrade the β-glucans and/or to prevent the hyper-inflammatory reaction observed in mice lacking a phagocytic oxidase.

Mice lacking the *Hvcn1* gene revealed another important and unanticipated function for proton channels in B-lymphocytes. Like phagocytic cells, B lymphocytes express an NADPH oxidase and produce ROS upon stimulation of the B cell antigen receptor (BCR). B lymphocytes produce about one order of magnitude less superoxide than neutrophils, and earlier studies suggested that these ROS are required for signalling and proper activation of B cells (Finkel, 2003). B lymphocytes also have a high density of proton currents (Schilling *et al.* 2002) and of VSOP/Hv1 protein (Okochi *et al.* 2009). A recent study from Hv1/VSOP-deficient mice revealed that proton channels are required for BCR-induced generation of ROS (Capasso *et al.* 2010). *Hvcn1* ablation impaired BCR-dependent oxidation of the tyrosine phosphatase SHP-1 and caused a severe signalling defect, slowing the proliferation of B cells and reducing the antibody responses *in vivo* after immunisation of mice. Furthermore, overexpression of Hv1/VSOP inhibits B cell proliferation and reduces B, but not T, cell numbers in transgenic mice, suggesting that proton channel expression inhibits B cell development (Suenaga *et al.* 2007). Whether proton channels sustain ROS production in B cells by providing a compensating charge or by extruding cytosolic acid is not known, but proton channels do not appear to be required for calcium signals in B cells (Capasso *et al.* 2010).

VSOP/Hv1 expression is not restricted to leukocytes and voltage-gated proton channels were recently shown to play an important role in sperm maturation (Lishko *et al.* 2010). As discussed in another article in this issue, the VSOP/Hv1 channel is expressed in human sperm flagella and regulates the functional maturation of sperm. In a 'tour de force' experiment, these authors managed to patch-clamp the plasma membrane of human sperm flagella, revealing a high proton conductance in sperm cells. Strong evidence suggest that proton channels control the onset of sperm capacitation by driving acid extrusion, and possibly also by regulating intracellular Ca^{2+} levels, in spermatozoids. Unfortunately, although robust proton currents were recorded from human sperm, no currents were recorded in mouse sperm flagella. The causal role of the VSOP/Hv1 protein could thus not be confirmed by genetic ablation, highlighting the limitation of animal models. The importance of VSOP/Hv1 for sperm activation makes proton channels an attractive target for the control of male fertility. Drug design could benefit from the similarity between VSOP/Hv1 channels with the voltage-sensing domain of potassium channels (Kv), whose structure has been solved (Long *et al.* 2007). The challenge will be to develop modulators of proton channels that do not affect the activity of potassium channels.

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