TOPICAL REVIEW

Voltage-gated proton channels: what's next?

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This review is an attempt to identify and place in context some of the many questions about voltage-gated proton channels that remain unsolved. As the gene was identified only 2 years ago, the situation is very different than in fields where the gene has been known for decades. For the proton channel, most of the obvious and less obvious structure–function questions are still wide open. Remarkably, the proton channel protein strongly resembles the voltage-sensing domain of many voltage-gated ion channels, and thus offers a novel approach to study gating mechanisms. Another surprise is that the proton channel appears to function as a dimer, with two separate conduction pathways. A number of significant biological questions remain in dispute, unanswered, or in some cases, not yet asked. This latter deficit is ascribable to the intrinsic difficulty in evaluating the importance of one component in a complex system, and in addition, to the lack, until recently, of a means of performing an unambiguous lesion experiment, that is, of selectively eliminating the molecule in question. We still lack a potent, selective pharmacological inhibitor, but the identification of the gene has allowed the development of powerful new tools including proton channel antibodies, siRNA and knockout mice.

A few years ago, a H^+-ATPase researcher made the astonishing remark that his field was finished – all the major questions had been answered! It had never even occurred to me that this was possible – I have worked mainly in areas that are rife with problems to solve. The voltage-gated proton channel is a poster child of unsolved problems! Before I begin to list several important unanswered questions, I will briefly introduce this little-known channel.

First, the voltage-gated proton channel is an ion channel. For reasons that escape me, some people seem to want to call it a 'proton pump.' It is nothing like a pump. The proton channel cannot move protons against an electrochemical gradient – it is a passive pathway across the membrane. It does not need or use ATP. Proton channels open and close just like other ion channels, generating noise and single-channel currents. They open with depolarization, like the classical Na^+, K^+ and Ca^{2+} channels of excitable cells. Although these properties define the proton channel as an ion channel, it does have a number of distinctive, if not unique properties. Its unitary conductance is $\sim 10^3$ smaller than most other channels, roughly 15 fS at room temperature and physiological pH (Cherny *et al.* 2003). Many channels are selective, but imperfectly so; the proton channel

has apparently perfect selectivity. Proton channels have stronger temperature dependence, both of conductance and gating kinetics, than almost any other ion channel. Reminiscent of inward rectifier K^+ channels, the voltage dependence of proton channel gating is not absolute, but varies with the permeant ion concentration. Inward rectifiers conduct mainly inward K^+ current; proton channels conduct mainly outward H^+ current. Finally, the proton channel may be unique in lacking a water-filled pore that acts as the conduction pathway. Protons can travel in ways that other ions cannot, such as Grotthuss conduction in water (de Grotthuss, 1806; Pomès, 2006) and hydrogen-bonded chain conduction through proteins (Nagle & Morowitz, 1978). It is virtually certain that the conducted species is the proton, H^+, not the hydronium ion, H_3O^+ (DeCoursey & Cherny, 1997, 1998). The lack of a continuous aqueous pore could explain the extreme selectivity of the proton channel.

The pH- and voltage-dependence of its gating makes the proton channel an exquisitely designed proton extrusion mechanism. Eliminating excess acid from cells is its general function. However, proton channel activity has other consequences, to be discussed later, that enable it to perform other specialized functions. The best known and best characterized function occurs in phagocytes. When

these cells engulf microbes, the enzyme NADPH oxidase begins to produce superoxide anion, the precursor to a host of bactericidal reactive oxygen species. NADPH oxidase is electrogenic (Henderson *et al.* 1987; Schrenzel *et al.* 1998), and proton channels provide the bulk of charge compensation (Henderson *et al.* 1988*a*,*b*; Murphy & DeCoursey, 2006), which prevents extreme depolarization that would stop enzyme function (DeCoursey *et al.* 2003). Recent evidence that proton channels are required for histamine release by human basophils (Musset *et al.* 2008*b*) might be explained by proton extrusion, charge compensation or some other mechanism.

Molecular and genetic properties

Has the right gene been identified? The historical controversies over whether the gp91^{phox} component of NADPH oxidase might function as a voltage-gated proton channel (Henderson *et al.* 1995; Henderson & Meech, 1999; Bánfi *et al.* 2000; DeCoursey *et al.* 2000, 2001*b*; Maturana *et al.* 2001) have been thoroughly discussed (Touret & Grinstein, 2002; DeCoursey *et al.* 2002; Henderson & Meech, 2002; Maturana *et al.* 2002; DeCoursey, 2003, 2008; Musset *et al.* 2008*a*) and little new can be added. What can be stated without any

doubt is that bona fide voltage-gated proton channel genes were identified in 2006. The Clapham lab identified and characterized the human proton channel gene, H$_V$1 (Ramsey *et al.* 2006). The Okamura lab (Sasaki *et al.* 2006) identified a voltage-gated proton channel, CiVSOP, in *Ciona intestinalis*, a sea squirt, in a project to classify the genome of this creature, and found a homologue in the mouse, mVSOP. A gene coding for a voltage-sensitive phosphatase had been identified previously (Murata *et al.* 2005); the proton channel was a fortuitous discovery in an effort to catalogue other homologues. The proton channel gene products have several remarkable and unexpected properties. As shown in Fig. 1, the proton channel has four putative membrane-spanning regions, S1–S4, which are generally similar to the S1–S4 regions of many voltage-gated ion channels (Sasaki *et al.* 2006; Ramsey *et al.* 2006) as well as the voltage-sensitive phosphatase in *Ciona* (Murata *et al.* 2005). However, the protein lacks the S5–S6 regions, which in ordinary ion channels form the conduction pathway. The proton channel is thus a voltage sensor without a conventional pore.

Genes that are highly homologous to the proton channel are present in at least two dozen species, with selected species given in Table 1. Other species with similar genes, listed in descending order of predicted protein similarity (to the human HVCN1) include: *Pan troglodytes* (chimpanzee), *Sus scrofa* (pig), *Equus caballus* (horse), *Monodelphis domestica* (grey, short-tailed opossum), *Rattus norvegicus* (rat), *Ornithorhynchus anatinus* (platypus), *Trichoplax adhaerens* (placozoan), *Nematostella vectensis* (starlet sea anemone), *Tetraodon nigroviridis* (puffer fish), *Macaca fascicularis* (crab-eating macaque), *Laccaria bicolor* (basidiomycete fungus), *Aspergillus niger* (black mold) and *Physcomitrella patens* (moss) which is 23% identical to H$_V$1 (NCBI BLAST). Each protein in Table 1 has four predicted transmembrane domains that have substantial homology, fairly long C and N terminal domains, and short linkers with the highest homology in the intracellular S2–S3 linker. In most species, the C terminus contains a long predicted intracellular coiled-coil region, where intersubunit interaction may occur (Koch *et al.* 2008; Lee *et al.* 2008; Tombola *et al.* 2008).
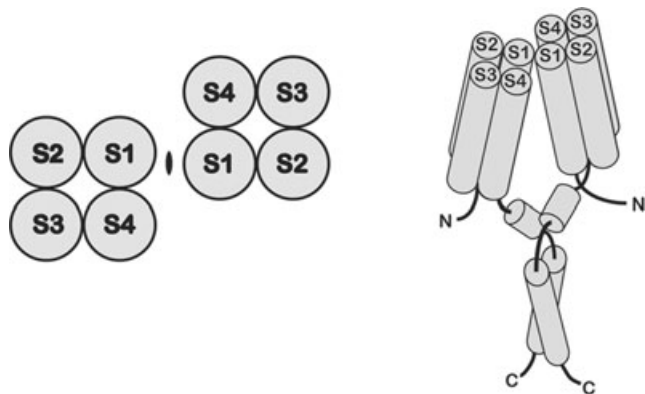
Figure 1. Topology of the voltage-gated proton channel
Voltage-gated proton channels resemble the voltage-sensing domain (VSD) of ordinary voltage-gated cation channels. Hydropathy plots of the protein coded for by the proton channel gene indicate four membrane-spanning regions that resemble S1–S4 of voltage-gated K$^+$ channels (Ramsey *et al.* 2006; Sasaki *et al.* 2006). The proton channel appears to assemble as a dimer (Koch *et al.* 2008; Lee *et al.* 2008; Tombola *et al.* 2008). The top view of the channel (with the membrane in the plane of the page) is on the left, the side view on the right. In the latter, the S1–S4 cylinders represent alpha helices spanning the membrane and both the N and C termini are intracellular. Proposed interaction sites are just external to S1 and within a predicted coiled-coil region in the C terminus. (From Lee *et al.* 2008, with permission.)

Is the functional channel really a dimer, and how independent are its pathways? This year, three groups presented diverse evidence indicating that the voltage-gated proton channel functions as a dimer, and each subunit has its own conduction pathway (Koch *et al.* 2008; Lee *et al.* 2008; Tombola *et al.* 2008). Before describing this evidence, it should be noted that these studies were exclusively based on the expressed proton channel gene product, not on measurements in native channels. In view of speculation that the oligomerization state might determine functional properties (Koch *et al.*

Table 1. Voltage-gated proton channel (HVCN1) family

GeneID	Protein identity (%)	Gene aliases	Organism	MW	Length (AA)
84329	100	VSOP, MGC15619, UNQ578/PRO1140	**Homo sapiens (Human)**	31 683	273
709745	93.4	LOC709745	*Macaca mulatta* (rhesus monkey)	31 523	273
616570	86.2	–	*Bos taurus* (cow)	31 872	272
608547	85.3	–	*Canis lupus familiaris* (dog)	31 216	268
74096	78.0	BTS, VSOP, 0610039P13Rik, AI450555	**Mus musculus (mouse)**	31 242	269
416871	53.5	RCJMB04–1c7	*Gallus gallus* (chicken)	27 599	235
496712	45.6	—	*Xenopus (Silurana) tropicalis* (western clawed frog)	26 575	230
496219	43.6	—	*Xenopus laevis* (African clawed frog)	26 596	230
436618	40.9	zgc:92181	*Danio (Brachydanio) rerio* (zebrafish)	27 110	235
778897	26.1	VSOP, VSX1	**Ciona intestinalis (transparent sea squirt)**	38 501	342
586317	22.3	VSOP	**Strongylocentrotus purpuratus (purple sea urchin)**	37 483	328

Characteristics of proteins coded by selected proton channel genes. Species for which the expressed gene product has been demonstrated by voltage clamp to function as a proton channel are in bold: human (Ramsey *et al.* 2006), mouse and *Ciona* (Sasaki *et al.* 2006), and sea urchin (personal communication, Y. Okamura). VSOP, voltage sensor domain-only protein. Identity with the human protein is from EMBL-EBI (http://www.ebi.ac.uk/Tools/es/cgi-bin/jobresults.cgi/needle). Some aliases are from UniProt (http://www.uniprot.org/). The isotopically averaged molecular weight (MW) of a monomer is given using Protein Calculator v. 3.3 (www.scripps.edu).

2008), a clear demonstration of the architecture of the assembled native proton channel, especially in phagocytes, would be most welcome.

Koch *et al.* (2008) tagged the C terminus of the mouse channel, mVSOP, with either HA or Myc, co-expressed the two constructs and then fished with antibodies to the tags. Each antibody detected the other tag, showing that the proton channel comprised multimers that contain both tags. They then used fluorescence resonance energy transfer (FRET) on the *Ciona intestinalis* proton channel, CiVSOP, engineering donor and acceptor fluorophores on the extracellular S3–S4 linker (at S242C), and determined a distance of 42.2 Å between them, a proximity consistent with multimerization. To determine the number of sub-units, they generated tandem dimers of CiVSOP in both sequences: WT-S242C and S242C-WT. Co-expression should lead to a FRET signal if tetramers formed, but there was no FRET, speaking against tetramerization, although not unequivocally ruling out a trimer, for example. Finally, Western blots of mVSOP revealed mainly monomers but with a faint band at the dimer position. After treatment with the cross-linker DSS (disuccinimidyl suberate), a strong dimer band was observed. There is evidently weak interaction in the cytoplasmic regions. This interaction was demonstrated by truncating N or C termini, or both. The channel still functioned (electrophysiologically) as a monomer with both C and N termini removed, although gating kinetics was altered. Evidently, the channel can function as a monomer, but usually occurs as a dimer. Clearly, each monomer contains an independent conduction pathway. The authors speculated that a trans-ition between monomer and dimer might underlie the 'activation' of proton channels in phagocytes.

MacKinnon's group (Lee *et al.* 2008) found that the human proton channel H_V1 migrated as a monomer on Western blots, but with increasing concentrations of the crosslinking agent DSS, predominantly occurred in dimer form. They introduced Cys residues to identify putative interaction sites. All mutants migrated as monomers in reducing conditions but mild to strong oxidation led to dimer formation, with key residues identified as C249 (one of two naturally occurring Cys in H_V1) in the C terminus (where coiled-coil interaction was proposed) and I^{127} near the external end of S1. The proposed interaction regions are shown in Fig. 1.

Tombola *et al.* (2008) used a completely different approach. They tagged the human proton channel with GFP and observed that photobleaching of the green fluorescent protein (GFP) occurred in two discrete steps, strongly suggesting that H_V1 assembles as a dimer. Next they introduced a Cys residue at an intracellular location (N214C, see Table 3) that proved to be accessible to the thiol modifying reagent MTSET; modification introduces a positively charged group that abolished current. Tandem dimers of all possible combinations, WT–WT, N214C–WT, WT–N214C and N214C–N214C, were compared. Currents were 40% inhibited with one mutant subunit and 90% with both. Two other MTS reagents were found to produce twice the inhibition in the double mutant as in a single mutant channel. These results are consistent with two independent pores, but conceivably (albeit improbably) could also occur if the

MTS reagents only partially reduced the current of a common pore. Dimerization was found to involve the cytoplasmic domain, using chimeras between H_V1 and CiVSP, a related phosphatase that is thought to exist as a monomer (Murata *et al.* 2005; Kohout *et al.* 2008). The N and C termini of H_V1 were required for dimer formation, assessed by photobleaching. Finally, enforcing monomeric expression by substituting both N and C termini from CiVSP into H_V1 resulted in proton currents that, like those reported by Koch *et al.* (2008), activated substantially more rapidly than the WT dimer.

In summary, the diversity of evidence indicating that the expressed proton channel exists as a dimer lends strong support to this conclusion. It also seems clear that each monomer forms a separate conduction pathway. As mentioned above, all of these studies were done on expressed proton channels, which in the next section are shown to have subtle but distinct differences from native channels. Furthermore, there is little or no evidence regarding whether the two protomers function independently or in a concerted manner (as speculated by Lee *et al.* 2008). Concerted gating might help explain the sigmoidal activation kinetics and certain peculiarities of gating charge measurements (below), and might conceivably be involved in the conversion of the resting channel to its 'enhanced gating mode' during phagocyte activation (as speculated by Koch *et al.* 2008).

Why are there differences between expressed and native proton channels? In almost every respect, the electrophysiological properties of the proton channel gene products resemble those of native proton channels. Features common to both include: (1) a depolarization-activated conductance; (2) sigmoidal activation kinetics; (3) generally slow activation kinetics; (4) potent inhibition by Zn^{2+} that, (a) slows current turn-on, (b) shifts the g_H-V relationship positively, and (c) exhibits profound pH dependence reflecting competition with H^+ for an external binding site; (5) a 40 mV shift of the g_H-V relationship when pH_o is increased by one unit or pH_i is decreased by one unit; (6) extraordinarily strong temperature dependence (Kuno *et al.* 1997; DeCoursey & Cherny, 1998; Ramsey *et al.* 2006); and (7) perfect selectivity for protons over all other ions. However, in a detailed study (Musset *et al.* 2008*a*), one difference was observed between expressed and native proton currents. In heterologous expression systems, expressed human and murine proton channels, H_V1 and mVSOP, both opened at potentials about 30 mV more negative at any given pH gradient, ΔpH, than did native proton channels in over a dozen cell types. As it turns out, this deviant behaviour affects a central characteristic of proton channels – the regulation of their gating by pH that results in only outward current in the steady state (ergo acid extrusion), as discussed below (Sensitivity

of gating to ΔpH). This aberrant voltage dependence may reflect a requirement for an additional co-factor or accessory protein, or a difference between the expression systems (COS-7 and HEK-293) and native cells, although the small endogenous proton currents in HEK-293 cells exhibit normal voltage dependence. Rather than speculate further, I will sit back and wait to see how this is resolved.

Permeation

How is perfect selectivity achieved? A hallmark of the voltage-gated proton channel is its extremely high selectivity. Selectivity determined from measurement of the reversal potential, V_{rev}, provides two clear results. First, when pH is changed, V_{rev} changes by an amount that approaches the Nernst potential for protons, E_H (DeCoursey, 2003). Second, when the predominant cation or anion in the bath is substituted, there is no detectable change in V_{rev} (Barish & Baud, 1984; Mahaut-Smith, 1989*a*; Bernheim *et al.* 1993; Demaurex *et al.* 1993; Kapus *et al.* 1993; DeCoursey & Cherny, 1993, 1994*a*, 1996*a*; Qu *et al.* 1994; Eder *et al.* 1995; Gordienko *et al.* 1996; Kuno *et al.* 1997) provided that liquid junction potentials are corrected (Neher, 1992) and that one avoids creating conditions in which Na^+-H^+ anti-port activity changes pH_i (DeCoursey & Cherny, 1994*a*; Demaurex *et al.* 1995; Klee *et al.* 1999). Calculated with the Goldman–Hodgkin–Katz voltage equation (Goldman, 1943; Hodgkin & Katz, 1949; Hille, 2001), the relative permeability of H^+ is 10^6-10^8 greater than that of any other ion (Demaurex *et al.* 1993; Kapus *et al.* 1993; Bernheim *et al.* 1993; DeCoursey & Cherny, 1994*a*,*b*, 1996*a*, 1997; Cherny *et al.* 1995, 2001*b*; Gordienko *et al.* 1996; DeCoursey *et al.* 2001*b*; Schilling *et al.* 2002), even under the improbable worst-case assumption that all deviation from Nernst is due to permeation of the other cation. This high relative permeability results in part from the extremely low proton concentration, typically $\sim10^6$ lower than the predominant cation. However, ion substitution experiments reveal that ion species other than H^+ have no detectable effect on V_{rev} and hence, the selectivity of the proton channel is effectively perfect.

Virtually all proton conduction occurs by a Grotthus-like mechanism (de Grotthuss, 1806; Pomès, 2006) in which the proton hops from one molecule to another. In the special case of a 'water wire', the proton hops from one water molecule to another, forming a hydronium ion, H_3O^+, at each stop along the way. A pivotal proposal by John Nagle and colleagues was that protons could cross membranes through proteins by a hydrogen-bonded chain (HBC) mechanism (Nagle & Morowitz, 1978; Nagle & Tristram-Nagle, 1983). The proton hops, as in the Grotthus mechanism, but the pathway may include hydroxyl, amino and carboxyl side groups of amino

Table 2. Proton conduction in water and through gramicidin and voltage-gated proton channels

Property	Bulk water	Gramicidin	Proton channel
Selectivity (P_{H^+}/P_{Na^+})	7[1]	38–60[2]	$>10^6$–10^{8}[3]
Deuterium isotope effect (I_{H^+}/I_{D^+})	1.41[4]	1.35[5]	1.9[6]
Activation energy (kcal mol^{-1})	2.6[7]	3–8[8]	18–27[9]

The activation energies listed are for proton permeation through open channels. The temperature dependence of gramicidin includes measurements of several modified dioxolane-linked channels. [1]Robinson & Stokes, 1959; [2]Myers & Haydon (1972); [3]DeCoursey (2003); [4]Lewis & Doody (1933); [5]Akeson & Deamer (1991); Chernyshev *et al.* 2003; [6]DeCoursey & Cherny (1997); [7]Robinson & Stokes (1959); [8]Akeson & Deamer (1991); Chernyshev & Cukierman, 2002; [9]DeCoursey & Cherny (1998).

acids as well as intervening water molecules. An HBC mechanism that included side chains would enable perfect proton selectivity; other ions are excluded (Nagle & Tristram-Nagle, 1983).

In Table 2, proton conduction through the voltage-gated proton channel is compared with proton conduction through the water-filled gramicidin channel, as well as with proton conduction in bulk water. The gramicidin channel has been useful as a model ion channel – it is relatively small, structurally simple and robust – it still functions at 5–6 M HCl (Eisenman *et al.* 1980; Cukierman *et al.* 1997)! The gramicidin channel has a narrow cylindrical pore that is filled with a single-file row of a dozen water molecules (Levitt *et al.* 1978). Gramicidin is a non-selective cation channel, but it conducts protons much better than any other ion, because protons can hop through a water wire without displacing the water molecules. In contrast a Na^+ or K^+ ion must wait patiently for the water molecules to diffuse through before it can permeate. For roughly the same reason, protons diffuse in bulk water 5 times faster than K^+ (Danneel, 1905; Robinson & Stokes, 1959). Gramicidin conducts up to 2×10^9 H^+ s^{-1} (Cukierman, 2000), faster than any other narrow-pore channel conducts any other ion. The impression that any proton that appears at the mouth of gramicidin permeates is supported by a nearly direct proportionality between proton current and proton concentration over 5 orders of magnitude (from pH 4.5 to pH −0.5) (DeCoursey, 2003). Proton conductivity of bulk water is similarly proportional to concentration up to pH ∼0 (Owen & Sweeton, 1941). In contrast, although the single-channel conductance of voltage-gated proton channels increases at lower pH_i, the increase is only 4-fold between pH_i 6.5 and 5.5 (Cherny *et al.* 2003). It would be intriguing to obtain single-channel currents at lower pH, but biological membranes seem unwilling to cooperate in this endeavour.

The impression from Table 2 is that proton permeation through gramicidin is not too different from conduction in bulk water, but permeation through voltage-gated proton channels is something completely different. Deuterons permeate both gramicidin and voltage-gated proton channels, but the isotope effect is substantially greater for permeation through voltage-gated proton channels. The temperature dependence of the open-channel conductance is stronger for the voltage-gated proton channel than for almost any other ion channel. Together with these factors, the concentration dependence mentioned above supports the conclusion that traversing voltage-gated proton channels is challenging for protons. The contrast between proton permeation through gramicidin, a water-filled ion channel, and voltage-gated proton channels has led to the proposal that the conduction pathway in the latter is not likely to comprise a simple water wire, but instead may comprise a HBC that includes at least one titratable group (DeCoursey & Cherny, 1994*a*,*b*, 1997, 1998; Henderson & Meech, 1999; Cherny *et al.* 2001*b*; Maturana *et al.* 2001; DeCoursey, 2003; Demaurex & Petheö, 2005).

Other proton selectivity mechanisms may exist, however. The perfect proton selectivity of the M_2 viral proton channel (Chizhmakov *et al.* 1996; Lin & Schroeder, 2001; Mould *et al.* 2000) has been explained by two different mechanisms. One is a traditional HBC mechanism in which protonation/deprotonation of a ring of His residues ensures selectivity (Pinto *et al.* 1997; Schweighofer & Pohorille, 2000; Shuck *et al.* 2000; Lear, 2003). Alternatively, channel opening might complete a water wire that experiences a constriction that is sufficiently narrow to prevent cation (or water) permeation, but still permits proton transfer between adjacent waters (Sansom *et al.* 1997; Kukol *et al.* 1999; Smondyrev & Voth, 2002; Kass & Arkin, 2005; Chen *et al.* 2007; Stouffer *et al.* 2008).

There are several examples of proton pathways that almost certainly involve protonation/deprotonation of a titratable group during proton conduction (DeCoursey, 2003, 2008). Strong candidates are produced by particular mutations of the voltage-sensing domain (VSD) of K^+ channels. Mutating any one of the first four Arg residues in S4 (the fourth putative membrane-spanning region, Table 3) to His produces proton selective transporters; R365H and R368H are proton carriers whereas R362H and R371H are voltage-gated proton channels with opposite voltage dependence (Starace *et al.* 1997; Starace & Bezanilla, 2001; Starace & Bezanilla, 2004). The central

Table 3. S4 regions of voltage-gated K$^+$ and H$^+$ channels and a voltage-sensing phosphatase (VSP)

Shaker	L	**R** 362	V	I	**R** 365	L	V	**R** 368	V	F	**R** 371	I	F	*K* 374	L	S	*R* 377	H	S	*K* 380	G	13 e_0
mSlo1	L	*R* 207	F	L	*R* 210	A	L	**R** 213	L	I	Q	F	S	*E* 219	I	L	Q	F	L	N	I	2.3 e_0
H$_V$1	L	I	L	L	**R** 205	L	W	*R* 208	V	A	*R* 211	I	I	N* 214	G	I	I	I	S	V	*K*	6 e_0
mVSOP	L	I	L	L	*R* 201	L	W	*R* 204	V	A	*R* 207	I	I	N	G	I	I	I	S	V	*K*	6 e_0
Ci-VSP	L	**R** 226	V	V	**R** 229	L	A	**R** 232	I	F	Y	S	H	Q	Q	M	K	A	S	S	R	1 e_0

A sequence of 21 amino acids in the putative S4 regions of the *Shaker* K$^+$ channel, the maxi-K calcium-activated K$^+$ channel (mSlo), the human proton channel (H$_V$1), the mouse proton channel (mVSOP), and a voltage-sensing phosphatase in *Ciona* (Ci-VSP). Potentially charged amino acids are indicated in bold. Single neutralization mutations at the numbered positions in red boxes reduce the gating charge; those in italics (in blue) do not affect gating charge, or were inconclusive (in green) (Aggarwal & MacKinnon, 1996; Seoh *et al.* 1996; Bezanilla, 2000; Murata *et al.* 2005; Ramsey *et al.* 2006; Sasaki *et al.* 2006; Ma *et al.* 2006; Musset *et al.* 2008a; Hossain *et al.* 2008). Total gating charge (e_0) estimates are listed in the final column; for H$_V$1 and mVSOP, these are from the limiting slope method (Musset *et al.* 2008a). The mouse R204Q mutant did not express well; R207Q was identical to wild type, and for R201Q activation was faster and the g_H–V relationship was shifted by −50 mV and had a slightly steeper slope (z = 1.9 *versus* 1.4 for wt) (Sasaki *et al.* 2006). In human proton channels, gating was faster for all three Arg mutants, and for R205A the midpoint was shifted positively, and the slope (from g_H–V relationships) was less steep by 1/3 (zδ = 0.57 *versus* 0.90 for wt) (Ramsey *et al.* 2006). The VSD of the Shaker K$^+$ channel can be transformed into a proton channel by the R362H mutation (Starace & Bezanilla, 2004), and R362X where X = Cys, Ala, Ser or Val produces non-selective cationic 'omega' current through the voltage sensor (Tombola *et al.* 2005). *The N214R mutation greatly attenuates conduction (Tombola *et al.* 2008). A ClustalW alignment of proteins containing S4 regions homologous to H$_V$1 was manually adjusted to reflect predicted transmembrane regions. In some cases, plausible alternative alignments can be obtained by shifting a sequence by three residues. These previously unpublished alignments were generously provided by S. M. E. Smith (Emory University).

Arg→His are alternately exposed to internal or external solutions during gating, whereas Arg→His positioned on either end apparently forms a solitary constriction that has simultaneous access to both sides of the membrane, one in the closed (R362H) and the other in the open (R371H) conformation of the central K$^+$ pore. If R^{362} is mutated into any of several other amino acids, the result is non-selective cation current through the VSD (Tombola *et al.* 2005, 2007). At two other locations in the VSD, Ile→His mutations (I241H in S1 and I287H in S2) produce proton channels (Campos *et al.* 2007). Finally, in the VSD of voltage-gated Na$^+$ channels, Arg→His mutation of one of the S4 residues (R663H) produces a proton selective channel (Struyk & Cannon, 2007), whereas Arg→X mutation produces non-selective cation current (Sokolov *et al.* 2005). It is difficult to imagine that the proton selectivity that is uniquely achieved by positioning a His residue at these locations does not result from protonation/deprotonation of the His during conduction. Unfortunately, it is also difficult to conceive an experiment that would reveal with certainty whether this is the case.

Finally, there are several recent examples of non-selective channels that conduct protons paradoxically well, including TRPV1 (Hellwig *et al.* 2004), TRP-ML1 (Soyombo *et al.* 2006), TRPM7 (Jiang *et al.* 2005; Numata & Okada, 2008) and colicin A (Slatin *et al.* 2008). The relative proton permeability, $P_{H+}/P_{Cs+} > 1000$ for TRPV1 and $P_{H+}/P_{Cs+} = 10^4 - 10^6$ for TRP-ML1, is astonishing, and that $P_{H+}/P_{K+} = 12\,000$ for colicin A is truly astounding in view of this channel having a lumen ∼10 Å in diameter (Slatin *et al.* 2008). Whether the proton permeability of these channels can be explained by conventional paradigms remains to be determined.

Where is the permeation pathway? Answering this question would be facilitated by knowing the structure. Although many multimeric channels form their conduction pathway at the interface between subunits, as discussed above, the proton channel appears to be a dimer and each subunit has its own pore (Koch *et al.* 2008; Tombola *et al.* 2008; Lee *et al.* 2008). At present, no published data exist that indicate which amino acids form the conduction pathway, although the N214R mutation greatly attenuates conduction, and thus may be near the inner mouth of the 'pore' (Tombola *et al.* 2008). The general similarity of the proton channel molecule to the voltage-sensing domain of a K$^+$ channel (Sasaki *et al.* 2006; Ramsey *et al.* 2006) is highly suggestive. The histidine scanning studies by Bezanilla and colleagues described above (Starace *et al.* 1997; Starace & Bezanilla, 2001; Starace & Bezanilla, 2004) demonstrate that the VSD has aqueous access channels that lead to a constriction that can provide access of a single (inserted) His residue to external

or internal solutions depending on protein movements that occur during gating (channel opening and closing). If the architecture of the proton channel follows this pattern, then the general framework for a proton pathway may reasonably occur in each monomer.

What is the concentration dependence of the proton conductance? Examination of macroscopic g_H data in many studies indicated that $g_{H,max}$ – the limiting g_H – increased only 2-fold per unit decrease in pH_i (DeCoursey, 1998). The prediction of the Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin & Katz, 1949; Hille, 2001) is a 10-fold increase, which presumes that entry into the channel is rate determining. This discrepancy suggested that permeation, rather than entry, was rate determining. The implicit assumption is that the same number of channels is open during a large depolarization at any pH_i. Surprisingly however, when determined at various pH_i values, the single-channel conductance increased from 38 fS to 140 fS at pH_i 6.5 and 5.5, respectively, nearly a 4-fold increase (Cherny *et al.* 2003). The open probability was 75% and 95%, respectively, in these conditions. The macroscopic and microscopic conductances can be reconciled if there is an inhibitory process at low pH_i that removes functional channels from the fray. Systematic study of this issue would be welcome, but has been hampered because biological membranes do not tolerate extremely low pH. Perhaps incorporation of proton channels into artificial lipid bilayers would enable direct observation over a wider pH range.

At the other extreme, it is curious at how high pH_i one could still detect proton currents. At high pH, free protons are exceedingly scarce and buffer (used at its pK_a) holds on to its proton tightly. At sufficiently high pH, deprotonation of buffer must become rate determining, at which point proton current should decrease in direct proportion to $[H^+]$ (Brönsted & Pedersen, 1923; Eigen & Hammes, 1963), assuming that buffer protonation is diffusion limited. Our attempts to probe this question have been limited by two factors: (1) cells do not survive well at extremely high pH; and (2) it is necessary to monitor V_{rev} to determine how well one has established the intended pH. Credible outward proton currents with Nernstian (i.e. reasonable) V_{rev} have been seen in excised patches at pH_i 8.5 with pH_o 7.5 (V. V. Cherny & T. E. DeCoursey, unpublished data), where only 3 nM permeant ion (H^+) was present.

Voltage gating

Is S4 the voltage sensor? It is well established that the S4 region contributes substantially to the ability of several voltage-gated ion channels to sense membrane potential (Noda *et al.* 1984; Stühmer *et al.* 1989). This said, it

should be emphasized that charged residues elsewhere in K^+ channels also contribute (Seoh *et al.* 1996), and the mechanism of gating remains controversial. As shown in Table 3, every third amino acid in S4 of the *Shaker* K^+ channel is a potentially charged Arg or Lys and each of the first four Arg residues appears to contribute to voltage sensing (Aggarwal & MacKinnon, 1996; Seoh *et al.* 1996; Bezanilla, 2000). Three of these Arg residues are conserved in the voltage-gated proton channel (Table 3). However, when these were mutated individually to neutral amino acids, only one appeared to reduce the effective charge moved during gating (channel opening) in the human channel (Ramsey *et al.* 2006), and none of them contributed in the mouse channel (Sasaki *et al.* 2006). One limitation of these studies is that they were based on Boltzmann fits to g_H–V relationships, which may not capture the entire gating charge moved (Sigworth, 1993). Preferable estimates are from the limiting slope of the g–V relationship (Almers, 1978; Sigworth, 1993). The gating charge movement estimated by this method is 12–14 e_0 for Na^+ or K^+ channels (Hirschberg *et al.* 1995; Schoppa *et al.* 1992; Aggarwal & MacKinnon, 1996; Seoh *et al.* 1996) and 5.4–8 e_0 for voltage-gated proton channels (DeCoursey & Cherny, 1996*b*, 1997; Musset *et al.* 2008*a*). Even more direct estimates of gating charge movement come from gating current measurements (Sigworth, 1993). Gating current occurs when channels open or close, but must be isolated from ionic current, for example by blockers. However, this measurement is nearly impossible for voltage-gated proton channels, because: (a) it is impossible to abolish ionic current by eliminating the permeant ion; (b) reducing the permeant ion concentration shifts the g_H–V relationship (see Sensitivity of gating to ΔpH) rather than permitting gating in the absence of ionic current; (c) attempts to measure gating currents at E_H (the Nernst potential for H^+), which would preclude ionic current, still did not reveal detectable gating current, probably because gating is very slow (V. V. Cherny, V. Sokolov & T. E. DeCoursey, unpublished data).

By the best available estimates, half as much gating charge moves in proton channels as in Shaker K^+ channels (Table 3). However, K^+ channels are tetramers, with four VSD and one pore; hence the charge moved is 3 e_0 per VSD. On the other hand, if proton channels are dimers with independently gated pores, the gating charge is 6 e_0 for each H^+ channel monomer, which is twice that for K^+ channels! If both monomers gate cooperatively, the charge would be 3 e_0 per proton channel monomer. Incidentally, if gating were cooperative but not precisely simultaneous, unitary H^+ currents roughly double the value estimated from noise measurements might be observed, as appears to be the case (Cherny *et al.* 2003). That only one Arg in S4 seems to contribute to gating charge movement presents a large discrepancy, and suggests that S4 may not be a

major participant in voltage sensing in proton channels. A similar conclusion was reached for BK channels, although their total gating charge is relatively petite (Ma *et al.* 2006). Proton current kinetics changed fairly dramatically even in the Arg mutations that did not affect total charge, however (Sasaki *et al.* 2006; Ramsey *et al.* 2006), suggesting that these groups do participate in gating. In summary, the voltage-sensing apparatus and its mechanism in proton channels are largely unknown.

How does gating work? Voltage-gated proton channels open and close like other ion channels. Single channel currents of ∼10 fA can just barely be resolved under highly favourable conditions: in excised patches, at low pH_i, with seal resistances in the teraohm range, and with no other conductances present (Cherny *et al.* 2003). Proton channel gating generates current fluctuations that exhibit archetypal stochastic behaviour. Distinct fluctuations (noise) appear at voltages at which the g_H is activated, and the variance of this noise increases to a maximum near the midpoint of the g_H–V relationship, precisely where one would expect the frequency of opening and closing transitions to be maximal. Beyond this, almost nothing is known about the gating mechanism. Given the likelihood of HBC conduction, a proton conduction pathway might appear or disappear in ways that would not be effective for gating conduction of other ions, so we need to keep an open mind. For example, subtle changes in the length or orientation of hydrogen bonds (Pauling, 1939; Scheiner, 1981) or in the pK_a of groups in the pathway could enable or disable a HBC.

The similarity between the proton channel and the VSD, combined with the demonstration that the K^+ channel VSD can be converted into a proton-selective channel by single mutations of Arg residues in S4 (Starace & Bezanilla, 2001, 2004) raises the obvious possibility that movement analogous to that which is thought to occur during VSD gating also occurs in the proton channel and results in channel 'opening.' Changes in accessibility of the four key Arg residues during K^+ channel gating led to the conclusion that they ratchet outwards past a solitary constriction, with the first and fourth Arg at the narrow point when the K^+ channel is closed or open, respectively (Larsson *et al.* 1996; Bezanilla, 2000; Horn, 2005; Tombola *et al.* 2006). Tombola *et al.* (2008) found that in H_V1, the N214R mutation abolished conduction and proposed that the proton channel opens when the polar Asn^{214} moves into this hypothetical constriction.

Some constraint on the gating process is placed by the extraordinarily strong temperature dependence of gating. Three parameters describing proton channel gating kinetics – the delay and activation time constant τ_{act} obtained from fitting proton current turn-on with a delay followed by a rising exponential, and τ_{tail} obtained by fitting tail current decay with a single exponential – all

had activation energies of 30–38 kcal mol^{-1} (Q_{10} values of 6–9) in six types of cells (DeCoursey & Cherny, 1998). Activation energies for the opening and closing of most ion channels, including K^+ channels (Bezanilla, 2000), are only about half this large. That all kinetic parameters for proton channel gating had similar large activation energies suggests that both opening and closing involve a single rate-limiting, energetically demanding transition that occurs in multiple independent subunits. Whether the two pores of the proton channel dimer gate independently or cooperatively has not been established.

What is the real kinetics of proton channels? From the beginning, it has always been evident that proton currents are prone to manifestations of depletion of the permeant ion. Since in many situations the purpose of proton currents is to change pH, this property should not come as a surprise. Nevertheless, someone who is familiar with K^+ currents for which the cellular concentration of permeant ion is 10^{-1} M is in for a rude awakening when studying channels for which the cellular concentration of permeant ion is 10^{-7} M. One should first of all be impressed that proton currents are as large as they are – in many cells they are as large or larger than any other conductance present, including K^+ currents (Byerly & Suen, 1989; DeCoursey, 1991; Demaurex *et al.* 1993; DeCoursey & Cherny, 1994*b*, 1996*a*; Femling *et al.* 2006). The conjunction of large currents and minuscule permeant ion concentration leads to depletion of the permeant ion. The most obvious manifestation of depletion is decay or droop of proton currents. Proton channels do not inactivate in any tissue studied to date (p. 532, DeCoursey, 2003). However, proton currents often decay during sustained depolarization. That the cause is proton depletion-induced pH changes has been verified by measuring pH_i by pH electrode (Thomas & Meech, 1982; Meech & Thomas, 1987), by fluorescent dye (Demaurex *et al.* 1993; Kapus *et al.* 1993; Schwiening & Willoughby, 2002), or by using the proton channel as a pH meter and measuring V_{rev} of proton currents (Barish & Baud, 1984; DeCoursey, 1991; Kapus *et al.* 1993; DeCoursey & Cherny, 1994*b*; Humez *et al.* 1995; Gordienko *et al.* 1996; Morihata *et al.* 2000; Schrenzel *et al.* 1996). There seems to be a spontaneous tendency for researchers to feel guilty about proton depletion, and to attempt to conceal droopy currents, for example. On the one hand, it makes sense to try to minimize depletion, because it compromises the measurements. On the other hand, it is dangerous to pretend that depletion is not occurring, because this can lead to gross misinterpretation of data.

Proton currents are more susceptible to depletion effects than other ion channels for several reasons. (1) The proton concentration is very low. (2) Buffer diffuses more slowly than small monovalent ions. Essentially all of the H^+ that carries current was immediately previously bound

to buffer. A 10 μm diameter spherical cell dialysed with 100 mM buffer at its pK_a contains only 315 000 free H^+ at pH 6 (DeCoursey, 2003). These protons can carry a 100 pA H^+ current for only 0.5 ms. Although there are 1.6×10^{10} protonated buffer molecules in the cell, 6.25×10^8 (4%) are consumed each second of the 100 pA H^+ current. If they are not replenished by diffusion from the pipette solution, pH_i will increase. (3) Finally, proton channels in most mammalian cells open quite slowly, with activation time constants, τ_{act}, in the range of seconds, in contrast with a few milliseconds in snail neurones (Byerly *et al.* 1984). By the time what appears to be a steady-state level of activation is achieved during the pulse, depletion has already occurred. Figure 2*A* illustrates this phenomenon. In order to determine the voltage dependence of 'steady-state activation' of the g_H, one typically applies a family of pulses. The current at the end of the pulse can be used to calculate the conductance, using V_{rev} measured previously by the tail-current method (Hodgkin & Huxley, 1952). These values are plotted as blue circles, and they appear to saturate and could be fitted by a Boltzmann function, as we are wont to do. However, the g_H ought to be identical at the start of the tail current, and many purists prefer measuring tail current amplitude, because this precludes worries about open-channel rectification. These values are plotted as red diamonds and they show little inclination to saturate. What has happened, of course, is that each pulse removed protons from the cytoplasm, so that pH_i was higher at the end of the pulse, and probably also at the start of the next pulse than it was for the previous pulse. The estimates of V_{rev} in Fig. 2*B* confirm a 9 mV depolarizing shift of V_{rev} by the end of the pulse family. Consequently, calculating g_H using a single V_{rev} value does not reflect the true situation. We can attack this problem by realizing that we could use these interpolated V_{rev} values for each pulse, and obtain the true g_H at that instant (green squares). The only problem is that our goal was to determine the g_H at one particular pH_i, not over a range of continuously changing pH_i that spans 0.15 pH units. This distinction is not trivial, because as we will see in the next section, the position of the g_H-V relationship depends strongly on pH_i.

Obviously, increasing the buffer concentration reduces the rate and extent of depletion (DeCoursey, 1991; Kapus *et al.* 1993; Demaurex *et al.* 1993; DeCoursey & Cherny, 1994*b*, 1996*b*; Kuno *et al.* 1997). Most labs routinely use $\geq$ 100 mM buffer, which reduces but does not prevent depletion; in the experiment in Fig. 2 there was 100 mM buffer in all solutions. Figure 3 illustrates the effects of buffer concentration on proton currents in an excised inside-out patch of membrane. Several features are remarkable. First, it is clear that depletion occurs even in excised patches. One can easily imagine that in whole-cell configuration depletion might be a problem, but both H^+

currents and unstirred compartment volumes ought to be much smaller in patches. Still, the membrane migrates some distance up from the tip of the pipette (Milton & Caldwell, 1990; Ruknudin *et al.* 1991), so there may be a substantial unstirred volume from which protons will be removed by depolarizing pulses (in inside-out patches). The impression from Fig. 3*A* is that the pulses begin well, but increasingly, the current at lower [buffer] sags below the one at higher [buffer]. Although frank droop is seen only during the pulse to +60 mV, it is glaringly obvious
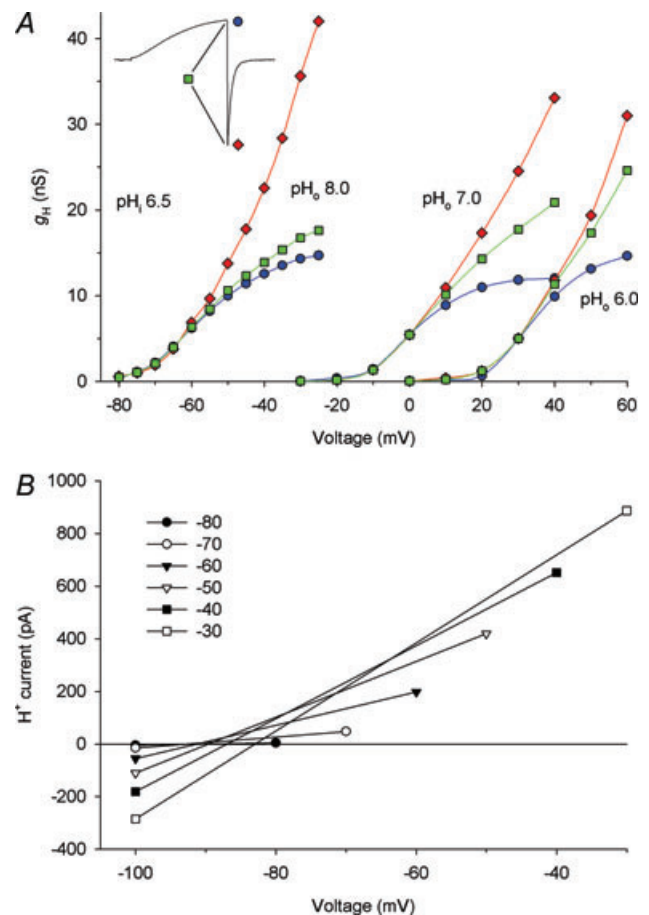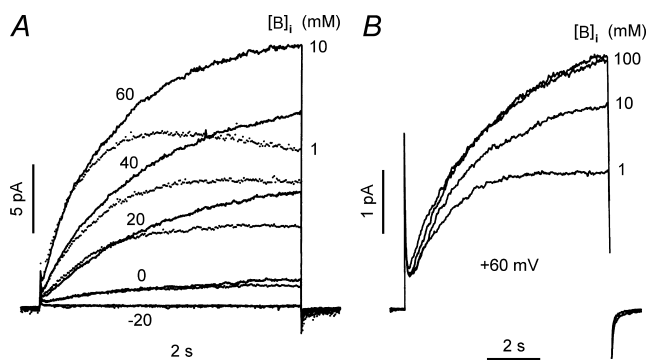

Figure 2. Proton depletion complicates measurement of the voltage dependence of the proton conductance, g_H

A, the g_H-V relationship estimated by three methods at each of three pH_o values in a COS-7 cell transfected with the mouse proton channel gene, mVSOP. The pipette contained pH_i 6.5 with 100 mM BisTris buffer. At pH_o 8.0, 2 s pulses were applied in 5 mV increments every 20 s from $V_{hold} = -100$ mV. At pH_o 7.0 or 6.0, V_{hold} was -60 or -40 mV, respectively, and 6 s pulses were applied every 20 s. As indicated in the inset, g_H was estimated from proton current measured at the end of each pulse (I_{end}, blue circles) or at the start of the tail current (I_{tail}, red diamonds) assuming a single constant measured value for V_{rev}. Alternatively, g_H was calculated as the slope conductance (green squares) between I_{end} and I_{tail}, which does not require an estimate of V_{rev}. *B*, actual V_{rev} estimated by the *X* axis intercept of a line connecting I_{end} and I_{tail} for the indicated pulses in the same cell at pH_o 8.0. (From Musset *et al.* 2008*a*.)

that the time course of the current is radically different at low [buffer], even at +20 mV where the current is much smaller. Clearly, lack of droop does not signify that the time course of the current reflects pure gating kinetics. At lower [buffer], τ_{act} will be artificially fast. In Fig. 3*B*, we see that 10 mM buffer was not enough, despite the normal appearance of the family in Fig. 3*A*, because the currents are larger and 'activate' even more slowly at 100 mM buffer. We sometimes use 200 mM buffer, after Gabor Petheö and Nic Demaurex showed us this was possible, but even then, can we be sure that the time course of the current reflects the true time course of gating? The deconvolution of the true gating kinetics of proton channels from diffusional effects on the time course of recorded currents may require serious modelling. In the meanwhile, it is best to remember that much of what you see is not a direct reflection of gating kinetics, and that one must guard against being seduced into over-interpreting data.

Depletion is more pronounced in whole-cell configuration. Proton accumulation outside the cell (Zifarelli *et al.* 2008*b*) and proton depletion inside the cell (Swietach *et al.* 2003) during constant proton efflux have been modelled. Certainly depletion during proton efflux is more severe inside the cell, and replenishment depends on diffusion of protonated buffer from the pipette solution, whose rate of equilibration has also been evaluated (Pusch & Neher, 1988). The effective diffusion coefficient for protons in cells is slowed by the effects of fixed and mobile intrinsic buffers (Junge & McLaughlin, 1987) by at least

two (Vaughan-Jones *et al.* 2002; Swietach *et al.* 2003) or even three orders of magnitude (Stewart *et al.* 1999), particularly at low pH (Swietach & Vaughan-Jones, 2005; Swietach *et al.* 2007) where the intrinsic buffering capacity is increased (p. 483, DeCoursey, 2003). In practical terms, establishing control of pH$_i$ by 100 mM buffer requires 8–10 min in large cells of diameter 120 μm (Byerly & Moody, 1986), and 1–3 min in small cells of diameter 10–20 μm (Demaurex *et al.* 1993; Kapus *et al.* 1993), depending on pipette geometry. Adequate control is never achieved at low (<20 mM) buffer concentration (Byerly & Moody, 1986; DeCoursey, 1991; Demaurex *et al.* 1993).

The inevitability of depletion has several consequences. As mentioned, one must be alert to this phenomenon and not over-interpret data. With depletion in mind, we often use the threshold for channel activation, $V_{threshold}$, as a rough indicator of the voltage dependence of gating. This parameter is arbitrary, because if g_H decreases exponentially with hyperpolarization, no true threshold exists; the $V_{threshold}$ detected will depend on $g_{H,max}$ and noise levels. However, $V_{threshold}$ is a useful and practical compromise for several reasons. It is easy to determine. It precludes the need for large depolarizations that cause extensive depletion. By definition, currents near $V_{threshold}$ are very small and produce little depletion. It does not assume any specific gating model, nor does it require force-fitting data to a Boltzmann function. In practice, $V_{threshold}$ gave results similar to more traditional methods of analysis (Musset *et al.* 2008*a*). We suspect that $V_{threshold}$ is a more reliable indication of the voltage dependence of proton channel gating than parameters obtained from Boltzmann fits, which are strongly influenced by depletion and the specific conditions of the measurements.

Sensitivity of gating to ΔpH

Which groups sense pH$_o$ and pH$_i$? A quintessential feature of voltage-gated proton channels is that their voltage dependence is strongly regulated by the pH gradient, ΔpH (defined as pH$_o$ – pH$_i$) (Cherny *et al.* 1995). The g_H–V relationship shifts 40 mV per unit change in ΔpH over a wide range encompassing all physiologically attainable values. The result of this extraordinary regulation by pH is that proton channels open only when the electrochemical proton gradient is outward, when opening will result in acid extrusion from the cell. Consequently, it is universally accepted that acid extrusion, especially in situations of excessive metabolic activity, is a major function of voltage-gated proton channels. All native voltage-gated proton channels share the property of ΔpH-dependent gating, and the voltage at which the g_H first turns on, $V_{threshold}$, can be predicted by: $V_{threshold} = 0.79\,V_{rev} + 18\,\text{mV}$ (DeCoursey, 2003). This behaviour can be modelled quite simply by assuming

Figure 3. High buffer concentration reduces but does not eliminate depletion

These records were obtained in inside-out patches of membrane from two rat alveolar epithelial cells, at pH$_o$ 7.5 and pH$_i$ 6.5. As one might expect, diffusion limitation is less severe in patches than in whole-cell configuration. However, depletion still occurs in patches. *A*, the currents in were recorded at the indicated voltages during pulses from a holding potential of −40 mV with [buffer] in the bath (corresponding to intracellular) either 10 mM (continuous lines) or 1 mM (dotted lines). *B*, proton currents in another patch during pulses to + 60 mV at the indicated BisTris concentrations. Note the radically different kinetics at different buffer concentration. Pulses were applied at 100 mM buffer before and after the other concentrations were applied. (From DeCoursey & Cherny, 1996*b*.)

that protonation of sites on the channel accessible to the external solution stabilizes the closed channel and protonation from the inside stabilizes the open channel. The model works only if the regulatory protonation sites are accessible to just one side of the membrane at a time, and accessibility is transferred by an unspecified conformational change in the protein (Cherny *et al.* 1995). At this moment, we have no idea what or where these protonation sites are, or whether these hypothetical sites exist at all.

An informal survey of amino acid mutations that significantly alter pH sensing in 35 membrane proteins revealed the following: 20 involved His residues, singly or in clusters of up to six, 15 involved Glu, 7 Asp, 6 Arg, 6 Lys, and 3 Gly. The apparent preference for Glu over Asp is surprising, but may be fortuitous. In some cases, a single residue seems to provide the entire pH sensitivity. In many others, the pH sensitivity seems to involve a complex inter-play between groups of nearby amino acids; sometimes mutation of only one residue of the group has no effect. The ClC-0 chloride channel is sensitive to pH_i, but when mutation of 22 candidate amino acids failed to reveal a sensor, the idea of a proton sensor was abandoned in favour of a different mechanism involving OH^- (Zifarelli *et al.* 2008*a*).

How do the pH sensors communicate with the voltage sensor? The mechanism of this coupling has not even been imagined. One can hardly speculate on this mechanism, despite its obvious importance, because we do not know what or where the sensors are or how gating works.

Pharmacology

Zinc's secrets revealed! One structure–function question has been solved, namely the location of the external Zn^{2+} binding site on the human proton channel. Zn^{2+} is among the most potent inhibitors of voltage-gated proton channels (Mahaut-Smith, 1989*b*). The effects of Zn^{2+} on proton currents closely resemble the effects of poly-valent cations on nearly all voltage-gated ion channels; namely, the g_H-V relationship is shifted positively and the turn-on of current during depolarizing pulses is slowed (larger τ_{act}). Qualitatively, these effects correspond to the electrostatic effects of positioning two positive charges at the outer end of the channel – with the result that the voltage sensor is tricked into thinking the membrane potential is more negative than it really is (Frankenhaeuser & Hodgkin, 1957). The efficacy of Zn^{2+} in inhibiting the proton channel is greatly reduced at low pH_o, and the details of the competition between H^+ and Zn^{2+} could be explained only by assuming that the external Zn^{2+} binding site comprised 2 or 3 (but not 1) titratable groups with pK_a

6.2–7.0, suggesting His residues (Cherny & DeCoursey, 1999). The human proton channel turns out to have two His residues that are accessible to the external solution, and their mutation individually to Ala lowers Zn^{2+} sensitivity by an order of magnitude; the double His→Ala mutant has only weak Zn^{2+} sensitivity (Ramsey *et al.* 2006).

To a rough approximation, mouse and human proton channels have similar Zn^{2+} sensitivity (Ramsey *et al.* 2006; Sasaki *et al.* 2006), but the *Ciona* proton channel is less sensitive (Sasaki *et al.* 2006). There are several His residues at suggestive locations in the mouse proton channel, and fewer in the *Ciona* channel (Sasaki *et al.* 2006; DeCoursey & Cherny, 2007), but whether this explains the weaker Zn^{2+} binding remains to be determined.

The internal Zn^{2+} binding site has not been identified. Applied intracellularly, Zn^{2+} has less dramatic effects that are qualitatively consistent with surface charge effects, namely shifting the g_H-V relationship negatively, slowing tail current decay (larger τ_{tail}) and decreasing the limiting g_H, $g_{H,max}$ (Cherny & DeCoursey, 1999; Petheö *et al.* 2003).

What about a potent, selective inhibitor? Unfortunately, Zn^{2+} is not a very selective inhibitor, which greatly hampers attempts to evaluate possible functions of proton channels. A potent and selective toxin or venom would be very handy, but so far, none has been discovered.

How are H+ channels regulated?

Where is(are) the phosphorylation site(s)? In phagocytes, voltage-gated proton channels can be transformed into an 'enhanced gating mode' that seems to coincide with NADPH oxidase activity (Bánfi *et al.* 1999). As illustrated in Fig. 4, agents that activate NADPH oxidase, such as PMA
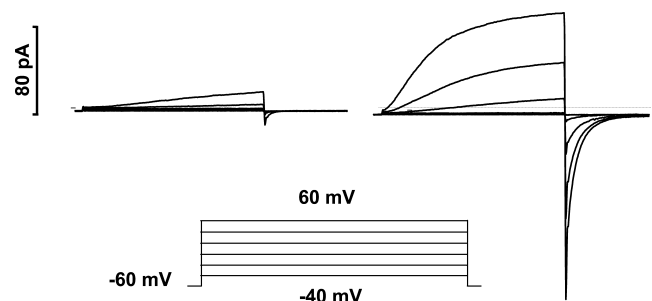
Figure 4. Agents that activate NADPH oxidase in phagocytes also greatly enhance the opening of proton channels
Identical families of pulses (inset) were applied to a human eosinophil before (left) and after (right) addition of PMA to the bathing solution. The enhanced gating response can be observed in perforated-patch configuration, but not whole-cell. This cell was exposed to a pH 7.0 bath solution with 50 mM NH_4^+ on both sides of the membrane to clamp pH_i near pH_o. (From Morgan *et al.* 2007.)

or arachidonic acid, profoundly alter the gating of proton channels. PMA shifts the g_H-V relationship by -40 mV, speeds activation, slows deactivation and increases $g_{H,max}$ (DeCoursey *et al.* 2000, 2001*a*,*b*; Bankers-Fulbright *et al.* 2001; Cherny *et al.* 2001*a*). These effects are prevented or largely reversed by the PKC inhibitors GF109203X (GFX) or staurosporine (Bankers-Fulbright *et al.* 2001; Mori *et al.* 2003; Morgan *et al.* 2007), which suggests that phosphorylation by PKC of either the channel or a regulatory molecule creates the enhanced gating mode. On a more phenomenological level, PKC stimulates both NADPH oxidase activity and proton efflux in intact human neutrophils (Henderson *et al.* 1987; Nanda & Grinstein, 1991; Kapus *et al.* 1992). Accepting that phosphorylation is a major mechanism of proton channel regulation, where is(are) the phosphorylation site(s)? The next question is, by what mechanism do these sites exert such a strong influence on proton channel gating? Does the level of 'activation' of proton channels reflect a balance between phosphorylation and dephosphorylation? If so, which kinases and phosphatases are involved?

Does phosphorylation account entirely for proton channel 'activation' in phagocytes? When GFX, a fairly selective PKC inhibitor, is applied to an activated eosinophil, the electron current turns off completely (Fig. 5), indicating that sustained NADPH oxidase activity requires ongoing PKC activity. At the same time, the enhanced features of proton currents are largely, but not completely reversed (Morgan *et al.* 2007). Two explanations for the less complete reversal of proton channel modulation are possible. First, it might be that deactivation reflects the activity of phosphatases, and those that dephosphorylate proton channels are less active then those that turn off NADPH oxidase. The phosphatase inhibitor, okadaic acid, does slow the reversal

of proton current enhancement by GFX (Morgan *et al.* 2007). Alternatively, the activation of proton channels may involve something more than PKC phosphorylation. If there are additional or alternative pathways of activating proton channels, what are they?

What is the relationship between proton channels and NADPH oxidase? This is one of the most intriguing mysteries regarding the activity of proton channels. As mentioned in 'Has the right gene been identified?' the preponderance of evidence speaks against gp91[phox] functioning as a proton channel. However, there remain several inexplicable interactions between these two molecules. The existence of these interactions contributed substantially to the attraction of the idea that gp91[phox] might be a proton channel.

1. The first indication of a liaison between proton channels and NADPH oxidase came from studies of neutrophils from patients with chronic granulomatous disease (CGD). In this hereditary disease, any of several hundred known mutations to one of the several critical components of the NADPH oxidase complex that results in enzyme dysfunction produces a severe susceptibility to infections that leads to early morbidity, unless the patient is actively managed (Babior, 1991). PMA-induced pH changes that were interpreted as reflecting conductive proton efflux were substantially diminished in CGD cells, compared to normal cells (Nanda *et al.* 1993). Examination of patients with a variety of specific mutations led to the conclusions that: (1) gp91[phox] is not a proton channel, because proton currents persist in its absence; (2) nevertheless, normal assembly of NADPH oxidase is required for activation of the proton conductance (Nanda *et al.* 1994*a*,*b*).
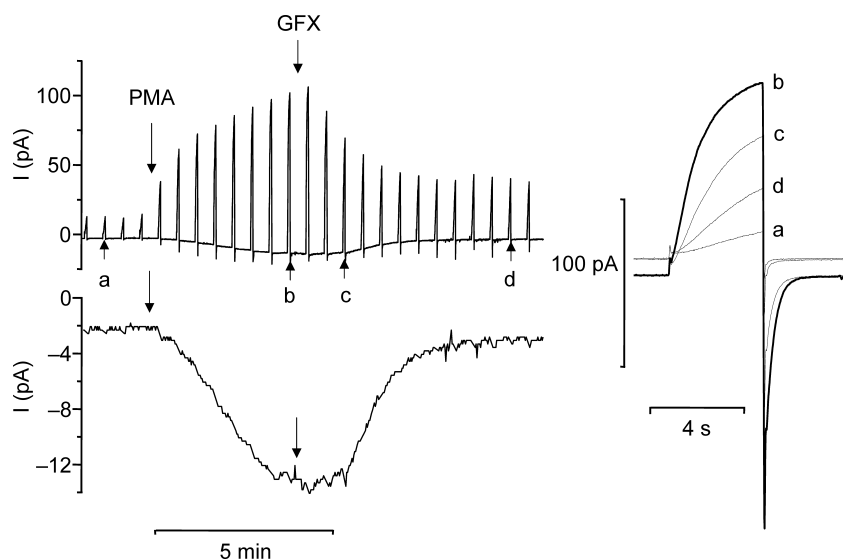
Figure 5. The enhanced gating mode is partially reversed by the PKC inhibitor, GFX
From a holding potential of -60 mV, test pulses were applied every 30 s to $+60$ mV, resulting in the upward deflections. Where indicated, 60 nM PMA was added, stimulating increased proton current as well as inward current at -60 mV. This inward current (shown at amplified gain below) is electron current that is generated by the activity of NADPH oxidase, and can be inhibited by the NADPH oxidase inhibitor DPI (diphenylene iodonium). Examples of proton currents during test pulses at right are labelled with lower case letters that show the time each was recorded. At the second arrow, 3 μM GFX was added. (From Morgan *et al.* 2007.)

2. Proton currents are converted into the enhanced gating mode by numerous activators of NADPH oxidase (Figs 4 and 5). NADPH oxidase activity was first associated with proton channel 'activation' in a whole-cell study in which ingredients supportive of NADPH oxidase activity were included in the pipette solution (Bánfi *et al.* 1999). In perforated-patch studies, agonists that activate both NADPH oxidase and proton channels include: PMA, AA (arachidonic acid), oleic acid, LTB4 (leukotriene B4), IL-5 (interleukin-5), fMLF (formyl-methionyl-leucyl-phenylalanine, a chemotactic peptide) and spontaneous activation presumably due to adherence (DeCoursey *et al.* 2000, 2001*a*,*b*; Bankers-Fulbright *et al.* 2001; Cherny *et al.* 2001*a*; Mori *et al.* 2003; Morgan *et al.* 2007).

3. Proton channels can sense whether NADPH oxidase is working or not. One of the characteristics of proton channels in the enhanced gating mode is profound slowing of tail current decay, with τ_{tail} increasing by 4- to 6-fold (Bánfi *et al.* 1999; DeCoursey *et al.* 2000, 2001*a*, 2001*b*; Morgan *et al.* 2007). When the NADPH oxidase inhibitor DPI is introduced, the electron current turns off and at the same time, τ_{tail} returns toward its original rapid kinetics (DeCoursey *et al.* 2000, 2001*a*). DPI only affects 'activated' proton channels, not resting channels in unstimulated cells (DeCoursey *et al.* 2000). However, there is no immediate effect of DPI on the other enhanced gating properties of proton channels. The association of slowing of τ_{tail} with electron currents is uncanny. In experiments on differentiated HL-60 cells, a fraction of cells responded to PMA stimulation with characteristic changes in proton currents seen in other phagocytes; τ_{tail} slowed appreciably only in cells in which electron current was detected (V. V. Cherny, D. Morgan and T. E. DeCoursey, unpublished data).

4. On the other hand, all of the features of enhanced gating except for τ_{tail} (including the -40 mV shift of the g_H-V relationship that produces inward currents) remain enhanced when NADPH oxidase is inhibited by DPI or by removal of substrate O_2 (Bánfi *et al.* 1999). Thus, most enhanced gating features do not require NADPH oxidase activity (electron current).

5. An extension of this phenomenon is observed in granulocytes from patients with CGD. Stimulation of neutrophils or eosinophils from CGD patients by PMA produces most of the enhanced gating features of proton channels that occur in normal cells, except the negative shift of the g_H-V relationship is smaller and τ_{tail} does not slow appreciably (DeCoursey *et al.* 2001*b*; DeCoursey, 2003). By definition, there is no electron current in these cells. Presumably the only difference between these and normal cells is the dysfunction of NADPH oxidase, yet the proton channel response is different.

6. A similar phenomenon occurs in human basophils. These leucocytes are developmentally related to eosinophils and, more distantly, to neutrophils, but they do not express detectable levels of NADPH oxidase (de Boer & Roos, 1986). In a recent study, we found that basophils respond to PMA almost identically to CGD cells: the shift of $V_{threshold}$ is only -20 mV instead of -40 mV, and τ_{tail} is slowed negligibly (Musset *et al.* 2008*b*).

7. In non-leukocytic cells that do not express high levels of NADPH oxidase, there is little evidence of the enhanced gating mode. So far, this has been tested only in rat alveolar epithelial cells (DeCoursey *et al.* 2000), and in HEK-293 and COS-7 cell lines (Musset *et al.* 2008*a*). Nevertheless, the coincidence of proton channel enhancement with NADPH oxidase expression is striking.

In the case of proton channels sensing NADPH oxidase activity (no. 3 above), one proposed mechanism is via local pH (DeCoursey *et al.* 2000). NADPH oxidase activity in phagocytes generates large quantities of cytoplasmic protons (van Zwieten *et al.* 1981; Gabig *et al.* 1984; Borregaard *et al.* 1984), and according to the model of proton channel gating, the effect of intracellular protons is to stabilize the open proton channel (Cherny *et al.* 1995). If sufficient protons were generated in the vicinity of proton channels, this could account for τ_{tail} slowing, at least qualitatively. Rough calculations do not support the likelihood of sufficiently large pH changes if random distribution of both molecules in the plasma membrane is assumed (p. 553–554, DeCoursey, 2003), but any number of *ad hoc* explanations could be offered. In summary, no mechanistic explanation for the cross-talk between proton channels and NADPH oxidase has been established.

How about PI(3,4)P$_2$? The stability of the enhanced gating mode of proton channels in inside-out patches appears to be supported by a combination of ATP, GTPγS and PI(3,4)P$_2$ (phosphoinositide 3,4-bisphosphate). The mechanism was hypothesized to include interaction with the p47^{phox} component of NADPH oxidase (Petheö *et al.* 2006), adding to the intriguing question of the nature of the relationship between proton channels and NADPH oxidase. The details of the interactions of these regulatory molecules with proton channels need further clarification.

What are the functions of proton channels?

What does the KO look like? What are the consequences of eliminating the voltage-gated proton channel? One could ask the function of proton channels in each of the cells where they occur. Specific functions that have been proposed in various cells are reviewed elsewhere (DeCoursey, 2003, 2008) and will not be reiterated here. With a few exceptions, the proposed functions are based mainly on pharmacological lesion experiments using Zn^{2+} in combination with varying amounts of pure speculation.

The identification of the proton channel gene now enables several new approaches to this question. Both groups who identified the gene (Ramsey *et al.* 2006; Sasaki *et al.* 2006) have generated knockout mice. We eagerly await reports of the manifestations of this genetic loss. Of course, in many knockouts, the functions performed by the missing protein are compensated by other molecules that may not normally act in this capacity. But this puts the apologetics before the horse. Antibodies to external epitopes on the proton channel that alter function in intact cells would be very useful, but to my knowledge do not yet exist. It is now also possible to use siRNA (small interfering RNA, or silencing RNA) to knock down the activity of the channel. The cautionary note for this approach is that the knockdown must be nearly complete. Ion channels are activated (i.e. they open) in a probabilistic manner. Even if 63% (e^{-1}) of all functional proton channels were eliminated, the cell would need only 4.2 mV additional depolarization – the limiting slope of the g_H–V relationship (Musset *et al.* 2008*a*) – to activate the same proton conductance. Whether this small extra depolarization would significantly affect anything else in the cell is not clear.

The converse of eliminating proton channel function is increased proton conductance. In brown fat cells, a proton leak across mitochondrial inner membrane generates heat. The inappropriate appearance of proton current occurs with certain mutations that insert His into the VSD of a Na^+ channel, and may be responsible for some of the consequences of hypokalaemic periodic paralysis (Struyk & Cannon, 2007).

In general, any of the consequences of proton channel activity may serve a useful function in a cell. These consequences include: (1) electrical effects, such as hyper-polarization of the membrane or charge compensation; (2) increasing pH_i; (3) decreasing pH_o; (4) osmotic effects; and (5) other specialized consequences. Specific situations have been identified in which each consequence of proton channel activity plays an important role.

Charge compensation. Opening proton channels will drive the membrane potential toward the Nernst potential for H^+. Since proton channels open mainly when the electrochemical gradient for protons is outward, their activity tends to hyperpolarize the membrane. The importance of electrical effects has been shown abundantly in phagocytes, where proton efflux balances the electrical consequences of the electrogenic electron flux through NADPH oxidase. NADPH oxidase works by translocating electrons from cytoplasmic NADPH across the membrane to reduce O_2 to superoxide anion, $O_2 \cdot^-$ (Henderson *et al.* 1987). As soon as NADPH oxidase is activated, there is rapid and profound depolarization (Seligmann & Gallin, 1980; Whitin *et al.* 1980; Lazzari *et al.* 1986; Di Virgilio *et al.* 1987; Henderson *et al.* 1987; Pugin *et al.* 1997; Geiszt *et al.*

1997; Jankowski & Grinstein, 1999; Bankers-Fulbright *et al.* 2003; Rada *et al.* 2004; Demaurex & Petheö, 2005), which is exacerbated by Zn^{2+} (Henderson *et al.* 1987; Bánfi *et al.* 1999; Bankers-Fulbright *et al.* 2003; Rada *et al.* 2004; Demaurex & Petheö, 2005). Zn^{2+} has no direct effect on NADPH oxidase (Yatsuyanagi & Ogiso, 1988; Schrenzel *et al.* 1998; DeCoursey *et al.* 2003), but potently inhibits proton channels (Thomas & Meech, 1982; Mahaut-Smith, 1989*b*; Cherny & DeCoursey, 1999; DeCoursey & Cherny, 2007). When enough proton channels are open, H^+ efflux balances electron extrusion, and there is no further change in membrane potential. If the electronic charge were not compensated, depolarization would reach levels that directly inhibit NADPH oxidase by opposing electron translocation (DeCoursey *et al.* 2003).

A recent speculative explanation for the need for proton channels in basophils is to compensate electrically for Ca^{2+} influx, which is required for anti-IgE-mediated histamine release (Musset *et al.* 2008*b*).

Acid extrusion (increasing pH_i). The classical way to demonstrate involvement of proton channels in acid extrusion is to load a cell with acid, either by the NH_4^+ pre-pulse method (Roos & Boron, 1981) or by direct injection of HCl (Thomas & Meech, 1982), and then observe the recovery. Under conditions in which other acid extrusion mechanisms are precluded, Zn^{2+} inhibits pH_i recovery in many cells (Nanda *et al.* 1992; Nordström *et al.* 1994, 1997; Demaurex *et al.* 1996; Kuno *et al.* 1997; Sheldon & Church, 2002; Murphy *et al.* 2005; Cheng *et al.* 2008). A complementary result is that transfecting mVSOP into HEK-293 cells greatly accelerated their recovery from an acid load (Sasaki *et al.* 2006).

Acid secretion (lowering pH_o). The distinction from acid extrusion is mainly teleological – the former has a 'goal' of maintaining pH_i in an optimal range for cellular functions, whereas the 'intent' of acid secretion is to regulate the acidity of the extracellular environment. In the case of respiratory epithelium, proton channel activity may serve to maintain the airway surface liquid at a moderately low pH (Fischer *et al.* 2002; Schwarzer *et al.* 2004), although additional functions have been proposed (Schwarzer *et al.* 2004; Fischer, 2007).

Osmotic effects. This function has been suggested to occur in the phagosome, where it might more properly be termed 'preventing osmotic effects.' Since NADPH oxidase activity extrudes enormous quantities of electrons that require charge compensation, as well as pH compensation, precisely which ionic species does the compensation becomes an important consideration. If all of the charge were compensated by K^+ flux into the phagosome, for example, the osmotic effect would be at least a 20-fold swelling (Murphy & DeCoursey, 2006). As the observed

swelling is much less (Reeves *et al.* 2002), it is clear that most of the compensation occurs by H^+ flux. The osmotic consequences of H^+ flux into the phagosome are mild because most of the products of reactions involving H^+ that occur in the phagosome (e.g. H_2O, H_2O_2, HOCl) are membrane permeable.

Other consequences. Another important function of proton flux in phagocytes is to provide the H^+ needed in large quantities as a substrate for the production of several reactive oxygen species (H_2O_2, HOCl, etc.) that kill microbes (Klebanoff, 2005; Nauseef, 2007; Rada *et al.* 2008). Proton flux into the phagosome delivers this substrate to the site of its utilization.

Conclusion

During the interval between the discovery of voltage-gated proton channels and the identification of their gene, many of the fundamental properties of these channels were determined. Now that the gene has been identified, a host of questions can be addressed, including the whole gamut of structure–function questions. Genetic approaches may facilitate further understanding of the functions of proton channels in the many cells that express them. The surprising similarity of the proton channel architecture to the VSD of other voltage-gated ion channels has the serendipitous effect that progress in understanding one type of channel has already and will continue to contribute in unpredictable ways to the understanding of other channels.

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