Detailed comparison of expressed and native voltage-gated proton channel currents

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Two years ago, genes coding for voltage-gated proton channels in humans, mice and *Ciona intestinalis* were discovered. Transfection of cDNA encoding the human *HVCN1* (H_V1) or mouse (mVSOP) ortholog of *HVCN1* into mammalian cells results in currents that are extremely similar to native proton currents, with a subtle, but functionally important, difference. Expressed proton channels exhibit high H^+ selectivity, voltage-dependent gating, strong temperature sensitivity, inhibition by Zn^{2+}, and gating kinetics similar to native proton currents. Like native channels, expressed proton channels are regulated by pH, with the proton conductance–voltage (g_H–V) relationship shifting toward more negative voltages when pH_o is increased or pH_i is decreased. However, in every (unstimulated) cell studied to date, endogenous proton channels open only positive to the Nernst potential for protons, E_H. Consequently, only outward H^+ currents exist in the steady state. In contrast, when the human or mouse proton channel genes are expressed in HEK-293 or COS-7 cells, sustained inward H^+ currents can be elicited, especially with an inward proton gradient ($pH_o < pH_i$). Inward current is the result of a negative shift in the absolute voltage dependence of gating. The voltage dependence at any given pH_o and pH_i is shifted by about −30 mV compared with native H^+ channels. Expressed H_V1 voltage dependence was insensitive to interventions that promote phosphorylation or dephosphorylation of native phagocyte proton channels, suggesting distinct regulation of expressed channels. Finally, we present additional evidence that speaks against a number of possible mechanisms for the anomalous voltage dependence of expressed H^+ channels.

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Two decades after the discovery of voltage-gated proton currents (Thomas & Meech, 1982), genes for voltage-gated proton channels were identified in humans (Ramsey *et al.* 2006), mice and *Ciona intestinalis* (Sasaki *et al.* 2006). The remarkable gene product closely resembles the S1–S4 domains of other voltage-gated ion channels, but lacks the S5–S6 domains that comprise the pore. When cDNAs encoding human or mouse proton channels are expressed in HEK-293 cells or COS-7 cells, the resulting currents exhibit nearly all of the characteristic behaviours of native voltage-gated proton channels (Ramsey *et al.* 2006; Sasaki *et al.* 2006). However, upon close inspection, one key property differs. The voltage-dependent gating of native proton channels is modulated profoundly by pH (Byerly *et al.* 1984). Lowering pH_i or increasing pH_o by one unit shifts the voltage dependence of channel opening by −40 mV (Cherny *et al.* 1995). The net result is that native proton channels open only when there is an outward electrochemical driving force, so that they only conduct outward current. The pH and voltage dependence of gating of proton channels appears to be identical in at least 15 species and cell types (DeCoursey, 2003). The *HVCN1* gene product, H_V1, responds to pH changes qualitatively like native proton channels (Ramsey *et al.* 2006); here we confirm this quantitatively. However, we show that over a wide range of pH, the absolute voltage dependence of opening is roughly 30 mV more negative than that of native channels. The result is that inward proton currents can be observed, especially with an inward pH gradient ($pH_o < pH_i$). Here we investigate several possible explanations for this behaviour.

Methods

Expression of H_V1 in HEK-293 or COS-7 cells

The coding sequence of human H_V1 (*HVCN1*) was cloned into either pcDNA3.1(–) or pQBI25-fC3 (to make

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GFP-H_V1) vectors as previously described (Ramsey *et al.* 2006). H_V1-HA was subcloned into pcDNA5/FRT/TO and expressed in F1p-In T-Rex-293 cells (Invitrogen) for stable, tetracycline-inducible expression. The mouse ortholog (mVSOP) was subcloned from RIKEN cDNA 0610039P13 as described (Sasaki *et al.* 2006). HEK-293 cells were grown to ∼80% confluency in 35 mm cultures dishes, usually by seeding cells 1 d ahead of transfection. Cells were transfected with 0.4–0.5 μg of the appropriate cDNA using Lipofectamine 2000 (Invitrogen). After 6 h at 37°C in 5% CO_2, the cells were trypsinized and re-plated onto glass coverslips at low density for patch clamp recording the following day. We selected green cells under fluorescence for recording. We detected no difference in the properties of proton channels expressed in COS-7 or HEK-293 cells. H_V1-HA expression was induced in previously-plated cells by addition of tetracycline.

Electrophysiology

The recording and data analysis setups were as previously described (Morgan *et al.* 2003). Pipettes were made from 8250 glass (Garner Glass Co., Claremont, CA, USA). Seals were formed with Ringer solution (mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.4) in the bath, and the potential zeroed after the pipette was in contact with the cell. For perforated patch recording, the pipette and bath solutions contained 130 mM $TMAMeSO_3$ (tetramethylammonium methanesulphonate), 50 mM NH_4^+ in the form of 25 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 10 mM Bes, 1 mM EGTA, and was titrated to pH 7.0 with TMAOH (tetramethylammonium hydroxide). The pipette solution included ∼500 μg ml^{-1} solubilized amphotericin B (∼45% purity; Sigma). For whole-cell recording, bath and pipette solutions contained 100–200 mM buffer, 1–2 mM $CaCl_2$ or $MgCl_2$ (pipette solutions were Ca^{2+} free), 1–2 mM EGTA, and $TMAMeSO_3$ to adjust the osmolality to roughly 300 mosmol kg^{-1}, titrated with TMAOH or methanesulphonate. Buffers used at various pH values were Homopipes at pH 5.0, Mes at pH 5.5–6.0, BisTris at pH 6.5, Bes at pH 7.0, Hepes at pH 7.5, and Tricine at pH 8.0–8.5. Experiments were done at 21°C or at room temperature (20–25°C). No leak correction has been applied to any current records.

The reversal potential (V_{rev}) was measured by two methods. When V_{rev} was negative to the threshold voltage at which the proton conductance (g_H) was first activated, $V_{threshold}$, V_{rev} was determined by the traditional tail current method (Hodgkin & Huxley, 1952). If V_{rev} was within the range of active proton conductance, it was determined by interpolation between time-dependent inward or outward currents during test pulses (after scaling according to the tail current amplitude). $V_{threshold}$ was defined as the voltage at which unambiguous time-dependent proton current was first elicited. Since $V_{threshold}$ is close to V_{rev} for H_V1, we used tail currents to corroborate activation of the g_H.

Alkaline phosphatase from porcine kidney and PMA (phorbol myristate acetate) were obtained from Sigma Chemical Co. (St Louis, MO, USA). GF109203X (GFX) was from Calbiochem (San Diego, CA, USA).

Results

Determining the voltage dependence of proton channel gating

The voltage dependence of ion channel gating is frequently quantified by fitting the g–V (conductance–voltage) relationship with a Boltzmann function. However, in very few of the nearly 100 voltage-clamp studies of proton currents has the g_H–V relationship been analysed in this way. Boltzmann fits have been avoided for two main reasons. First, as best as it can be determined, the g_H–V relationship is not described very well by a simple Boltzmann function or one raised to some particular exponent. The second reason, which complicates the first, is that proton flux during each pulse changes the pH on both sides of the membrane, thereby altering both the driving force and the position of the g_H–V relationship, which is itself extremely sensitive to the pH gradient, ΔpH (Cherny *et al.* 1995). Perhaps proton currents are more severely afflicted with these problems than other types of currents because (1) the permeant ion is present at exceedingly low concentration, (2) the diffusion of protonated buffer, which provides essentially all of the permeating protons, is relatively slow, (3) activation of the g_H is very slow in most mammalian cells (time constants of several seconds), so that genuine steady-state current is rarely achieved, and (4) small pH changes have profound effects on the position of the g_H–V relationship.

Figure 1*A* illustrates g_H–V relationships obtained from the same data using three approaches. Families of voltage pulses were applied at three pH_o values to a COS-7 cell transfected with the mouse proton channel gene. The direct method of measuring the current at the end of each pulse (I_{end}) and dividing by the driving force ($V_{test} - V_{rev}$) provides a g_H–V relationship that appears to saturate (circles), and which resembles a Boltzmann function. However, the apparent saturation is largely an artifact that results from proton depletion from the cell (despite there being 100 mM buffer in the pipette solution). The actual V_{rev} at the end of the pulse can be estimated by interpolation between the H^+ current at the end of the pulse (I_{end}) and at the start of the tail current (I_{tail}) (Humez *et al.* 1995). By this method (Fig. 1*B*), V_{rev} at pH_o 8.0 was shifted positively by 9 mV during the family of pulses from −80 mV (V_{rev} = −92 mV) to −30 mV (V_{rev} = −83 mV). Between the pulses to −40 mV and −30 mV, V_{rev} was depolarized by 3.9 mV, reflecting an increase in pH_i of

0.067 units. This value can be compared with the pH_i change due to proton efflux during each pulse, using the Henderson–Hasselbalch equation from the cell diameter of 20 μm, containing 100 mM BisTris buffer at its pK_a of 6.5. Integrating the outward current at −35 mV and −30 mV gives 1.05 and 1.24 nC, corresponding to an increase in pH_i of 0.045 and 0.054 units, respectively. Both estimates agree and demonstrate that the H^+ current removes enough protons from even a well-buffered intracellular solution to change pH_i substantially.

The voltage dependence of H^+ channel opening can also be evaluated by the 'tail current method', which avoids the need to correct for rectification of the instantaneous current–voltage relationship. Rectification introduces errors into the g_H calculated from currents measured at different voltages, but the tail current, I_{tail}, is measured at a single voltage and thus precludes this source of error. In the case of the proton currents in Fig. 1*A*, however, g_H estimated from I_{tail} did not saturate even with large depolarizing pulses. Just as the true g_H is underestimated using I_{end}, it is overestimated using I_{tail}, with values for the larger pulses in Fig. 1*A* differing by more than twofold. Proton depletion from the cell increases pH_i and shifts V_{rev} to more positive voltages, decreasing the driving force for outward current and increasing it for inward current. Both estimates of g_H are incorrect because the V_{rev} used in the calculation is measured separately, and does not reflect the true ΔpH during each pulse.

An estimate that does not require knowing V_{rev} can be obtained by $(I_{end}-I_{tail})/(V_{test}-V_{hold})$ (Fig. 1*A*, squares). The g_H estimated by this method falls between the other two, and is correct in the sense that it accurately gives the g_H in the cell at that particular moment. The problem is that during each pulse, pH_i increases, hence ΔpH for each pulse is larger and the resulting g_H–V relationship sampled by each pulse is shifted progressively toward more positive voltages. Consequently, the result is not a single g_H–V relationship, but rather a Frankenstein monster amalgamated from many different g_H–V relationships. 'Correcting' each g_H value for the increased pH_i would lessen the apparent saturation of the curve, making a Boltzmann fit even less well.

One can try to minimize depletion effects by using short pulses, but then the current does not reach steady state. One can fit an exponential to currents during short pulses and extrapolate to estimate the steady-state value, but this assumes that the underlying time course is exponential. In some cells, very slow components of H^+ current turn-on persist during depolarizations sustained for several minutes (DeCoursey & Cherny, 1993; Cherny *et al.* 2003). We have not discovered an error-free method of quantifying the voltage dependence of proton channel gating. However, for proton currents, $V_{threshold}$ is a very useful parameter that avoids or minimizes many of the sources of systematic error. In Fig. 3, we evaluate the g_H–V relationship by estimating $V_{threshold}$. This parameter is to some extent arbitrary because the amplitude of H^+ current that can be detected varies with noise, leak current and the stability of recording, but if data are treated and analysed consistently, $V_{threshold}$ provides an excellent way to compare different cells. For proton currents, $V_{threshold}$ has five advantages over other approaches: (1) $V_{threshold}$ occurs at voltages where there is very little current flow, and hence the ΔpH is close to its nominal value; (2) It is not necessary to assume any specific time course for the turn-on of current, nor any specific shape for the g_H–V relationship;

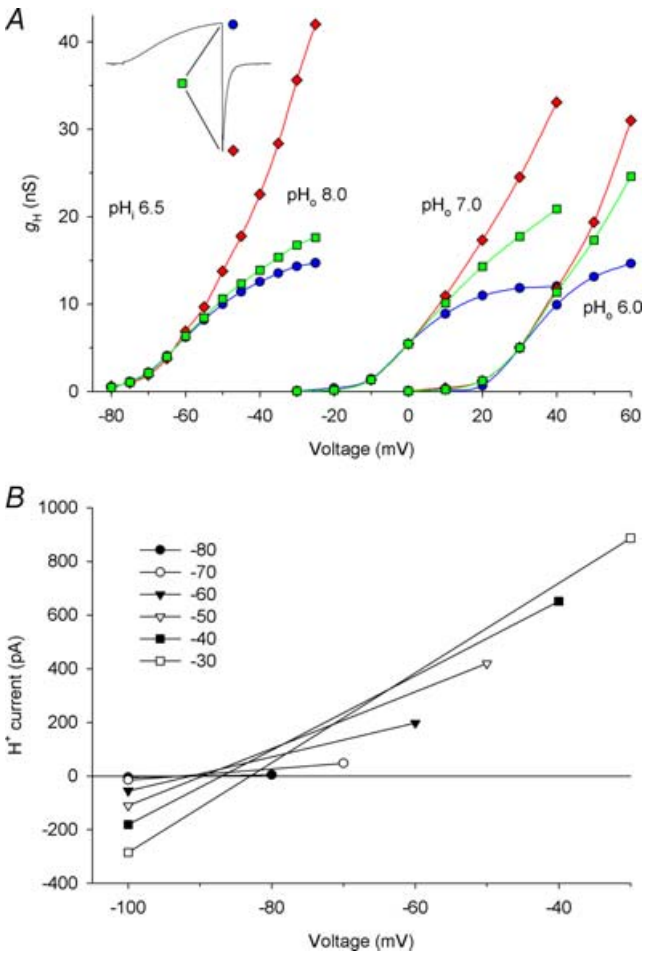

Figure 1. Measured g_H is altered profoundly by flux and cannot be uniquely determined
A, the g_H–V relationship estimated by three methods at each of three pH_o values in a COS-7 cell transfected with the mouse proton channel gene. The pipette contained pH_i 6.5 with 100 mM BisTris buffer. At pH_o 8.0, 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 and –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}, circles) or at the start of the tail current (I_{tail}, diamonds) assuming a single constant measured value for V_{rev}. Alternatively, g_H was calculated as the slope conductance (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.

(3) Data spanning a large range of pH_o and pH_i values can be compared simultaneously by plotting $V_{threshold}$ against V_{rev}. A comparison of g_H–V relationships can be done only at identical pH; (4) The data are automatically corrected for offsets and junction potential errors, because both parameters are equally affected; (5) From the viewpoint of cell physiology, $V_{threshold}$ is an important parameter that indicates when the channel will open.

H_V1 expressed in HEK-293 cells and mVSOP expressed in COS-7 cells

Figure 2 illustrates families of proton currents at three pH_o values in a HEK-293 cell transfected with the human proton channel H_V1 and studied at pH_i 6.5 in whole-cell configuration. The g_H was the only time-dependent conductance consistently identified in transfected cells. Proton selectivity of the conductance was confirmed by measuring the reversal potential, V_{rev}, in all solutions (not shown), which was usually in reasonable agreement with the Nernst potential for H^+. In the cell in Fig. 2*A*, V_{rev} was −23 mV at pH 7.0//6.5 (pH_o//pH_i). A small inward current, producing a distinct inward tail current after repolarization, is seen at −30 mV, which is therefore designated $V_{threshold}$. At pH 6.5//6.5 (Fig. 2*B*) $V_{threshold}$ was shifted by 20 mV to −10 mV, where inward current is unambiguous. Inward proton currents at symmetrical pH have never been reported in any cell expressing native H^+ currents, except in activated phagocytes in which the g_H–V relationship is shifted by −40 mV compared to unstimulated cells (Bánfi *et al.* 1999; DeCoursey *et al.* 2000). When ΔpH was decreased further by 1.0 unit (Fig. 2*C*), inward currents were elicited at +30 to +50 mV, with $V_{rev} = +56$ mV. A consistent observation was that the amplitude and voltage range over which inward currents were elicited increased as pH_o (or ΔpH) decreased. Thus, pronounced inward currents were also observed in cells studied at pH 6.0//7.5.

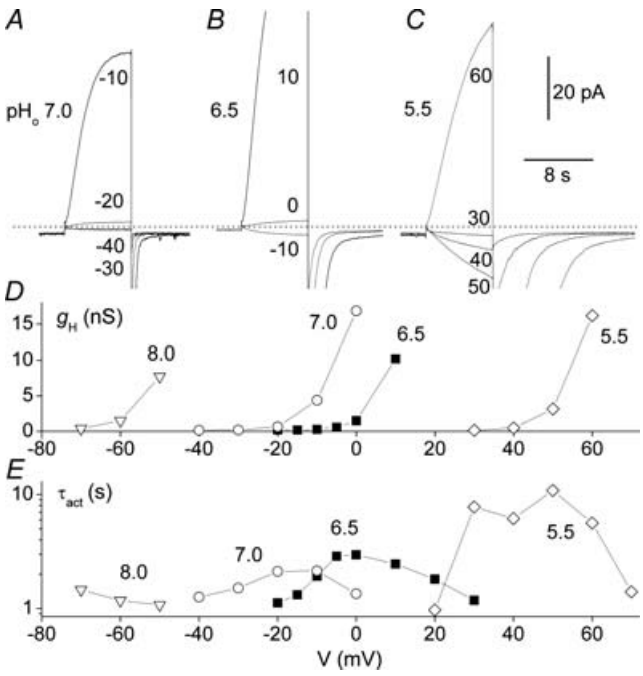

Figure 2. Inward proton currents are large at inward ΔpH
Whole-cell currents near V_{rev} in a HEK-293 cell transfected with H_V1. Pipette pH was 6.5, pH_o was 7.0 (*A*), 6.5 (*B*) or 5.5 (*C*). No leak correction has been applied. Illustrated currents are for pulses in 10 mV increments from −40 to −10 mV (*A*), −10 to +10 mV (*B*) or +30 to +60 mV (*C*), from V_{hold} −100 mV (*A*), −50 mV (*B*) or −20 mV (*C*). *D*, chord conductance–voltage (g_H–V) relationships from this cell, calculated by extrapolation of a single exponential fitted to the tail currents to the start of the pulse, using V_{rev} measured in each solution. *E*, activation time constants, τ_{act}, in the same cell, from single exponential fits. For pH_o 6.5 and 7.0, values for τ_{act} from two different families of pulses were averaged; other data are individual measurements.

Like native proton channels, the H_V1 g_H–V relationship shifted with changes in pH_o by roughly 40 mV unit^{-1} (Fig. 2*D*). Time constants of H^+ current activation, τ_{act}, in this cell (Fig. 2*E*) reveal another surprising difference. The τ_{act}–V relationship for H_V1 expressed in HEK-293 cells is bell-shaped, whereas that for native channels, τ_{act} usually becomes faster monotonically with depolarization.

In Fig. 3, the voltage- and pH-dependence of H_V1 channels expressed in HEK-293 cells and studied at various pH_o and pH_i values, are compared with native channel behaviour using $V_{threshold}$ (Methods). All native voltage-gated proton channels appear to share identical voltage- and pH-dependence (DeCoursey, 2003), with data from 15 different types of cells summarized as the continuous green line. H_V1 data obtained in whole-cell measurements in HEK-293 cells at pH_i 6.5 (black triangle) or 7.5 (blue diamonds) as well as in inside-out patches (green squares) are plotted. All of the H_V1 data fall below the line for native channels. It is clear that H_V1 channels expressed in HEK-293 cells differ from native proton channels in having a more negative $V_{threshold}$ at all ΔpH studied. The relationship for native proton channels is $V_{threshold} = 0.79\ V_{rev} + 23$ mV (DeCoursey, 2003). Linear regression on the three data sets for H_V1 (Fig. 3, legend) indicate identical slopes of 0.67 to 0.71 and offsets of −9 to −11 mV. It is noteworthy that the slopes are identical for data sets in which pH_i was fixed, and pH_o was varied (black triangle, blue diamond), as well as when pH_o was fixed and pH_i was varied (green square). This means that the sensitivity of the g_H–V relationship to changes in both pH_o and pH_i are identical. In summary, the slope of the ΔpH dependence of H^+ channel opening is similar or slightly lower in H_V1 than in native channels. More significantly, $V_{threshold}$ of H_V1 is about 30 mV more negative.

In Fig. 4, traditional g_H–V relationships for expressed human or mouse proton currents are compared with data from several native cells, all studied in whole-cell

configuration at pH_o 7.0 and pH_i 6.5. The slopes of the g_H–V relationships appear similar for native and expressed channels, but the position of the curve is 20–40 mV more negative for expressed channels. This difference is not due to variations in pH because V_{rev} was similar in native or expressed H^+ currents: -27.4 ± 3.7 mV (mean ± S.D., $n = 9$) and -24.8 ± 5.6 mV ($n = 9$), respectively. The -30 mV shift of the normalized g_H–V relationship for expressed proton channels is consistent with the shift of $V_{threshold}$ in Fig. 3.

Total gating charge movement can be estimated from the limiting slope of g_H–V relationships plotted semilogarithmically (Almers, 1978; Sigworth, 1993). Replotting the H_V1 and mVSOP data in Fig. 4 provides a rough estimate of 4.2 ± 0.2 mV per e-fold change in g_H (mean ± S.E.M., $n = 8$). This corresponds to 6.0 elementary charges, e_0. This value should be considered a lower limit because our inability to reliably detect single-channel currents limits the range of open probabilities that can be explored. Similar estimates were obtained previously for the gating charge translocated by native proton channels in alveolar epithelial cells: 6–8 e_0 (DeCoursey & Cherny, 1996*b*) and 5.4 e_0 (DeCoursey & Cherny, 1997).

Possible explanations

A number of explanations for the negative $V_{threshold}$ of H_V1 were considered. Abnormal voltage- and pH-dependence of gating might reflect abnormal sensitivity to pH_o or pH_i or both. However, the slope and positions of the data for changes of pH_o at fixed pH_i of 7.5 (blue diamonds) or 6.5 (black triangles) in Fig. 3 are identical to those for changes of pH_i at fixed pH_o in excised inside-out patches (green squares, Fig. 3). Therefore, like the native channel, H_V1 has the same sensitivity to pH_o and pH_i.

Part of the more negative $V_{threshold}$ of expressed channels might be due to higher resolution of the larger currents in cells with overexpression. This question was addressed with a cell line in which H_V1-HA expression could be deliberately varied. Figure 3 includes data from a number of these cells, in which the g_H was roughly an order of magnitude smaller than that in typical transfected cells. Linear regression gives $V_{threshold} = 0.82\ V_{rev} - 8.5$ mV,

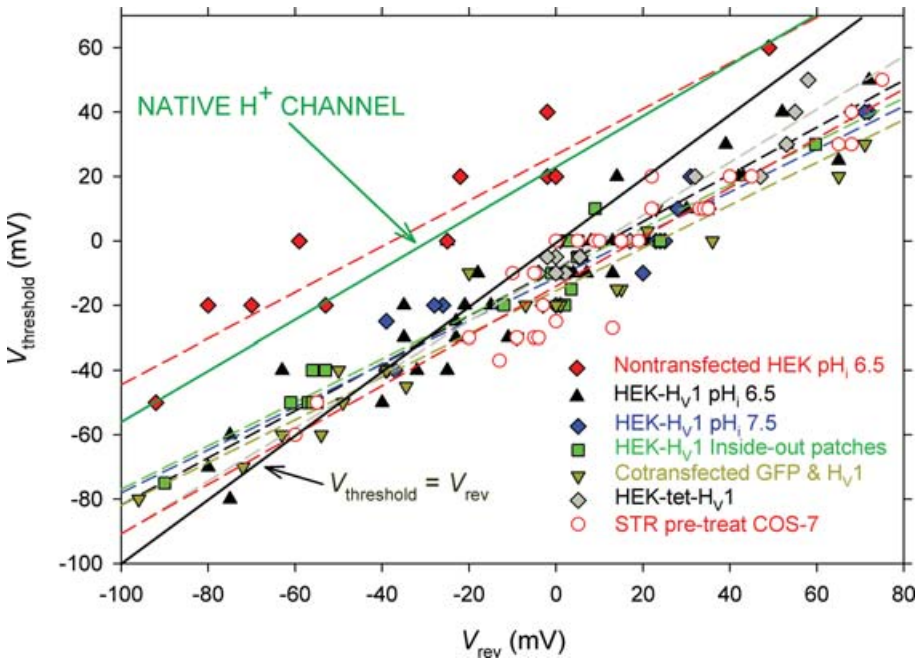

Figure 3. Relationship between $V_{threshold}$ and V_{rev} for H_V1 expressed in HEK-293 or COS-7 cells compared with native proton channels in HEK-293 and other cells
Endogenous proton currents in 5 non-transfected HEK-293 cells (◆) studied in whole-cell configuration with pH_i 6.5, fitted by the red dashed line: $V_{threshold} = 0.71\ V_{rev} + 27$ mV. Data for H_V1 at various pH_o values with pH_i 7.5 or 6.5 in whole-cell configuration, or at various pH_i values with pH_o 7.5 in inside-out patches. Dashed lines are drawn by linear regression on the points, according to $V_{threshold} = 0.66\ V_{rev} - 11$ mV for pH_i 7.5 (◆), $V_{threshold} = 0.73\ V_{rev} - 9$ mV for pH_i 6.5 (▲), and $V_{threshold} = 0.67\ V_{rev} - 10$ mV for pH_o 7.5 (■) in inside-out patches. Data are from 21 cells and 8 patches for H_V1. The continuous green line indicates the relationship for native proton currents that includes published data from 15 different types of cells (from DeCoursey, 2003). The continuous black line indicates equality between $V_{threshold}$ and V_{rev}; data below this line exhibit inward H^+ current at $V_{threshold}$. HEK-293 or COS-7 cells co-transfected with H_V1 and GFP (▼), fitted by: $V_{threshold} = 0.66\ V_{rev} - 16$ mV. HEK-293 cells expressing low levels of H_V1, 'tet' (◇), fitted by: $V_{threshold} = 0.82\ V_{rev} - 9$ mV. HEK-293 cells expressing H_V1 after pretreatment with 10 nM staurosporine (○), fitted by: $V_{threshold} = 0.77\ V_{rev} - 14$ mV. See text for more details.

which is similar to that in cells transiently transfected with GFP-H_V1.

The GFP tag, which was linked to the channel protein, might influence gating or pH sensitivity. However, in HEK-293 and COS-7 cells co-transfected with GFP and H_V1 cDNA in separate plasmids, the voltage- and ΔpH-dependence was indistinguishable from that of the tandem H_V1-GFP fusion ($V_{threshold} = 0.66\ V_{rev} - 16$ mV, Fig. 3). In addition, τ_{act} in co-transfected H_V1 channels exhibited a bell-shaped voltage dependence (data not shown) like that seen with the tandem construct (Fig. 2*E*).

Another possibility is that HEK-293 cells have a unique membrane composition or process the proton channel protein anomalously. We took advantage of the fact that non-transfected HEK-293 cells exhibit small endogenous voltage-gated proton currents (Maturana *et al.* 2001; DeCoursey, 2003). One might expect that native proton channel proteins in HEK-293 cells would be processed in a similar fashion as H_V1 channel proteins, and exist in a similar membrane microenvironment. However, $V_{threshold}$ data from native proton currents in HEK-293 cells (Fig. 3, red diamonds) have normal voltage- and pH-dependence.

Another way to evaluate whether there is something peculiar about HEK-293 cells is to use a different expression system, COS-7 cells (Fig. 5*A* and *C*). COS-7 cells have the advantage that they exhibit no measurable endogenous proton currents (Maturana *et al.* 2001; Morgan *et al.* 2002). Figure 5*A* illustrates a family of H_V1 currents in an inside-out patch from a COS-7 cell at pH 7.5//7.5. Inward currents are evident at −30, −20 and −10 mV, with distinct tail currents after repolarization. Similar voltage dependence was observed for H_V1 currents in an inside-out patch from a HEK-293 cell (Fig. 5*B*), also at pH 7.5//7.5. The mouse ortholog (mVSOP) also exhibited a negative $V_{threshold}$ when expressed in COS-7 cells and studied in excised patches (Fig. 5*C*) or whole-cell configuration (Fig. 4). Plotting data at various pH values from a number of COS-7 cells in the format of Fig. 3 indicated that mVSOP or H_V1 expressed in COS-7 cells had a negatively shifted $V_{threshold}$ indistinguishable from that of H_V1 expressed in HEK-293 cells. Linear regression on 31 measurements of mVSOP (6 cells and 1 patch) and 16 of H_V1 (6 cells and 2 patches) expressed in COS-7 cells gave $V_{threshold} = 0.69\ V_{rev} - 15$ mV (data not shown). Thus, the voltage dependence was not detectably different in two expression systems.

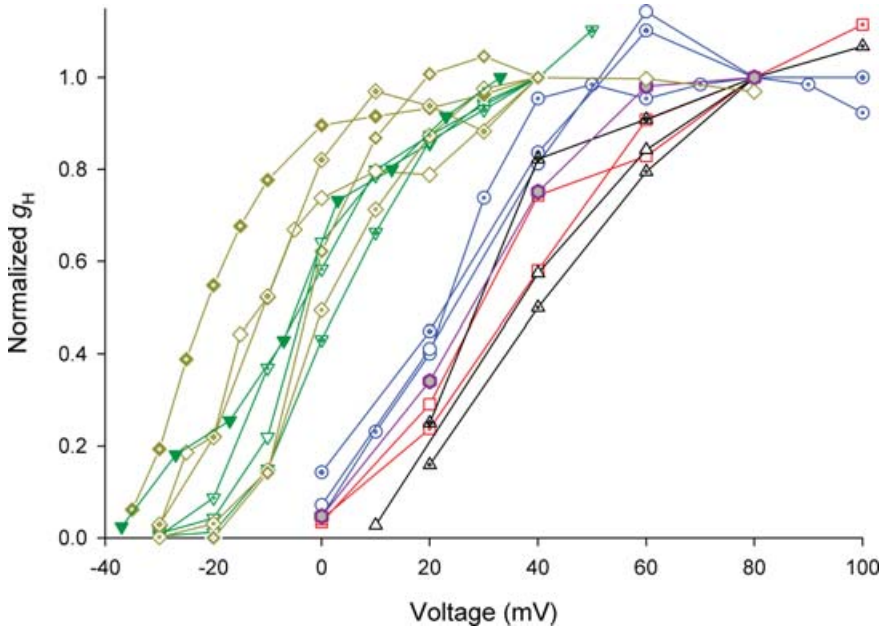

Figure 4. The g_H–V relationship is shifted negatively by about 30 mV in expressed proton channels compared with native proton channels
The human proton channel was expressed in HEK-293 cells (downward green triangles), the mouse channel in COS-7 cells (diamonds). Native proton currents are from other studies in THP-1 cells (upward triangles, DeCoursey & Cherny, 1996*a*), PLB-985 cells (squares, DeCoursey *et al.* 2001*b*), human basophils (open circles, unpublished studies by B. Musset, V. V. Cherny, D. Morgan and T. E. DeCoursey), and mean values from 6 to 9 rat alveolar epithelial cells (hexagons, taken from Fig. 6 in Cherny *et al.* 1995). Proton currents were fitted with single exponential curves extrapolated to infinite time to obtain the current amplitude, and chord conductance was calculated using V_{rev} measured in each solution, all studied at pH_o 7.0 and pH_i 6.5 in whole-cell configuration. Native g_H was normalized to its value at +80 mV, expressed g_H at +40 mV. Except for alveolar epithelium, data are from individual cells.

Phosphorylation

Native proton channels can present with inward current in phagocytes that are stimulated with PMA or other agonists of the respiratory burst. Among several profound changes in gating kinetics in 'activated' cells, the g_H–V relationship is shifted 40 mV toward more negative voltages (Bánfi *et al.* 1999; DeCoursey *et al.* 2000, 2001*a*), with the result that inward H^+ current may be observed negative to V_{rev}. The negatively shifted gating and inward currents of expressed proton channels suggest the possibility that they might already be 'activated.' The enhanced gating mode of the proton channel occurs by protein kinase C (PKC)-dependent phosphorylation, either of the channel itself or an accessory protein, and is partially reversed by the PKC inhibitors GF109203X or staurosporine (Morgan *et al.* 2007). Exposure of H_V1-transfected HEK-293 cells in perforated-patch configuration to PMA enhanced proton currents in some cells; and subsequent exposure to GF109203X appeared to reverse this effect. Although these changes were qualitatively like those that occur in human eosinophils under similar conditions, the effects in HEK-293 cells were quantitatively much smaller. Most transfected HEK-293 cells either failed to respond or responded weakly. In cells that appeared to respond to PMA, I_H increased only $48 \pm 33\%$ (mean ± S.E.M., $n = 13$), compared with a 470% increase in eosinophils (DeCoursey *et al.* 2001*a*). Changes in kinetics were also weak: τ_{act} was $38 \pm 14\%$ faster ($n = 14$) and τ_{tail} was $21 \pm 6\%$ slower ($n = 15$), compared with 420% and 540% changes, respectively, in eosinophils. Finally, $V_{threshold}$ shifted only −5.5 mV (±2.5 mV, $n = 13$), far less than −42.6 mV seen in eosinophils. Addition of GF109203X after PMA shifted $V_{threshold}$ by $+6.9 \pm 3.4$ mV ($n = 11$). Even if the response of H_V1 in HEK-293 cells were related to phosphorylation, it is small and unlikely to account for the negative g_H–V relationship of expressed proton channels.

The possibility that expressed proton channels are constitutively phosphorylated in HEK-293 or COS-7 cells, but with different kinetics or by a different kinase than exists in phagocytes was addressed by pretreating these cells for 3–6 h ($n = 6$) or 3 days ($n = 4$) with 10 nM staurosporine, a non-specific kinase inhibitor. In seven HEK-293 cells and three COS-7 cells treated in this manner, the voltage dependence of g_H was $V_{threshold} = 0.77\, V_{rev} - 14.1$ mV (linear regression on 30 measurements at various pH values, Fig. 3, open red circles), which is indistinguishable from that of untreated cells.

Finally, HEK-293 cells expressing H_V1 were studied in whole-cell configuration with 100 units ml^{-1} alkaline phosphatase in the pipette solution. This broad spectrum phosphatase had no discernible effect on the voltage dependence or any other property of proton currents in four cells studied for 40–75 min. From the beginning and throughout the duration of these experiments at symmetrical pH 7.5, $V_{threshold}$ was at −30 mV and distinct inward proton currents were also observed at −20 and −10 mV. In conclusion, there was no evidence that the voltage dependence or kinetics of expressed H_V1 channels can be regulated by phosphorylation or dephosphorylation, in sharp contrast with native phagocyte proton currents (Morgan *et al.* 2007).

Discussion

The characteristic dependence of proton channel gating on pH, specifically ΔpH (Cherny *et al.* 1995), has been universally considered to indicate that the primary function of the channel is acid extrusion. Here we report that the recently identified mammalian proton channels expressed in HEK-293 cells or COS-7 cells exhibit voltage-dependent gating at pH_o 5.0–8.0 and pH_i 5.5–7.5 that is roughly 30 mV more negative than in

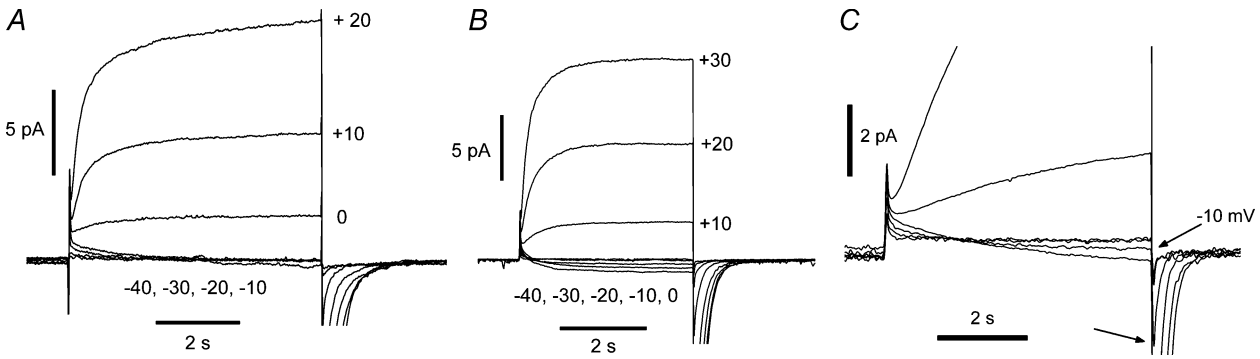

Figure 5. Inward proton currents also occur in excised patches
Proton currents in inside-out patches from a COS-7 cell (*A*) and a HEK-293 cell (*B*), both transfected with H_V1, and a COS-7 cell transfected with the mouse proton channel mVSOP (*C*). Pipette and bath solutions were at pH 7.5 for all. Pulses are in 10 mV increments as indicated. In *C* the arrows indicate the inward current during the pulse to −10 mV as well as the corresponding tail current.

all cells studied to date that express endogenous proton channels. As a result, inward currents can be elicited. It is noteworthy that the sensitivity of the g_H–V relationship to changes in both pH_o and pH_i was identical, thus the pH sensing mechanisms (whatever they are) appear to be functional. Inward H^+ currents probably were not previously described because they are small; however, their existence represents an important mechanistic distinction. Intriguingly, the R201Q mutation shifted the g_H–V relationship by −50 mV, resulting in robust inward currents (Sasaki *et al.* 2006). Another difference is that the τ_{act}–V relationship is bell-shaped for expressed H_V1, whereas for native proton currents, τ_{act} decreases monotonically with depolarization in snail neurons (Byerly *et al.* 1984), *Ambystoma* oocytes (Barish & Baud, 1984), mouse macrophages (Kapus *et al.* 1993), rat alveolar epithelial cells (DeCoursey & Cherny, 1995), THP-1 cells (DeCoursey & Cherny, 1996*a*), human eosinophils (DeCoursey *et al.* 2001*a*), PLB-985 cells (DeCoursey *et al.* 2001*b*), human basophils (Cherny *et al.* 2001), and Jurkat cells (Schilling *et al.* 2002). The shifted gating of expressed proton channels has physiological relevance, because inward and outward H^+ currents have opposite effects on pH_i and membrane potential. When proton currents are opened by strong depolarization, as in activated phagocytes, the proton efflux prevents cytoplasmic acidification and limits membrane depolarization during electrogenic NADPH oxidase activity (Murphy & DeCoursey, 2006). However, if the g_H were activated by low pH_i when $V_{threshold}$ is negative to V_{rev}, the resulting inward proton currents would decrease pH_i and depolarize the membrane potential toward E_H. From the viewpoint of cellular homeostasis, $V_{threshold}$ is an important parameter.

The abnormal gating behaviour of expressed H_V1 is not due to the GFP tag, because identical behaviour was observed when the channel and GFP were co-transfected using separate vectors. The difference persists if cells with comparable current density and leak conductance are compared. When hH_V1-HA expression was reduced by using a tetracycline-inducible cell line, $V_{threshold}$ was similar to that in cells with 10-fold larger currents. Furthermore, $V_{threshold}$ was abnormally negative even in leaky HEK-293 cells, whereas native proton currents from unstimulated eosinophils never exhibited inward currents despite the seal/leak resistance in excised patches routinely being in the teraohm range (Cherny *et al.* 2003). The only report of inward native proton current is at pH 6.5//8.0 in *Rana pipiens* renal proximal tubule cells (Gu & Sackin, 1995). At such a large inward ΔpH, $V_{threshold}$ approaches V_{rev} (Fig. 3). Much effort has gone into confirming that the native proton channel does not normally conduct inward current (Thomas, 1989).

The negatively shifted $V_{threshold}$ occurs both in HEK-293 cells and in COS-7 cells, two mammalian cell lines. A number of possible explanations (such as differential post-translational modification or trafficking to different lipid microdomains) appear to be ruled out by the observation that endogenous proton currents in non-transfected HEK-293 cells have voltage dependence like that of other native proton currents (Fig. 3, red diamonds). However, it is conceivable that overexpression itself might have unexpected effects on processing or trafficking. Differential N-linked glycosylation of native *versus* transfected channel protein is unlikely given that H_V1 channels lack a consensus glycosylation sequence (-NXS/T-) (Asn-X-Ser/Thr) (Kornfeld & Kornfeld, 1985) and both the native and expressed proteins (with or without the GFP or HA tags) migrate as sharp bands at their predicted molecular mass (Ramsey *et al.* 2006).

Another possible explanation is that expressed proton channels are regulated differently in cultured cell lines and phagocytic leucocytes. Native proton channels in granulocytes that are stimulated by respiratory burst agonists (PMA, fMetLeuPhe, arachidonic acid, opsonized zymosan) exhibit radically altered gating properties that are prevented or reversed by PKC inhibitors (Morgan *et al.* 2007). In HEK-293 or COS-7 cells expressing H_V1, responses to PMA were small and in many cells undetectable. PKC inhibitors reversed these small effects, but did not shift $V_{threshold}$ much beyond its initial value. Thus, the unusually negative $V_{threshold}$ of H_V1 cannot be explained in terms of the channel already being 'activated' by pathways like those that operate in phagocytes. Pretreatment for 3 days with staurosporine, a broad spectrum kinase inhibitor, had no detectable effect on the position of the g_H–V relationship. Finally, including alkaline phosphatase in the pipette solution had no detectable effect on expressed H_V1 channels. Thus, no evidence was found to support the idea that expressed proton channels are already 'activated.' In summary, we have tested a number of explanations for the aberrant voltage dependence of H_V1 gating, and found none of them convincing.

H_V1 and mVSOP function as voltage-gated proton channels, but with gating kinetics that are distinct from native, unstimulated proton channels. The reason for this difference remains unclear; the possibilities that remain are not so easily tested. Although other channel isoforms conceivably remain to be discovered, genes encoding homologous voltage-sensor domain proteins like Ci-VSP fail to reconstitute a g_H when overexpressed in HEK-293 cells (Murata *et al.* 2005; data not shown). Another possibility seems more likely. An as yet unidentified protein may associate with or regulate the activity of native proton channels, but is absent or underexpressed in the

expression systems used here. For example, the $V_{threshold}$ of heterologously expressed H_V1 is similar to that of the PKC-activated native channel. Perhaps phosphorylation of H_V1 is aberrant in heterologous expression systems and it remains in a phosphorylated state, although our attempts to demonstrate such a mechanism failed. This could occur if, for example, expressed H_V1 fails to associate with a specific phosphatase that normally acts to reverse PKC-mediated phosphorylation of the native channel. The failure of expressed H_V1 channels to respond to interventions designed to promote phosphorylation or dephosphorylation indicates different regulation than in phagocytes. However, native proton channels in non-phagocytes do not respond to PMA (DeCoursey *et al.* 2000). Perhaps the differences between proton channels in different tissues might provide clues to the novel behaviour of proton channels in expression systems described here.

One might ask whether the correct gene has been identified. Previous candidates for proton channel genes were $gp91^{phox}$ (Henderson *et al.* 1995) and other homologs in the NOX family of proteins (Bánfi *et al.* 2000; Bánfi *et al.* 2001; Maturana *et al.* 2001). The gene products studied here are present in leucocytes, but are structurally unrelated to $gp91^{phox}$ or other NOX proteins (Ramsey *et al.* 2006; Sasaki *et al.* 2006). However, the suggestion that phagocytes have two types of proton channel, one of which is $gp91^{phox}$ (Bánfi *et al.* 1999), is difficult to rule out. Currents that in some respects resemble proton currents have been reported when $gp91^{phox}$ was transfected into various expression systems (Henderson & Meech, 1999; Bánfi *et al.* 2000; Maturana *et al.* 2001; Murillo & Henderson, 2005). However, we observed normal proton currents and a normal increase of proton conductance upon activation by PMA in $gp91^{phox}$ knockout PLB-985 cells (DeCoursey *et al.* 2001*b*) and in granulocytes from human patients with chronic granulomatous disease who lacked $gp91^{phox}$ expression (DeCoursey *et al.* 2001*b*). Conversely, expression of $gp91^{phox}$ together with other essential NADPH oxidase components in COS-7 cells did not result in detectable proton currents, despite a demonstrated ability of the expressed NADPH oxidase components to generate superoxide anion (Morgan *et al.* 2002). Furthermore, several $gp91^{phox}$ homologs, including Nox1, Duox-1 and Duox-2, appear not to function as proton channels (Geiszt *et al.* 2004; Schwarzer *et al.* 2004; Gaggioli *et al.* 2007). Finally, in the proposal that two types of H^+ channel exist, the channel formed by $gp91^{phox}$ was interpreted to activate at more negative voltages (Bánfi *et al.* 1999); however, H_V1 and mVSOP clearly are unrelated to $gp91^{phox}$, yet open at anomalously negative voltages. Future demonstration of electron currents and a complete absence of proton currents in granulocytes from knockout mice before and after stimulation, would provide strong evidence that $gp91^{phox}$ does not function as a proton channel.

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