Voltage-gated proton channel in a dinoflagellate

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Fogel and Hastings first hypothesized the existence of voltage-gated proton channels in 1972 in bioluminescent dinoflagellates, where they were thought to trigger the flash by activating luciferase. Proton channel genes were subsequently identified in human, mouse, and Ciona intestinalis, but their existence in dinoflagellates remained unconfirmed. We identified a candidate proton channel gene from a Karlodinium veneficum cDNA library based on homology with known proton channel genes. K. veneficum is a predatory, nonbioluminescent dinoagellate that produces toxins responsible for fish kills worldwide. Patch clamp studies on the heterologously expressed gene confirm that it codes for a genuine voltage-gated proton channel. KHV1 is proton-specific and activated by depolarization, its g_{H^+}-V relationship shifts with changes in external or internal pH, and mutation of the selectivity filter (which we identify as Asp51) results in loss of proton-specific conduction. Indirect evidence suggests that KHV1 is monomeric, unlike other proton channels. Furthermore, KHV1 differs from all known proton channels in activating well negative to the Nernst potential for protons, E_{H^+}. This unique voltage dependence makes the dinoflagellate proton channel ideally suited to mediate the proton influx postulated to trigger bioluminescence. In contrast to vertebrate proton channels, whose main function is acid extrusion, we propose that proton channels in dinoflagellates have fundamentally different functions of signaling and excitability.

Results

To identify a gene for a voltage-gated proton channel in dinoflagellates, we searched a cDNA library from K. veneficum (National Science Foundation Microbial Genome Sequencing Program). Although analysis of a large alignment of voltage sensor domains (VSDs) identified only a handful of sequence elements unique to the KHV1 family, KHV1s occupy a branch of a phylogenetic tree distinct from branches comprising the close homologs C15orf27 and voltage-sensitive phosphatases (VSPs) (24). To capture features of the S1–S4 transmembrane helices that comprise the core structure of the VSD, we constructed sequence logos (25) based on a multiple sequence alignment of 37 H+1s with 15 C15orf27s, 11 VSPs, and 38 KVs (Fig. 1). The logos highlight differences between the various families in spacing, identity, and level of conservation of positions in each transmembrane (TM) helix and reveal some sequence patterns unique to the H+1 family, which presumably correlate with functions not shared by other VSDs. In addition, we noticed that H+1 C-terminal domains are significantly shorter than the domains of other VSD-containing orthologs (Fig. 1).

Using the amino acid sequences of Ciona intestinalis H+1 as a probe, we used tBLASTn to search sequence libraries of full-length splice leader-primed cDNAs of K. veneficum; these libraries are especially deep, estimated to represent ∼80% to 90% of the genes in the K. veneficum genome. The search identified a candidate proton channel gene, which we call KHV1, in a cDNA library from dark-feeding K. veneficum. The protein sequence exhibits a good, although imperfect, match to the sequence patterns found in bona fide H+1s (Fig. 1), giving us enough confidence to proceed. The use of cDNA rather than genomic sequence information provides very high confidence that the predicted protein sequence corresponds to the sequence expressed in the organism. Express-
sion of kHV1 mRNA was confirmed using RT-quantitative PCR (RT-qPCR; primers in Table S2) on mRNA extracted from cultures of *K. vene* grown autotrophically. For reference, expression of actin was also examined. kHV1 mRNA was detectable in all samples but at lower levels than actin (by 78- and 71-fold in the middle of the light and dark phases, respectively), consistent with the typically low abundance of ion channel mRNA.

The kHV1 Proton Channel Carries Large Inward Currents. We used heterologous expression in standard mammalian systems for characterization of the candidate kHV1 and site-directed mutagenesis studies. Because dinoflagellates exhibit a significantly different pattern of codon use than mammals (26), we ordered the commercial synthesis of a gene coding for the same protein but with a DNA sequence optimized for human expression. Because dinoflagellates exhibit a significantly different pattern of codon use than mammals (26), we ordered the commercial synthesis of a gene coding for the same protein but with a DNA sequence optimized for human expression cloned into a mammalian expression plasmid. Two mammalian cell lines, HEK-293 or COS-7, were transfected with the expression plasmid, and membrane currents were examined by tight seal whole-cell voltage clamp. The family of currents recorded during a series of depolarizing voltage pulses in Fig. 2A reveals the most distinctive feature of kHV1 currents. The proton conductance, $g_{\text{H}^+}$, activated well negative to the reversal potential, $V_{\text{rev}}$, and therefore, inward currents were observed over a wide voltage range. The corresponding current–voltage relationship in Fig. 2B is reminiscent of analogous curves for voltage-gated sodium channels. All previously described voltage-gated proton channels open only when the electrochemical gradient for protons is outward, and therefore, only outward current is observed (27).

The kHV1 Proton Channel Is Perfectly Proton-Selective. The ion species that permeates a channel can be identified by comparing the zero current potential ($V_{\text{rev}}$) with the Nernst potential for each ion present. The negative range of kHV1 activation enabled measuring $V_{\text{rev}}$ directly in families of currents. Fig. 2C illustrates that $V_{\text{rev}}$ was very close to the Nernst potential for protons, $E_{\text{H}^+}$, over a wide range of external pH ($p\text{H}_e$ 4.5–8.5) and internal pH ($p\text{H}_i$ 5.5–8.5). In fact, kHV1 seems to be even more proton-selective than other H$_2$O-1s, although the small deviations of $V_{\text{rev}}$ from $E_{\text{H}^+}$ in the latter are considered to reflect imperfect pH control rather than finite selectivity for other ions (27). We suspect that the proximity of $V_{\text{rev}}$ to $E_{\text{H}^+}$ in kHV1 reflects the ability to measure $V_{\text{rev}}$ without applying a prepulse to first activate the conductance. In other species, $H^+$ efflux during the prepulse changes local pH sufficiently to shift $V_{\text{rev}}$ a few millivolts positive to $E_{\text{H}^+}$.

Gating of the kHV1 Proton Channel Is Regulated by the pH Gradient. A property shared by every H$_2$O-1 thus far identified is the exquisite regulation of the absolute position of the proton conductance–voltage ($g_{\text{H}^+}$–$V$) relationship by the pH gradient, $\Delta pH$, defined as $p\text{H}_e - p\text{H}_i$. The $g_{\text{H}^+}$–$V$ relationship shifts negatively by 40 mV/unit increase in $p\text{H}_o$ or decrease in $p\text{H}_i$ (27, 28). Fig. 3 illustrates that kHV1 behaves in the same way. The effect of changing $p\text{H}_i$ at constant $p\text{H}_o$ was studied in inside-out patches of membrane (Fig. 3A–C). The corresponding $g_{\text{H}^+}$–$V$ relationships in Fig. 3E reveal that, at lower $p\text{H}_i$ channel opening occurs at more negative voltages. Activation was substantially faster at lower $p\text{H}_i$ as in other cells (29). In addition, the limiting $g_{\text{H}^+}$ approximately doubles per unit decrease in $p\text{H}_i$ consistent with $H^+$ rather than $OH^-$ flux (in the opposite direction). When $p\text{H}_o$ was varied in whole-cell measurements, the $g_{\text{H}^+}$–$V$ relationship also shifted ~40 mV/unit, with little change in the maximum $g_{\text{H}^+}$. Thus, the effects of changes of $p\text{H}_e$ and $p\text{H}_i$ on the $g_{\text{H}^+}$–$V$ relationship in kHV1 are quite similar to the effects in other proton channels. In most respects, the properties of kHV1 resemble the properties of other proton channels.

However, kHV1 is unique in one key respect. At each $p\text{H}_i$ studied, inward current was activated well negative to $E_{\text{H}^+}$ (Fig. 3A–C), which is also evident in current voltage curves (Fig. 3D). This negative voltage range of activation, resulting in inward current, is unprecedented. In Fig. 3F, the voltage at which kHV1 current is first evident, $V_{\text{threshold}}$, is plotted against $V_{\text{rev}}$. In every

Fig. 1. The primary sequence of kHV1 (line 1) compared with sequence logos of TM regions S1 to S4 of families of homologs that were created as described in Materials and Methods. The height of each letter in a stack indicates its relative representation at that location. The total stack height at each position indicates its information content, which for proteins, has a theoretical maximum of 4.3 bits (25) and depends both on the number of sequences in the alignment and the number of substitutions observed at a position. Numbers on the left and right sides indicate the length of N and C termini, respectively, (mean ± SD) of the sequences included in the alignment from which the logos were created (H$_1$ n = 37, C10af27 n = 15, VSP n = 11) or in the case of K$_v$, from a subset of 13 sequences drawn at random from the 38 sequences included in the alignment. All sequences used for the logos are listed in Table S1. One-way ANOVA followed by Tukey’s test indicates that the length of the H$_1$ C terminus differs significantly from the length of the C terminus of each other family (P < 0.001). Numbering of TM residues is for kHV1, C10af27, CIVSP, and Shaker (K$_v$). Although kHV1 displays some significant differences from the most common H$_1$ sequence, 30 of 87 TM residues match the predominant H$_1$ pattern.
fundamentally from their function in other cells. Opening kHV1 openings of these relationships were identical, and thus, kHV1 simply by (Eq. 1).

The current–voltage relationship from this family illustrates that inward currents occur over a wide voltage range negative to the Nernst potential for protons, $E_\text{N}$. (C) The measured $V_\text{rev}$ is extremely close to $E_\text{N}$. $V_\text{rev}$ was determined directly from the reversal of current during depolarizing pulses. Data from 79 whole-cell and excised patch measurements are included. The dashed line shows $E_\text{N}$.

cell at all $\Delta pH$ explored, $V_\text{threshold}$ was negative to $V_\text{rev}$ (the dashed line in Fig. 3F shows equality). Consequently, inward proton currents occur over a wide voltage range. In contrast, other proton channels are regulated by $\Delta pH$ in such a way that they conduct only outward current over the entire physiological pH range (27). The dotted line in Fig. 3F shows the average relationship reported for voltage-gated proton channels in 15 different types of cells (27). These relationships can be described simply by (Eq. 1)

$$V_\text{threshold} = \text{slope} \times V_\text{rev} + V_\text{offset},$$

in which $V_\text{offset}$ is $V_\text{threshold}$ at symmetrical pH. For example, when $pH_o = pH_i$, native proton currents activate 23 mV positive to $V_\text{rev}$, whereas kHV1 activates 37 mV negative to $V_\text{rev}$. The slopes of these relationships were identical, and thus, kHV1 activates at any given $\Delta pH$ at potentials $\sim$60 mV more negative than all proton channels described to date. The identical slope suggests that, despite the 60-mV difference in $V_\text{offset}$, a similar mechanism is involved in the regulation of gating by $\Delta pH$ in Hv1 from all species. The profound difference in $V_\text{threshold}$ portends that the function of proton channels in dinoflagellates differs fundamentally from their function in other cells. Opening kHV1 will result in inward current that will tend to depolarize the membrane regeneratively, potentially both producing an action potential and acidifying the cytoplasm. In bioluminescent species, Hv1 would conduct protons from the vacuole into the cytoplasm, propagating the action potential, and when the action potential invades the scintillon, from vacuole into the scintillon, triggering a flash.

**Aspartate**$^\text{51}$ **Is the Selectivity Filter.** Recently, we reported that mutation of Asp$^{112}$ in human Hv1 converted the channel to anion selectivity, identifying Asp$^{112}$ as the selectivity filter (24). Our alignment (Fig. 1) predicts the corresponding residue in kHV1 to be Asp$^{51}$. We mutated this Asp to Glu, Ala, Ser, or His. Fig. 4 shows that the D51A mutant was anion permeable. Whole-cell currents in symmetrical pH 5.5 TMA$^+$ (tetrathymethylammonium$^+$) CH$_3$SO$_3^-$ (methanesulfonate$^-$) solutions were small, but they activated at roughly the same voltage as WT channels and reversed near 0 mV (Fig. 4A). Replacing all external CH$_3$SO$_3^-$ with Cl$^-$ increased the outward current substantially (Fig. 4B), consistent with Cl$^-$ influx-mediated outward current, indicating that Cl$^-$ is more permeant than CH$_3$SO$_3^-$, $V_\text{rev}$ shifted to near $-40$ mV in the Cl$^-$ solution (Fig. 4C), showing that the channel is no longer proton-selective, and in fact, it has substantial permeability to Cl$^-$. Fig. 4D summarizes the Cl$^-$ permeability of several Asp$^{51}$ mutants. As in Hv1, the D51S mutant was highly permeable to Cl$^-$, and D51H was moderately permeable. In contrast, D51E had no detectable permeability to Cl$^-$, and its $V_\text{rev}$ was near $E_\text{H}$, consistent with perfect H$^+$ selectivity (H$^+$ specificity) like the WT channel.
what complicated by the observations that proton channels retained function after mutagenesis of individual R residues in S4 (40–42) and also after deletion of the entire C terminus starting immediately after the second Arg residue in S4 (43).

Asn\textsuperscript{214} was proposed to move into the narrowest portion of the conduction pathway in the open hHV1 channel and facilitate proton conduction (44). His occupies the equivalent position in kHV1, EhHV1, and CpHV1 (45). The work by Sakata et al. (43) showed that the murine equivalent N → R mutant retained proton conductance. We recently showed that N214D mutants of hHV1 are proton-selective (24). Combined, these observations show that this position can be occupied at least by other polar residues with little obvious effect on function.

Discussion

Distinctive Properties of kHV1. Most of the properties of kHV1 resemble the properties of other voltage-gated proton channels. The conductance seems to be perfectly selective for protons. The channel opens with depolarization and activates more rapidly at more positive voltages. In three other species—human, mouse, and C. intestinalis—hHV1 seems to exist as a dimer, in which each monomer contains its own conduction pathway (44, 46–48). The dimer is held together mainly by coiled-coil interactions in the C terminus (44, 46, 48–50). The C terminus of kHV1 lacks any predicted coiled-coil region and thus, kHV1 may exist as a monomer. Interestingly, a putative H\textsubscript{2}O from Phaeodactylum tricornutum, a diatom, lacks a significant predicted coiled-coil region in the C terminus, whereas a weak, short region of predicted coiled coil is present in putative H\textsubscript{2}O from Thalassiosira pseudonana, another diatom, and Trichoplax adhaerens, a primitive single-layer multicellular organism. The C termini of two (unicellular) coccolithophore H\textsubscript{2}O\textsubscript{1}s (45) also show a region of high-probability coiled-coil interactions as do all 28 H\textsubscript{2}O\textsubscript{1}s provisionally identified by sequence similarity in multicellular species. Evidently, dimeric architecture is a feature of H\textsubscript{2}O\textsubscript{1} in multicellular species but may be variably present in unicellular species. Dimerization slows proton channel opening (44, 48, 49); perhaps dinoflagellate H\textsubscript{2}O\textsubscript{1}s are built for speed.

The position of the \(g_{\text{f2}} \cdot V\) relationship depends on \(\Delta \text{pH}\) in kHV1, the same as in all other known proton channels. The slope of the \(V_{\text{rev}} \cdot V_{\text{thresh}}\) relationship (Fig. 3F) was identical, supporting a similar gating mechanism. However, the position of the \(g_{\text{f2}} \cdot V\) relationship at any given \(\Delta \text{pH}\) is 60 mV more negative in kHV1 than in all other species. This unique property could reside in the sequence differences within the VSD (Fig. 1) but could also be modulated by the N or C terminus, both of which differ significantly in kHV1 compared with other confirmed H\textsubscript{2}O\textsubscript{1}s (Fig. S1). A full explanation of this property awaits elucidation of the \(\Delta \text{pH}\) gating mechanism. The functional consequence of the negatively shifted voltage dependence, however, is clear. Whereas other proton channels apparently evolved to extrude acid from cells, kHV1 seems to be optimized to enable H\textsuperscript{+} influx. In bioluminescent species, such as Noctiluca, depolarization-activated H\textsuperscript{+} flux from vacuolar sap into the scintillon is the postulated trigger for the flash (10). A proton channel with normal properties that opens only when the proton electrochemical gradient is outward (27, 28) would not permit the H\textsuperscript{+} influx (from the vacuole into the scintillon) required to trigger a flash. However, the uniquely negative voltage range seen in kHV1 is perfectly suited to this task and indeed, to propagating an action potential into the scintillon membrane.

In nonbioluminescent mixotrophic species like K. veneficum, H\textsuperscript{+} influx might be involved in prey digestion (e.g., signaling prey capture) or prey capture (e.g., extrusion of trichocysts). A phylogenetic analysis of VSD regions from high-confidence H\textsubscript{2}O\textsubscript{1} sequences (Fig. S2) indicates high sequence diversity among the single-celled species and among invertebrates, suggesting the possibility of other novel functions of H\textsubscript{2}O\textsubscript{1}. As in multicellular
organisms, ion channels in dinoflagellates play various roles in regulating basic life functions, which make them targets for controlling dinoflagellate populations and behavior.

**Mechanism of Proton Selectivity.** Here, we identify Asp51 as a crucial element in the selectivity filter of kHV1. Mutation of Asp51 (D51S, D51A, and D51H) converted the kHV1 channel to anion permeability. In contrast, the conservative replacement D51E preserved proton selectivity. These results are closely analogous to the effects of Asp112 mutation in kHV1 (24). In addition to identifying a residue crucial to proton selectivity in each channel, our results generalize two surprising results from the previous study in kHV1: (i) a perfectly proton-selective channel can be made anion-permeable by mutation of a single amino acid, and (ii) replacement of Asp by His did not preserve proton selectivity. The latter point is remarkable in view of several examples of proton-conducting molecules in which His seems to be the residue responsible for proton selectivity. The M2 viral proton channel loses its proton selectivity when His17 is mutated (51, 52). Even more remarkable, Arg → His mutations to the K+ channel VSD (53, 54) or the Na+ channel VSD (55) produce a proton-selective conductance pathway. Extensive measurements led to the conclusion that, in the Arg → His mutations to the K+ channel VSD, the His residue is located at a constriction, where it is accessible to both external and internal solutions (53, 54, 56). Given the structural parallels of other ion channel VSDs with kHV1, we envision Asp51 in kHV1 to exist at or near a similar constriction at the focus of an hourglass of water molecules. The reason that His mediates proton conduction in K+ or Na+ channels but anion conduction in kHV1 is obscure.

There are substantial differences in sequence and even in overall architecture (e.g., major differences in N terminus, S1–S2 loop, and C terminus) between kHV1 and kHV1 (Fig. S1). The fact that a single amino acid substitution converts a proton-specific channel to an anion-permeable channel in both species must, therefore, reflect an essential design feature of the proton channel. There are several possible interpretations of this phenomenon. For example, after elimination of the negative charge at Asp51 or Glu51, the remainder of the conducting pore prefers anions, which may serve as a mechanism to exclude other cations from approaching the selectivity filter. In this view, Asp51 then provides both charge and proton selectivity. Elucidation of the precise mechanism of H+ selective conduction may require calculations using a structural model.

In summary, we have identified a gene encoding a voltage-gated proton channel in the dinoflagellate, *K. veneficum*. We describe key elements that comprise the signature of a proton channel. There are several possible interpretations of this proton channel in the dinoflagellate. In contrast with other proton channels at voltages 60 mV more negative than other proton channels at +40 mV, inward currents occur. In contrast with other proton channels, the function of kHV1 is evidently proton influx, which could mediate re-generative action potentials or locally acidify the cytoplasm.

**Materials and Methods**

**Alignments and Homology.** Additions were made to a previous set of HV1s and other VSD-containing proteins (24) by searching the updated eggNOG (57) database, which uses criteria that provide high-confidence homology. The full set of VSDs was realigned using Promals3D (58) as previously described (24). The resulting alignment was divided into subsets corresponding to four families of homologs: H1, C15orf27, VSP, and Kc, members of these subsets are listed in Table S1. Sequence logos of TM regions S1 to S4 were created from the individual family alignments using the Weblogo site (59). The length of the N terminus was taken as the number of residues before the first residue in S1, and the length of the C terminus was taken as the number of residues after the last residue in S4, which was defined in Fig. 1. The lengths of the C termini were compared by one-way ANOVA followed by Tukey’s test.

Both full-length and 4 regions of *C. intestinalis* and human H2 were used as templates to search a database of full-length cDNAs cloned from *K. veneficum*. Resulting sequence hits were compared with the sequence logos and the full sequence alignment of the VSD, and kHV1 (National Center for Biotechnology Information accession no. JN255155) was identified.

**Dinoflagellate Cultures.** *K. veneficum* strain CCMP2778 was grown in Corning Cell Culture Flasks (150 cm²) in 250 mL slightly modified EASW (enriched seawater, artificial water) medium (60) at 30 psu (practical salinity units) with the concentration of Hesper reduced to 1 μM at 24 °C. The photoperiod was shifted to 14:10 light to dark light cycle with 138 μM m⁻² s⁻¹ photon flux on the surface of the vessel closest to the light (measured with a Li-Cor QUANTUM probe attached to a Li-Cor LI-250 light meter). The dark period started at 4:00 PM Eastern Standard Time. Cell abundance was measured on a Coulter Counter (Beckman Coulter) using the narrow size range (4–30 μm). After 19 d of growth, duplicate flasks were removed at 4-hour intervals starting at 4:00 AM; a 1-mL aliquot was taken from each flask to which 50 μL glutarddehyde (50%) were added to fix cells for cell counts, and the remaining culture was poured into prechilled conical centrifuge bottles. After centrifugation at 1,500 rpm (500 × g) for 10 min at 25 °C, the supernatant was decanted, and 1 mL TrisReagent (Molecular Research Center) was added to the pellet on ice. The pellet was triturated to an even suspension, and it was placed in a 15-mL centrifuge tube and stored at 80 °C until RNA isolation.

**RNA Isolation and cDNA Preparation.** Total RNA from each pooled sample was prepared using TriReagent according to the manufacturer’s protocol. After precipitation, the RNA was resuspended and purified using a Qiagen RNeasy column. Total RNA was quantified by UV spectroscopy and qualified on an Agilent 2100 Bioanalyzer; 1 μg total RNA from individual cultures was reverse-transcribed in triplicate using an oligod(T) primer and the RetroScript reverse transcription kit (Ambion).

**qPCR Primers and Conditions.** The expression of mRNA for a select number of genes was determined by RT-qPCR using primers generated from the EST sequences obtained from library screening (Table S2). At the time of sampling, the average cell density was 10,466 ± 1,374 (n = 12) cells/mL. Preliminary experiments verified that all primer/template sets showed high PCR efficiency; PCR product sizes were all about 100 to 120 nt. All real-time assays were performed on an Applied Biosystems 7500 Real-Time system using the iTaq Fast SYBR Green supermix with ROX from Bio-Rad. A protocol: 95 °C for 15 min, 94 °C for 15 s, gene-specific annealing temperature (58–62 °C) for 40 s, and 72 °C for 1 min for 40 cycles followed by a gradual increase in temperature from 60 °C to 95 °C during the dissociation (melt) stage. The dissociation stage was performed to confirm the presence of a single PCR product.

**Gene Expression.** A version of the kHV1 gene optimized for human codon use but preserving the amino acid sequence was commercially synthesized (GenScript); the synthesized DNA, including a 5′ BamHI restriction site and Kozak sequence and 3′ Not1 restriction site (complete sequence in Dataset S1), was cloned commercially (GenScript) into pCDA3.1+ (Invitrogen). We subcloned the gene by PCR into pEGFP-C3 (Clontech) using 5′ EcoRI and 3′ BamHI restriction sites. Site-directed mutants were created using the Stratagene Quickchange (Agilent) protocol according to the manufacturer’s instructions. Clones were sequenced commercially to confirm the mutation. HEK-293 or COS-7 cells were grown to ∼80% confluence in 35-mm cultures dishes. Cells were transfected with 0.4–0.5 μg appropriate cDNA using Lipofectamine 2000 (Invitrogen) or polyethyleneimine (Sigma). After 6 h at 37 °C in 5% CO₂, cells were trypsinized and replated onto glass coverslips at low density for patch clamp recording the next day. We selected green cells under fluorescence for recording.
Electrophysiology.
Whole-cell or excised patch variants of the patch clamp technique were carried out as described previously (61). The main pipette solution (also used externally) contained 130 mM TMACl, 2 mM MgCl₂, 2 mM EGTA, and 80 mM Mes titrated to pH 5.5 with ~20 mM TMAOH. In the pH 5.5 TMACl solution, TMACl replaced TMACl₂. Solutions at pH 7.0 had 90 mM TMACl₂, 2 mM MgCl₂, 3 mM CaCl₂, 1 mM EGTA, 100 mM BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), and 36 to 40 mM TMAOH. Except where noted, experiments were done at 21 °C or room temperature (20–25 °C). No leak correction has been applied to current records. V_m data were corrected for liquid junction potentials measured in each solution.

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Table S1.

The sequence of the DNA synthesized (Genscript, Piscataway, NJ) and cloned into pcDNA3.1+ for heterologous expression in primate cells is given below. Start and stop codons are in bold, restriction sites are underlined, and the Kozak sequence is in italics. The coding sequence of the synthesized DNA was optimized for human codon usage, while preserving the protein sequence predicted by the kHV1 cDNA (NCBI accession number JN255155).

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GGATCCGCCACCATGGATAGAATCTCTGCATCACGCTGTCATACGTGACATACATCA
AAGTCCGCAAGAGACGCTGAAGGGAACGGGAACCTGCGAGAAGCAAGCTGAACGAAG
CCCTGAATAGCTCCAAAAGTCCACACACTACCTGACTGCTGCTGATTTTGCGACCTGA
TGACCGTCATCATATTGGGATGGCAGTACATATTTCTGATAGTCAGGTGAGG
GACTGACCGAGGCGCTTCAAGGACGTGCCTGGAGAAACGCACATTTTTGTCGCCGATCCTA
GTCACCTGGACACATTACGGGAACCAGCCTGGCATGAGTGGGCGAAGCAGAATGGAG
TATGGCATCCCTGGCCCATCCCTGCTGATTTTCTCTGGGAGAACATCGCTGCTGTCCCTGG
CTAATTGCTGTAGATTTCTTTCGAAACCCTTCCACATCCCTGGACATTTGTTCTGTGTCTG
CTGAGCCTGGGAATTGAACTGACGAGCTCTGGGGAGAAGGACATGATGCAAGGAA
TTGGCCCTGGATGCAATTTGGGTGCATCTGGGAGGCGATACCGCTGCCGACCGCACTCC
ACGGAAATGCAACGAGAACATGAGGCCGAGAACACGGGAGGAGCATCGGGGTTCAGGA
TGCCGCTGGAAAGCTCCCTGCCAGAACAGGGCTCTTCTGGAGCAGCAGCAAGAACCGACGGTA
AGAGGAGGATGTTGCTTCAGATGCGGCGGC
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Fig. S1. Alignment of six H$_{\text{V1}}$ sequences that have been confirmed by heterologous expression and electrophysiological characterization.
Fig. S2. Phylogenetic analysis of 37 Hv1 sequences. A maximum likelihood phylogenetic tree with 100 bootstraps was constructed using PhyML 3.0 (1) at the Mobyle portal (2) [JTT (Jones, Taylor, Thornton) substitution model, four relative substitution rate categories] from a multiple sequence alignment of the voltage sensor domain portion of 37 Hv1s. The tree was visualized using iTOL (3). Branch lengths are displayed to scale and are proportional to the distance between sequences. Bootstrap values > 60 are shown.

Table S2. Primers used for quantitative PCR

<table>
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<td>Kven_actin_QRev</td>
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Table S1. Sequences from which the family sequence logos in Fig. 1 were created

<table>
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<th>Sequences</th>
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<tr>
<td>C15orf</td>
<td>Ornithorhynchus GI:149410687; Danio GI:123703002; Monodelphis GI:12627230 ; Sus GI:194039682; Homo GI:118442841; Pan GI:114658268; Equus GI:149692210; Mus GI: 27370422 ; Rattus GI:157817759 ; Meleagris, GI:326926430; Taeniopygia, GI:224061716; Pongo, GI:297697189; Callithrix, GI:296236847; Alluropodia, GI:301780212; Oryctolagus, GI: 291410741</td>
</tr>
<tr>
<td>VSP</td>
<td>Ciona GI:76253898; Mus GI:0549440; Homo GI:213972591; Homo GI: 40549435; Canis GI:73993164; Rattus GI:157820295; Xenopus tropicalis GI: 62859843; Canis laevis GI:148230800; Danio GI:70887553; Gallus GI: 118084254; Ornithorhynchus GI:149635858</td>
</tr>
<tr>
<td>Kv</td>
<td>Homo, sp_Q8TDN2; Homo, sp_Q9H3M0; Homo, sp_Q14721; Canis, sp_Q95167; Drosophila, sp_P17970; Pongo, sp_Q5RC10; Homo, sp_Q9BQ31; Saimiri, sp_A4K2X4; Gallus, sp_Q73606; Homo, sp_Q8TDN1; Rattus, sp_Q1956; Homo, sp_Q96PR1; Drosophila, sp_P17972; Homo, sp_Q9470; Rattus, sp_P17659; Homo, sp_P22460; Rattus, sp_P15384; Canis GI: 57088651; Bos sp_Q05037; Homo sp_Q16322; Rattus GI: 16087779; Homo GI: 4826782; Rattus PDB:2A79; Canis, sp_Q28293; Drosophila, GI: 288442; Oryctolagus, sp_Q9TT55; Aeropyrum, PDB:1ORS; Oncorhynchus, sp_Q91830; Danio, TR_B0V2U3; Danio, TR_E0R78; Brugia, TR_A8NE89; Brugia, TR_A8QFU4; X. laevis, TR_Q91593; X. laevis, TR_Q91592; X. laevis, TR_BZ9Q5; Dicentrarchus, TR_E6ZH08; Squalus, TR_O73925; Zonochoria, TR_D8KW77</td>
</tr>
</tbody>
</table>

GI, accession numbers from the National Center for Biotechnology Information; ENS, accession numbers from Ensembl; JGI, numbers from Joint Genome Institute; TR or sp, accession numbers from Uniprot. VSP, voltage-sensitive phosphatase.

Other Supporting Information Files

Dataset S1 (PDF)