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Multiple modalities converge on a common gate to control K_{2P} channel function

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Members of the K_{2P} potassium channel family regulate neuronal excitability and are implicated in pain, anaesthetic responses, thermosensation, neuroprotection, and mood. Unlike other potassium channels, K_{2P}s are gated by remarkably diverse stimuli that include chemical, thermal, and mechanical modalities. It has remained unclear whether the various gating inputs act through separate or common channel elements. Here, we show that protons, heat, and pressure affect activity of the prototypical, polymodal K_{2P}, K_{2P}2.1 (*KCNK2*/TREK-1), at a common molecular gate that comprises elements of the pore-forming segments and the N-terminal end of the M4 transmembrane segment. We further demonstrate that the M4 gating element is conserved among K_{2P}s and is employed regardless of whether the gating stimuli are inhibitory or activating. Our results define a unique gating mechanism shared by K_{2P} family members and suggest that their diverse sensory properties are achieved by coupling different molecular sensors to a conserved core gating apparatus.

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Introduction

K_{2P} (*KCNK*) potassium channels produce 'background' currents that stabilize the membrane resting potential and that have a critical role in control of cell excitability (Bayliss and Barrett, 2008; Enyedi and Czirjak, 2010). K_{2P}s are members of the superfamily of voltage-gated ion channels (Yu *et al*, 2005) but have a unique topology; the channels are dimers of subunits that each has four transmembrane segments and two pore regions per polypeptide (Goldstein *et al*, 2005). Although well known as 'leak' channels that are constitutively open at rest, many K_{2P}s are polymodal and respond to a wide range of diverse regulatory inputs that include extracellular and intracellular pH, temperature, membrane stretch, polyunsaturated fatty acids, volatile anaesthetics, and noxious chemicals (Honore, 2007; Enyedi and Czirjak, 2010). Numerous studies have established that K_{2P}s have major roles in the brain, cardiovascular system, and somatosensory neurons (Duprat *et al*, 2007; Honore, 2007; Bayliss and Barrett, 2008; Folgering *et al*, 2008; Sabbadini and Yost, 2009; Enyedi and Czirjak, 2010). Further, mutations in K_{2P}s have been linked to mental retardation (Barel *et al*, 2008) and migraine (Lafreniere *et al*, 2010). Nevertheless, our understanding of K_{2P} gating mechanisms lags far behind other potassium channel classes (Cohen *et al*, 2009; Mathie *et al*, 2010) and presents a barrier to explaining how such diverse types of inputs modulate K_{2P} function. Thus, defining K_{2P} functional mechanisms remain an important objective for establishing how these channels influence excitation.

K_{2P}2.1 (*KCNK2*/TREK-1) (Fink *et al*, 1996; Honore, 2007) is the best-studied polymodal K_{2P} and responds to both extracellular (Cohen *et al*, 2008; Sandoz *et al*, 2009) and intracellular (Maingret *et al*, 1999b; Honore *et al*, 2002) acidosis, heat (Maingret *et al*, 2000; Noel *et al*, 2009), mechanical forces (Maingret *et al*, 1999a; Noel *et al*, 2009), and anaesthetics (Patel *et al*, 1999; Heurteaux *et al*, 2004). K_{2P}2.1 (TREK-1) is important in sensory neuron pain responses (Alloui *et al*, 2006; Noel *et al*, 2009) and vasodilation (Bryan *et al*, 2006, 2007; Blondeau *et al*, 2007; Garry *et al*, 2007). Its activity is linked to chronic pain, thermosensation (Alloui *et al*, 2006; Noel *et al*, 2009), response to general anaesthetics (Heurteaux *et al*, 2004), and depression (Gordon and Hen, 2006; Heurteaux *et al*, 2006; Perlis *et al*, 2008; Dillon *et al*, 2010). Consequently, K_{2P}2.1 (TREK-1) along with other K_{2P}s present attractive targets for the development of new agents directed at treating ischaemic injury, pain, and depression (Honore, 2007; Bayliss and Barrett, 2008).

In general, potassium channels contain two major points of control or 'gates' that are used to varied degrees to control activity depending on the particular channel (Yellen, 2002). One gate, called the 'outer' or 'C-type' gate involves the selectivity filter, which makes the direct contacts with the permeant ions, and is sensitive to external potassium concentration. The other gate, a constriction of the pore-lining transmembrane segments, is known as the 'inner gate' and can block access to the channel pore from the cytoplasmic side. Both K_{2P}2.1 (TREK-1) and the drosophila K_{2P}, KCNK0, have been shown to have a C-type gate (Zilberberg *et al*, 2001; Cohen *et al*, 2008). Because of the extracellular placement of key proton sensing residues, this type of gate has been implicated in the response of a number of K_{2P}s to external pH (Kim *et al*, 2000; Rajan *et al*, 2000; Lopes *et al*, 2001; Morton *et al*, 2005; Sandoz *et al*, 2009). Recent studies also suggest the presence of an inner gate in mutant KCNK0

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channels (Ben-Abu *et al*, 2009), although the extent to which such a gate operates in native K_{2P}s is unclear. Nevertheless, the gating mechanisms that control K_{2P} function remain obscure and many fundamental questions remain unanswered regarding the generality of these gating elements within the K_{2P} family and how such elements are coupled to the diverse types of gating inputs (Cohen *et al*, 2009; Mathie *et al*, 2010).

Such questions are magnified in the archetypal polymodal K_{2P} K_{2P}2.1 (TREK-1). This channel is controlled by physically diverse signals that act on different channel elements. For example, a histidine in the first extracellular loop is the main sensor for extracellular protons (Cohen *et al*, 2008; Sandoz *et al*, 2009), whereas the C-terminal cytosolic domain contains elements that are involved in the response to temperature and mechanical inputs (Patel *et al*, 1998; Maingret *et al*, 1999b, 2000; Honore *et al*, 2002). It has remained unclear whether these diverse modulatory inputs and sensors control K_{2P}2.1 (TREK-1) via separate or common gating mechanisms. Additionally, because of the high sequence divergence in the K_{2P} family, the extent to which gating mechanisms are conserved among K_{2P}s has remained unresolved. Here, we examine these issues with regard to external proton, heat, and pressure-evoked K_{2P}2.1 (TREK-1) gating and demonstrate that these three gating inputs act via a common gate that has the characteristics of a C-type gate. Further, we find that regardless of whether the gating signal activates or inhibits channel function, this mechanism is conserved among diverse K_{2P} channels.

Results

A yeast potassium uptake selection identifies mammalian K_{2P}2.1 (TREK-1) gain-of-function mutations

Complementation of potassium-uptake-deficient yeast has been a fruitful approach for studying a variety inward rectifier and viral potassium channels (Minor, 2009) but has not yet been applied to other types of potassium channels. Because of their ability to conduct 'leak' currents, we reasoned that K_{2P} channels such as K_{2P}2.1 (*KCNK2*/TREK-1) might be functional in this system. We found that unlike the inward rectifier Kir2.1 (Minor *et al*, 1999; Chatelain *et al*, 2005), mammalian K_{2P}2.1 (TREK-1) complemented growth of a potassium-uptake-deficient yeast strain SGY1528 (Tang *et al*, 1995) under the mild potassium limited conditions (1 mM KCl) but not under the most stringent complementation conditions (<1 mM KCl) (Figure 1A). To identify key K_{2P}2.1 (TREK-1) gating elements, and following precedents set with G-protein activated inward rectifiers (Sadja *et al*, 2001; Yi *et al*, 2001), we constructed a randomly mutagenized K_{2P}2.1 (TREK-1) library that had mutations throughout the channel sequence (Supplementary Figure S1A) and selected for gain-of-function (GOF) mutants that would rescue growth in the presence of 0.5 mM KCl. The selections yielded GOF mutations in four channel regions (Figure 1B): the extracellular portion of the first selectivity filter (I148T), the second P-loop (L267P), the N-terminal part of transmembrane segment 4 (M4) (W275S and F276L), and the C-terminal cytoplasmic domain (E306G, E309A, S333G, and S333R). We also isolated a double mutant that combined the two changes in the extracellular vestibule (I148T/L267P). The identified C-terminal domain positions have been shown previously to cause K_{2P}2.1 (TREK-1) GOF (Maingret *et al*, 2000; Honore *et al*, 2002; Murbartian *et al*, 2005) and thus, validate the general selection strategy. As the GOF positions in the extracellular region and the M4 positions had not been previously implicated in K_{2P} function, we turned our attention to these.

Measurement of current–voltage relationships in *Xenopus* oocytes injected with equivalent amounts of mRNA for each of the GOF mutants or wild-type K_{2P}2.1 (TREK-1) revealed that all tested GOF mutants had increased activity relative to wild type (Figure 1C and D). The effects of the I148T/L267P double mutant were additive compared with the individual mutants and suggest that the selectivity filter and second P-loop sites act independently. Importantly, the GOF mutants retained potassium selectivity (Supplementary Figure S1B). Three parameters affect whole cell current (I) as expressed by the equation: $I = N \times P_O \times i$, where N is the active channel number, P_O is single channel open probability, and i is single channel current amplitude (Hille, 2001). Therefore, we examined the effects of the GOF mutations on each of these parameters. Single channel analysis, under conditions in which similar numbers of channels were observed (Supplementary Figure S1C), demonstrated that all of the GOF mutations increase single channel open probability (mean ± s.e., $n = 5$–9): wild type (0.04 ± 0.01), I148T (0.10 ± 0.03), L267P (0.12 ± 0.07), I148T/L267P (0.17 ± 0.037), W275S (0.17 ± 0.08), F276L (0.16 ± 0.07) (Figure 1E and F), and that all but F276L cause a slight increase in single channel conductance (Supplementary Figure S1D and E). Furthermore, none of the tested GOF mutants increased surface expression, as judged either by biotinylation of surface proteins in COS7 cells (Supplementary Figure S2A) or by immuno-detection of the channels on the surface of the oocytes (Supplementary Figure S2B). Thus, taken together, the data indicate that the GOF mutations affected the gating machinery of the channel.

Protons, heat, and mechanical force control K_{2P}2.1 (TREK-1) activity via a common gate

The K_{2P}2.1 (TREK-1) gating apparatus includes a C-type-like outer gate that encompasses the selectivity filter that closes in response to extracellular acidosis (Cohen *et al*, 2008; Sandoz *et al*, 2009) as well as sensory elements in the C-terminal cytoplasmic tail that respond to temperature and mechanical inputs (Maingret *et al*, 1999b, 2000). It has remained unclear, however, whether activating stimuli such as increased temperature (Maingret *et al*, 2000) and mechanical force (Chemin *et al*, 2005) act at the same outer gate or elsewhere.

To probe how the gating changes affected K_{2P}2.1 (TREK-1) responses to both inhibitory and activating inputs, we challenged each of the GOF mutants by three different stimuli: extracellular acidosis, temperature, and mechanical stress. Strikingly, the GOF mutants I148T, L267P, I148T/L267P, and W275S affected the responses to each of the three stimuli to some degree (Figure 2; Supplementary Figure S3 and Table S1). The largest effects were caused by I148T/L267P and W275S. I148T/L267P blunted the response to all three modalities (Figure 2B, D, and F), whereas W275S had large effects on extracellular pH (pH_O) and temperature responses (Figure 2B and D). In stark contrast, the reaction of the F276L GOF mutant to pH_O, temperature, and mechanical stress was not significantly different from that of wild type. Even though I148T, L267P, I148T/L267P, and W275S could not be completely inhibited by pH_O under the limits of our experimental conditions, their sensitivity to external magnesium inhibition

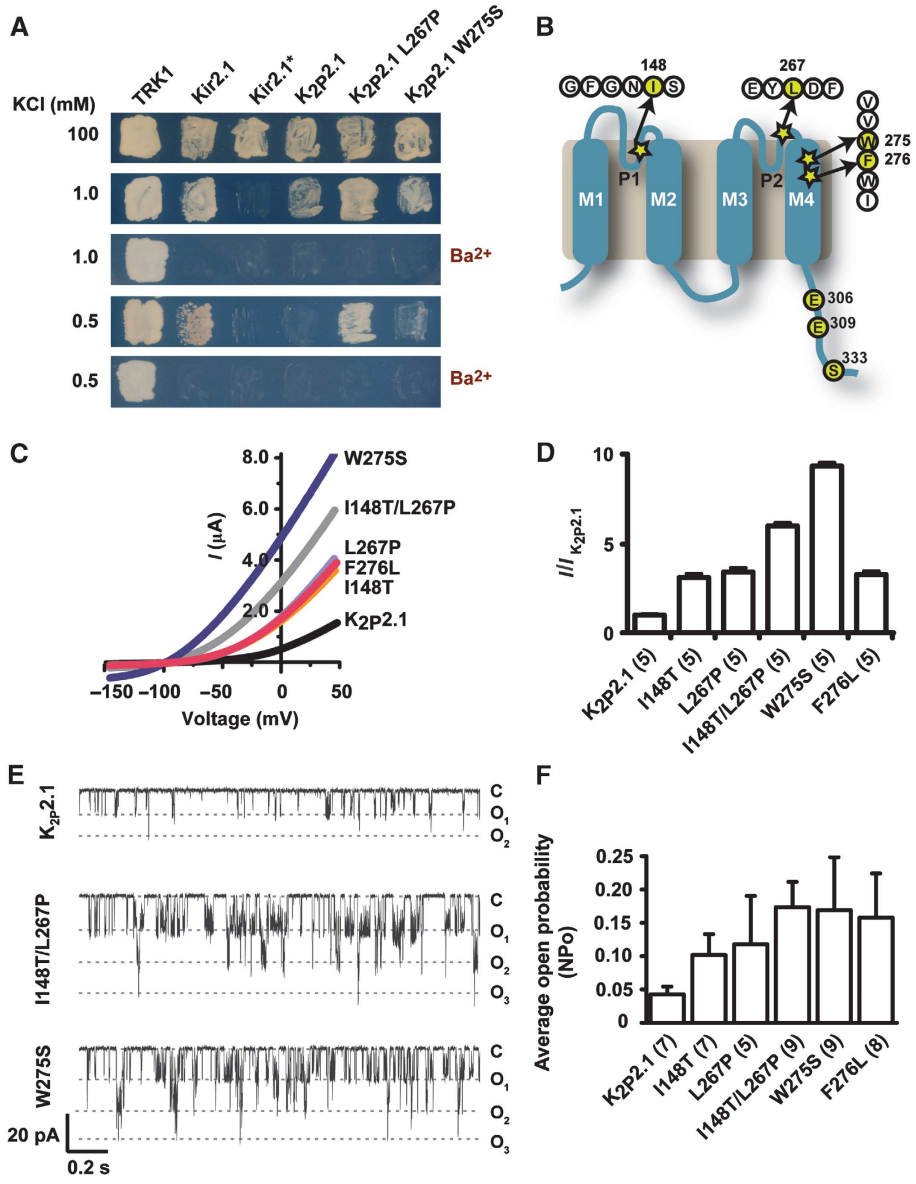

Figure 1 Functional selection identifies GOF mutations in a mammalian $K_{2P}2.1$ (TREK-1). (**A**) Growth of potassium-transport-deficient yeast (SGY1528) expressing the yeast potassium transporter TRK1, Kir2.1, an inactive Kir2.1 mutant (Kir2.1*), $K_{2P}2.1$ (TREK-1), and two exemplar $K_{2P}2.1$ (TREK-1) GOF mutants, L267P and W275S under non-selective conditions (100 mM KCl), and two different selective conditions (1 mM and 0.5 mM KCl). Rows indicated with 'Ba^{2+}' show growth in the presence of the Kir2.1 and $K_{2P}2.1$ (TREK-1) inhibitor 8 mM $BaCl_2$. (**B**) $K_{2P}2.1$ (TREK-1) subunit topology diagram. Locations of GOF mutations are indicated in yellow. Transmembrane segments M1, M2, M3, and M4 and the two P-loop domains are labelled. (**C**) Exemplar current–voltage traces from whole cell recordings of *Xenopus* oocytes injected with 0.3 ng of $K_{2P}2.1$ (TREK-1) or GOF mutant mRNA. Currents were elicited in solutions containing 2 mM potassium (ND96) by a ramp protocol from −150 to +50 mV from a −80 mV holding potential. Values for the average current (in μA, mean ± s.e., $n = 5$) at 0 mV were $K_{2P}2.1$ (TREK-1) (0.52 ± 0.40), I148T (1.61 ± 0.106), L267P (1.77 ± 0.121), I148T/L267P (3.11 ± 0.092), W275S (4.86 ± 0.099), F276L (1.70 ± 0.097). (**D**) Quantification of normalized current amplitudes at 0 mV from *Xenopus* oocytes injected with 0.3 ng of mRNA for the indicated channels. (**E**) Cell-attached mode single channel recordings of $K_{2P}2.1$ (TREK-1), I148T/L267P, and W275S expressed in COS7 cells. O_1, O_2, and O_3, indicate the first, second, and third open states, respectively. C indicates the closed state. (**F**) Open channel probabilities from single channel analyses calculated on recordings of ~30 s duration. $K_{2P}2.1$ (TREK-1) ($n = 7$), I148T ($n = 7$), L267P ($n = 5$), I148T/L267P ($n = 9$), W275S ($n = 9$), F276L ($n = 8$). Data represent mean ± s.e.

(Maingret *et al*, 2002) remained similar to wild type (Supplementary Figure S4). This result indicates that the GOF mutations did not create channels that were generally resistant to inhibition. The fact that neither the open probability nor the surface expression of F276L exceeds that of I148T/L267P or W275S eliminates the possibility that the blunted responses of I148T/L267P and W275S arise simply because of the increased open probability or differences in expression levels. Instead, these data support the idea that the I148T, L267P, and W275S mutants act by uncoupling the sensors for the various stimuli from the gating apparatus. Further, when taken together, our data indicate that despite the radically different physical natures of the stimuli, the actions of all three modalities converge on the parts of $K_{2P}2.1$ (TREK-1) in which these GOF mutations reside, the extracellular vestibule and the outer M4 region, to control gating.

The effect of pH_O on $K_{2P}2.1$ (TREK-1) is antagonized by increases in the permeant potassium ion concentration

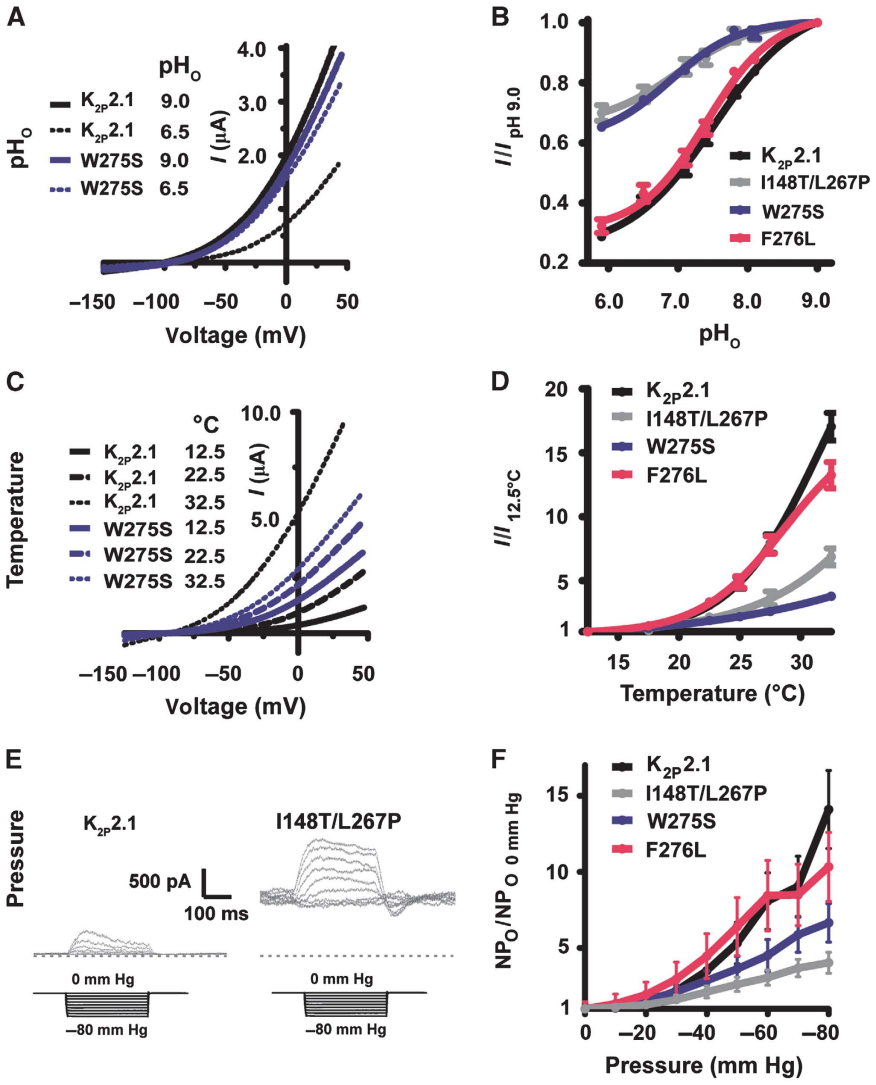

Figure 2 K_{2P}2.1 (TREK-1) GOF mutations affect response to extracellular acidosis, heat, and pressure. (**A**) Exemplar two-electrode voltage-clamp recordings of the response of K_{2P}2.1 (TREK-1) and the W275S GOF mutant to external pH (pH_O) changes in 2 mM $[K^+]_O$ solutions. (**B**) Normalized pH_O responses (at 0 mV) for the indicated channels. (**C**) Exemplar two-electrode voltage-clamp recordings of the response of K_{2P}2.1 (TREK-1) and the W275S GOF mutant to temperature in 2 mM $[K^+]_O$, pH 7.4 solutions. (**D**) Normalized temperature responses (at 0 mV) for the indicated channels. (**E**) Exemplar mechanical force (cell-attached mode, 150 mM KCl pH 7.2 in the bath, 5 mM KCl pH 7.4 in the pipette) responses of K_{2P}2.1 (TREK-1) and the I148T/L267P GOF mutant stimulated by negative pressure applied to the extracellular side of the plasma membrane through the patch pipette. (**F**) Normalized pressure responses for the indicated channels. In (**A**, **C**) currents were elicited by a ramp from −150 to +50 mV from a holding potential of −80 mV. Lines for (**B**, **D**) show fits to the equations $I = I_{min} + (I_{max}–I_{min})/(1 + ([H^+]_O/K_{1/2})^H)$ and $I = I_{min} + (I_{max}–I_{min})/(1 + e^{(T_{1/2}–T)/S})$, respectively. Data in (**B**, **D**, **F**) show mean ± s.e. ($n = 8$–30). $N \geqslant 2$ for all experiments.

(Cohen *et al*, 2008; Sandoz *et al*, 2009) and suggests a gating mechanism that relies on conformational changes in the selectivity filter. To probe whether the GOF mutations affected this process, we measured how an increase in the extracellular potassium concentration, $[K^+]_O$, from 2 to 90 mM affected pH_O inhibition. We found that I148T, I148T/L267P, and W275S had significantly diminished responses to changes in $[K^+]_O$ in comparison with wild-type K_{2P}2.1 (TREK-1) (Figure 3A and B; Supplementary Figure S5). Notably, the two GOF mutants that had the largest effects on eliminating the responses to the various gating inputs, I148T/L267P and W275S, also had the largest impact on $[K^+]_O$ sensitivity (Figure 3D). Further, the pH_O response of these mutants in both high and low $[K^+]_O$ conditions was very similar to that of wild-type K_{2P}2.1 (TREK-1) under high $[K^+]_O$ conditions. In contrast, the F276L GOF mutant was not different than wild type (Figure 3C and D). Thus, three properties indicate that I148T/L267P and W275S GOF mutations act by stabilizing the core gating mechanism of the channel to promote the conductive conformation of the selectivity filter: the large increase in channel activity, the resistance to changes in $[K^+]_O$, and the similarity of the pH_O response to that of the wild-type channel in high external potassium. These properties are consistent with a C-type gating mechanism that involves changes in the conformation of the selectivity filter.

C-type gating of K_{2P} potassium channels is accompanied by an increase of the relative permeability of sodium over potassium (Yuill *et al*, 2007; Cohen *et al*, 2008). To apply a further test to the idea that the conductive conformation of the pore is stabilized by the I148T/L267P and W275S GOF mutations, we measured how pH_O changes affected the ion

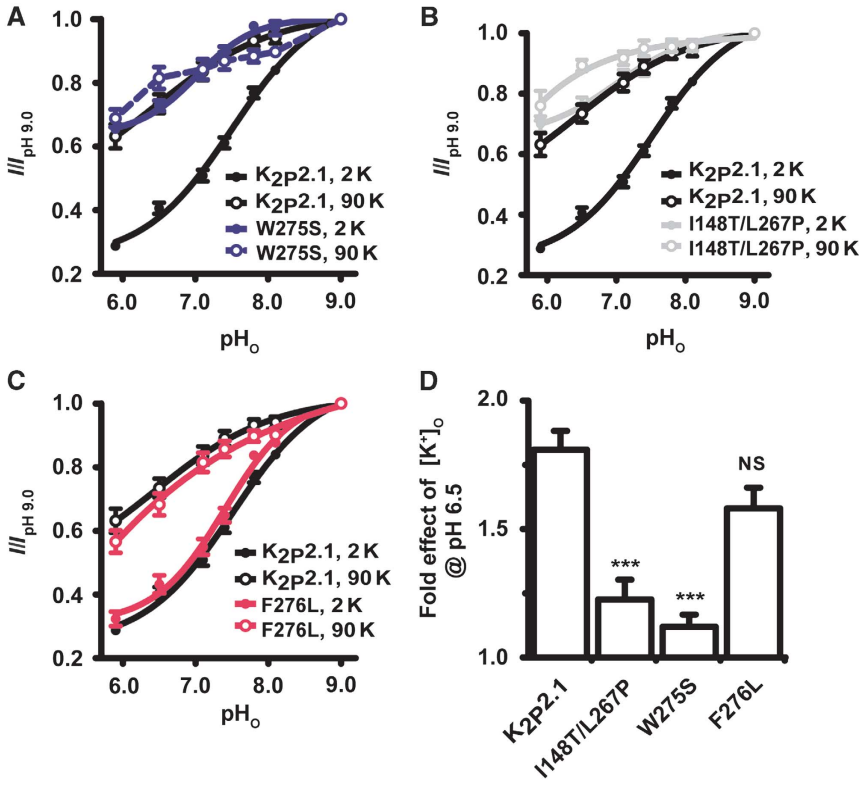

Figure 3 Extracellular loops and extracellular proximal portion of M4 control $K_{2P}2.1$ (TREK-1) gating. (**A–C**) Normalized response to pH_O in low (2 mM, data are from Figure 2B) and high (90 mM) $[K^+]_O$ (2 K and 90 K, respectively) for $K_{2P}2.1$ (TREK-1) and the indicated GOF mutants. Whole cell currents were elicited in *Xenopus* oocytes by a ramp from −150 to +50 mV from a holding potential of −80 mV (2 K) or 0 mV (90 K). (**D**) Quantification of the effect of high (90 mM) external potassium on the pH_O response from the curves in (**A–C**) $I_{6.5norm}(90\,K)/I_{6.5norm}(2\,K)$. Lines for (**A–C**) show fits to the Hill equation $((I = I_{min} + (I_{max} - I_{min})/(1 + ([H^+]_O/K_{1/2})^H))$. Data represent mean ± s.e. ($n = 6$–30). Statistical analysis: *t*-test. ***$P < 0.001$, NS, not significant ($P > 0.05$). $N \geqslant 2$ for all experiments.

selectivity of the mutants in comparison with wild-type $K_{2P}2.1$ (TREK-1) (Figure 4). Lowering pH_O from 9.0 to 6.9 in a recording solution containing 100 mM sodium caused a significant right shift in the reversal potential values measured in oocytes expressing either wild-type or F276L channels ($\Delta E_{rev} = 31.1$ and 21.3 mV, respectively; Figure 4A–D) and had no effect on the reversal potential recorded in 100 mM potassium (Figure 4E). Of note, the change in the reversal potential in 100 mM sodium at pH_O 6.9 did not simply arise from a decrease in the number of active potassium channels (Supplementary Figure S6). These results are consistent with prior studies of wild-type $K_{2P}2.1$ (TREK-1) (Cohen *et al*, 2008) and are indicative of C-type gating. In contrast, under the same recording conditions, I148T/L267P and W275S were significantly less sensitive to the pH_O change ($\Delta E_{rev} = 8.8$ and 12.7 mV, respectively). Accordingly, pH_O changes from 9.0 to 6.9 steadily increased Na^+/K^+ permeability ratio of wild-type and F276L channels, but had a minimal effect on I148T/L267P and W275S (Figure 4F). These data provide further support for the idea that I148T/L267P and W275S mutations stabilize the potassium-selective conductive conformation of the selectivity filter and act on a C-type-like gate.

The extracellular region of M4 is a key element of the $K_{2P}2.1$ (TREK-1) gating apparatus

Of all the GOF mutations we isolated, W275S stood out. In contrast to the Ile148 site, which is in a region that is similar to a portion of voltage-gated potassium channels that is crucial for control of the C-type gate (Lopez-Barneo *et al*, 1993) and the Leu267 site, which is not conserved within the K_{2P} family, Trp275 is largely conserved in K_{2P}s (Supplementary Figure S7). More strikingly, it occurs in a channel element that is rather intolerant to amino-acid changes in other potassium channel classes (Collins *et al*, 1997; Minor *et al*, 1999; Irizarry *et al*, 2002). These observations, together with our data demonstrating that a single change at this site blunted different gating modality responses and $[K^+]_O$ sensitivity, strongly suggested that this M4 region might have a special role in K_{2P} function. As the Trp→Ser substitution causes a dramatic sidechain volume change, we tested the tolerance of the $K_{2P}2.1$ (TREK-1) Trp275 position to substitution with a set of amino acids having diverse physicochemical properties. All non-aromatic substitutions examined, W275A/T/L/N/Q/D/E, resulted in channels with increased activity as assessed by two-electrode voltage-clamp (Figure 5A). In contrast, aromatic substitutions, W275Y and W275F, resulted in channels that were similar to wild type. Accordingly, investigation of how the Trp275 substitutions affect gating by pH_O and temperature revealed that all non-aromatic substitutions produced channels having reduced responses to both gating stimuli (Figure 5B and C; Supplementary Table S1). These results further support the integral role of the Trp275 position in $K_{2P}2.1$ (TREK-1) gating and the key role of the extracellular proximal portion of M4.

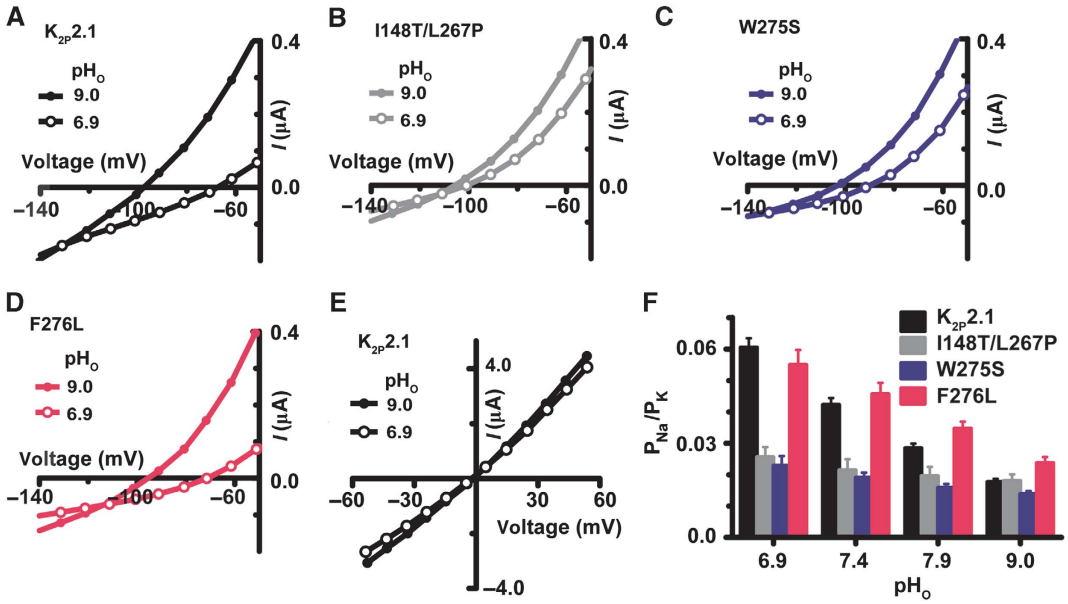

Figure 4 Extracellular loops and extracellular proximal portion of M4 control ion selectivity of $K_{2P}2.1$ (TREK-1). (**A**–**D**) Exemplar two-electrode voltage-clamp recordings of the response of the wild-type and mutant $K_{2P}2.1$ (TREK-1) channel to pH_O changes in 100 mM external sodium solutions. Currents were evoked by 60 ms long pulses from −150 to −50 mV in 10 mV increments from a −80 mV holding potential. Cells were injected with different amounts of mRNA to yield comparable current amplitudes. (**E**) Exemplar two-electrode voltage-clamp recordings of the response of the wild-type $K_{2P}2.1$(TREK-1) channel to pH_O changes in 100 mM external potassium solutions. Currents were evoked by 60 ms long pulses from −50 to 60 mV in 10 mV increments from a 0-mV holding potential. (**F**) Quantification (mean ± s.e., $n = 8$–11) of apparent permeability ratios at different pH_O using the equation $pNa/pK = e^{F\Delta Erev/RT}$, where pNa and pK are permeabilities for sodium and potassium, respectively, and ΔErev is a difference between the reversal potentials measured in 100 mM sodium and 100 mM potassium solutions. $N \geqslant 2$ for all experiments.

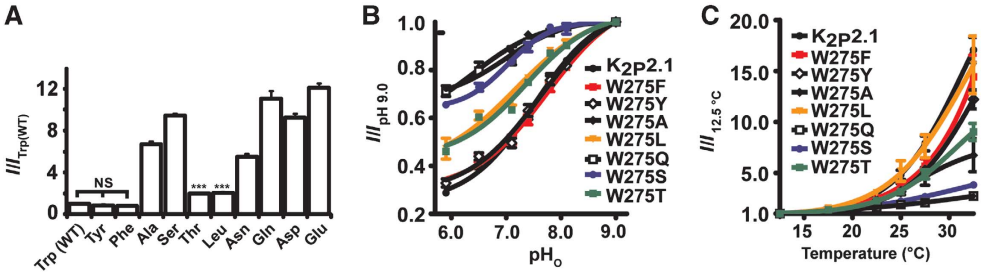

Figure 5 Tests of the impact of amino-acid changes at Trp275 on $K_{2P}2.1$ (TREK-1) function. (**A**) Normalized whole cell current amplitude (at 0 mV) recorded in 2 mM $[K^+]_O$ pH 7.4 from *Xenopus* oocytes injected with equivalent amounts of mRNA for the indicated $K_{2P}2.1$ (TREK-1) Trp275 substitutions. Statistical analysis: *t*-test. ***$P < 0.001$; NS, not significant ($P > 0.05$). (**B**) Normalized pH_O responses in 2 mM $[K^+]_O$ for the indicated Trp275 mutants. (**C**) Normalized temperature responses in 2 mM $[K^+]_O$ for the indicated W275 mutants. Curves show fits to the Hill equation $I = I_{min} + (I_{max} - I_{min})/(1 + ([H^+]_O/K_{1/2})^H)$ or $I = I_{min} + (I_{max} - I_{min})/(1 + e^{(T_{1/2} - T)/S})$. Data represent mean ± s.e. ($n = 4$–30). $N \geqslant 2$ for all experiments.

The extracellular region of M4 is functionally conserved across the K_{2P} family

To test whether the Trp275 equivalent position might have a general role in controlling gating within the K_{2P} family, we examined how aromatic→Ser mutations at the analogous M4 position in other pH_O-sensitive K_{2P} channels affected function (Figure 6A). Strikingly, equivalent changes at the Trp275 homologous position in $K_{2P}10.1$ (*KCNK10*/TREK-2), W301S; $K_{2P}9.1$ (*KCNK9*/TASK-3), F225S; and $K_{2P}3.1$ (*KCNK3*/TASK-1), F225S, profoundly affected pH_O gating (Figure 6B–D). This effect occurred even though these pH_O-gated K_{2P} channels use different extracellular elements as $[H^+]_O$ sensors (Kim *et al*, 2000; Rajan *et al*, 2000; Lopes *et al*, 2001; Morton *et al*, 2005; Sandoz *et al*, 2009) and was independent of whether $[H^+]_O$ is inhibitory, as for $K_{2P}9.1$ (TASK-3) and $K_{2P}3.1$ (TASK-1), or activating, as for $K_{2P}10.1$ (TREK-2). Therefore, despite diverse $[H^+]_O$-sensor placement and the non-uniform direction of gating responses to $[H^+]_O$, our data indicate that this gating stimulus converges on a common site in M4 and strongly suggests that the gates of $K_{2P}2.1$ (TREK-1), $K_{2P}9.1$ (TASK-3), $K_{2P}3.1$ (TASK-1), and $K_{2P}10.1$ (TREK-2) work by a shared mechanism. As a further test, we examined substitution of a homologous position that is not natively an aromatic residue, Glu228, in the $[H^+]_O$-gated channel $K_{2P}5.1$ (*KCNK5*/TASK-2). E228S resulted in non-functional channels; however, introduction of an aromatic residue, E228W, profoundly inhibited $K_{2P}5.1$ (TASK-2) pH_O gating (Figure 6E). In accord with our $K_{2P}2.1$ results, M4 mutations that make the channel less responsive to pH_O gating ($K_{2P}10.1$ (TREK-2) W301S and $K_{2P}5.1$ (TASK-2) E228W) increase basal activity

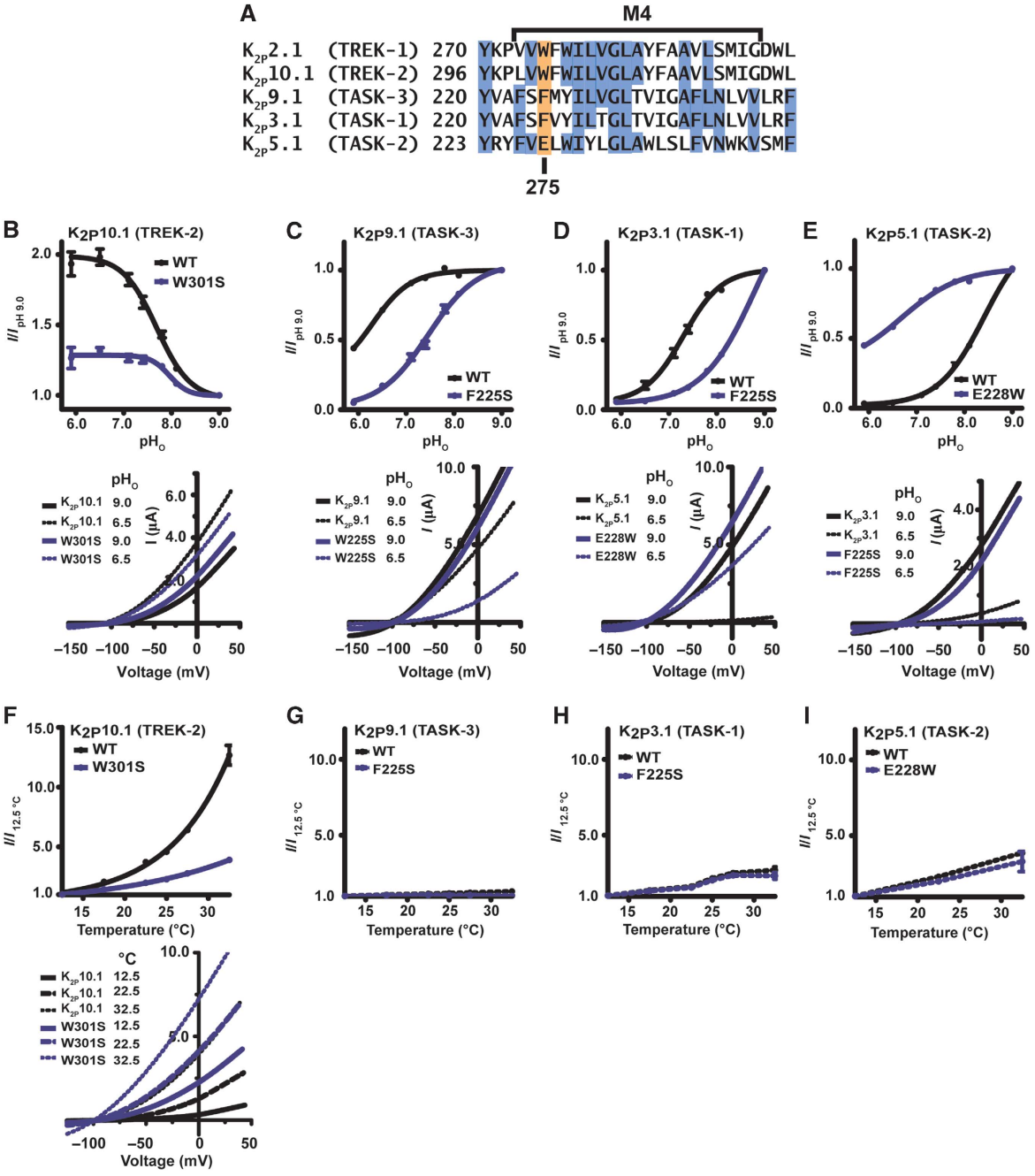

Figure 6 Importance of the Trp275 position is functionally conserved among K_{2P} channels for both pH_O and temperature induced gating. (**A**) Amino-acid alignment of the M4 region from the indicated K_{2P} channels. Residues conserved in three or more of the indicated sequences are highlighted in blue. The K_{2P}2.1 (TREK-1) Trp275 homologous position is indicated in orange. (**B**–**E**) Normalized pH_O responses and exemplar two-electrode voltage-clamp recordings of *Xenopus* oocytes for the indicated channels and mutants (2 mM $[K^+]_O$). Injected mRNA amounts for the exemplar traces in ng are as follows for wild type and mutant, respectively: K_{2P}10.1 (TREK-2) 5.0, 1.0; K_{2P}9.1 (TASK-3) 0.08, 0.20; K_{2P}3.1 (TASK-1) 1.5, 5.0; K_{2P}5.1 (TASK-2) 2.0, 1.0. (**F**–**I**) Normalized temperature responses for the indicated channels and mutants (2 mM $[K^+]_O$, pH 7.4). Exemplar two-electrode voltage-clamp records are shown only for K_{2P}10.1 (TREK-2). Data represent mean ± s.e. ($n = 6$–15) and is fitted to $I = I_{min} + (I_{max}–I_{min})/(1 + ([H^+]_O/K_{1/2})^H)$ or $I = I_{min} + (I_{max}–I_{min})/(1 + e^{(T_{1/2}–T)/S})$. Dashed lines connect data points. $N \geqslant 2$ for all experiments.

(Supplementary Figure S8). In contrast, mutations that facilitate pH_O responses (K_{2P}3.1 (TASK-1) F225S and K_{2P}3.1 (TASK-3) F225S) suppress macro-current amplitude at pH_O 7.4. Together, our studies of the effects of mutations at the K_{2P}2.1 (TREK-1) W275 equivalent M4 position provide further evidence for the general role of this element in K_{2P} channel gating and establish that various K_{2P} channels utilize a conserved gating apparatus to respond to extracellular acidosis regardless of the location of the sensor or direction of the response.

Our data show that in K_{2P}2.1 (TREK-1) different modalities act on the same gate even though the gating inputs produce opposite effects (i.e. $[H^+]_O$ inhibition versus temperature activation (Figure 2)). To test whether this property is a shared feature of the core gating apparatus within the K_{2P} family, we examined how the Trp275 equivalent changes

affected temperature responses of other K_{2P} channels. We found that in K_{2P}10.1 (TREK-2), in which both $[H^+]_O$ and increased temperature activate the channels (Maingret *et al*, 2000; Kang *et al*, 2005), the M4 mutant W301S profoundly diminished temperature-induced gating (Figure 6F) in addition to reducing the $[H^+]_O$ response (Figure 6B). In contrast, aromatic→Ser substitution at the Trp275 position in channels that are $[H^+]_O$ gated but that lack a strong temperature response, K_{2P}9.1 (TASK-3), K_{2P}3.1 (TASK-1), K_{2P}5.1 (TASK-2), had no effect on temperature sensitivity (Figure 6G–I). These results suggest that gating machinery alterations are not sufficient to affect the temperature response of a K_{2P} channel that lacks strong intrinsic temperature sensitivity. Importantly, the data demonstrate that $[H^+]_O$ and increased temperature both act on a common gate in channels that respond to both modalities regardless of the direction of the response.

The C-type gate controls diverse gating inputs in multiple K_{2P} family members

In K_{2P}s that respond to both pH_O and temperature, the above experiments support the idea that these diverse inputs act at a common gate that is affected by Trp275 or its equivalent. Because of these observations and the fact that the K_{2P}2.1 (TREK-1) W275S GOF mutant reduces the sensitivity of pH_O gating to extracellular potassium concentration (Figure 3) in a way that supports the presence of a C-type gate, we decided to test whether the K_{2P} temperature response is also sensitive to extracellular potassium. In line with the idea that the C-type-like gate responds to both pH_O and temperature, we found that high extracellular potassium significantly blunted the temperature response of both K_{2P}2.1 (TREK-1) (Figure 7A) and K_{2P}10.1 (TREK-2) (Figure 7B). The K_{2P}2.1 (TREK-1) temperature response in high external potassium was very similar as that caused by the W275S mutation. In contrast, the temperature response of the GOF mutant F276L, which has a pH_O and $[K^+]_O$ response like wild type (Figure 3C), followed the changes in external potassium in a way that resembled wild type. Together, these results strongly support the idea that the C-type gate controls both proton and temperature responses in the two channels and that the M4 Trp275 position has an important role in this process.

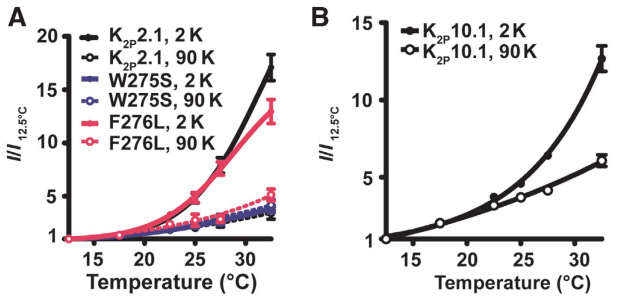

Figure 7 Temperature gating responds to changes in external potassium concentration. Normalized temperature responses of (**A**) K_{2P}2.1 (TREK-1), K_{2P}2.1 (TREK-1) W275S, and K_{2P}2.1 (TREK-1) F276L and (**B**) K_{2P}10.1 (TREK-2) from two-electrode voltage-clamp recordings in 2 and 90 mM $[K^+]_O$ (2 K and 90 K, respectively) elicited by a ramp from −150 to +50 mV from a holding potential of −80 mV (2 K) or 0 mV (90 K) at indicated temperatures at pH 7.4. The K_{2P}2.1 and K_{2P}10.1 responses to heat in 2 mM $[K^+]_O$ are the same as in Figures 2D and 6F, respectively. Data (mean ± s.e., $n = 8$–15) were taken at 0 mV (2 K) or +40 mV (90 K) and fitted with the equation $I = I_{min} + (I_{max} - I_{min})/(1 + e^{(T_{1/2}-T)/S})$. $N \geqslant 2$ for all experiments.

Discussion

K_{2P} channels are the most diverse potassium channel family in terms of both sequence (Goldstein *et al*, 2005; Yu *et al*, 2005) and gating inputs (Cohen *et al*, 2009; Mathie *et al*, 2010). This high sequence diversity together with the radically different physical natures of the gating commands, such as pH, temperature, and pressure, have posed a challenge to the identification of unifying models that could explain the mechanisms by which K_{2P}s act. To address this question in an unbiased manner, we sought to identify the key gating elements in the archetypal polymodal K_{2P} channel, K_{2P}2.1 (TREK-1), by exploiting a GOF selection in potassium-transport-deficient yeast. This initial effort was based on the premise that changes in components of the channel that contribute to gating should be particularly sensitive to mutations. The rationale builds on the ample precedents in which both functional screens and selection experiments have provided unexpected insights into the mechanisms of permeation and gating of a variety of other ion channel types such as inwardly rectifying potassium channels (Minor *et al*, 1999; Sadja *et al*, 2001; Yi *et al*, 2001; Bichet *et al*, 2004; Chatelain *et al*, 2005, 2009), voltage-gated potassium channels (Lai *et al*, 2005), and TRP channels (Su *et al*, 2007; Zhou *et al*, 2007; Grandl *et al*, 2008, 2010; Myers *et al*, 2008; Hu *et al*, 2009).

Prior studies have identified a variety of K_{2P}2.1 (TREK-1) elements that have principal roles in sensing different input types. His126 in the first extracellular loop acts as the external pH sensor (Cohen *et al*, 2008; Sandoz *et al*, 2009), whereas heat and mechanical force gating require elements from the cytosolic C-terminal domain (Maingret *et al*, 2000; Chemin *et al*, 2005; Kang *et al*, 2005). Although these different stimuli have different molecular sensors, it has not been obvious whether such components act via a common gate or by independent mechanisms. We expected that some of the GOF mutations from the selection experiment would target such sensor elements. Indeed, we found most of the known GOF mutations that hit key residues in the C-terminal tail (e.g. E306G, E309A, S333G, and S333R). These outcomes validated the concept behind the selection and focused our attention on a set of GOF mutations at K_{2P}2.1 (TREK-1) positions that had not previously been implicated in channel function. These sites included one in the external part of the selectivity filter (I148T), one in the distal part of the second P-loop (L267P), a double mutant combining both (I148T/L267P), and two sites on the N-terminal end of the M4 transmembrane segment (W275S and F276L). All of these mutations increase channel activity and affect channel open probability. The properties of the F276L mutant are important. This mutant affects open probability to a similar degree as the most effective GOF mutants, I148T/L267P and W275S, is expressed on the surface at similar levels as the other GOF mutants, but responds to the various gating inputs in a manner that is similar to wild type. Thus, simply increasing channel open probability is insufficient for causing the change in the responses to the various gating modalities. This result points to another explanation

for how I148T/L267P and W275S act and indicates that in addition to increasing the open probability, changes at these sites affect elements central to the integration of the gating commands.

Many K_{2P}s respond to extracellular pH (pH_O) changes (Enyedi and Czirjak, 2010; Mathie *et al*, 2010). Although, pH_O is a common gating modality, the locations of the proton sensors in the K_{2P} extracellular loops are not conserved (Kim *et al*, 2000; Rajan *et al*, 2000; Lopes *et al*, 2001; Morton *et al*, 2005; Sandoz *et al*, 2009). Interestingly, the direction of the response to pH_O changes is also not shared. This response diversity is best exemplified by two closely related K_{2P}s that use the equivalent extracellular histidine for proton sensing but that have opposite pH_O responses (Sandoz *et al*, 2009), K_{2P}2.1 (TREK-1), and K_{2P}10.1 (TREK-2). Extracellular protons inhibit K_{2P}2.1 (TREK-1) but activate K_{2P}10.1 (TREK-2). pH_O is thought to affect K_{2P}s by a C-type-like mechanism that involves the channel selectivity filter (Lopes *et al*, 2001; Cohen *et al*, 2008; Sandoz *et al*, 2009). Because the selectivity filter region is the most conserved K_{2P} feature, such a mechanism could provide a general means by which different pH_O sensing determinants could affect gating. We found that mutations in the K_{2P}2.1 (TREK-1) extracellular loops and at the Trp275 M4 position reduce the inhibitory effect of low pH_O on channel activity. In other types of potassium channels, C-type gating is tightly linked to changes in the ion occupancy of the selectivity filter (Cordero-Morales *et al*, 2006; Clarke *et al*, 2010; Cuello *et al*, 2010). Accordingly, we also found that the sensitivity of the K_{2P}2.1 (TREK-1) pH_O response was markedly reduced by increased extracellular potassium (Figure 3). This sensitivity was effectively lost in the I148T/L267P and W275S GOF mutants but not in the F276L GOF mutant. Further, closure of the C-type-like gate has been shown to lead to a loss in ion selectivity (Yuill *et al*, 2007; Cohen *et al*, 2008). In accord with the idea that the I148T/L267P and W275S GOF mutants act on a C-type-like gate, these mutants did not display the pH_O-induced changes in selectivity that are prominent in both wild-type K_{2P}2.1 (TREK-1) and the F2767L GOF mutant (Figure 4). Thus, we infer that the I148T/L267P and W275S blunt the channel response by favouring a conformation that is equivalent to that of an activated C-type gate.

Discovery of GOF mutants in the N-terminal end of the M4 region is striking. In other potassium channel classes, drastic alterations in the homologous region are generally not tolerated (Collins *et al*, 1997; Minor *et al*, 1999; Irizarry *et al*, 2002). Investigation of the mutational tolerance at the Trp275 position showed that aromatic substitutions produced channels that were very similar to wild type, whereas changes to small or hydrophobic residues increased whole cell currents and reduced responses to both pH_O and temperature (Figure 5). Deeper probing of the equivalent position among other K_{2P}s revealed the general importance of the Trp275 equivalent M4 position in both pH_O-mediated and temperature-evoked gating (Figure 6). Explicit tests of the effects of extracellular potassium show that, similar to pH_O responses, temperature activation of K_{2P}2.1 (TREK-1) and K_{2P}10.1 (TREK-2) are blunted to a level that is equivalent to that caused by the W275S change. Taken together, these observations provide overwhelming support for a model in which the selectivity filter acts as the common gate used in diverse K_{2P}s to respond to radically different input modalities and indicate that this gate involves components of the extracellular loops and the N-terminal portion of M4.

In many ways, a C-type gate is well suited to act as a common means to respond to different physical inputs detected by separate channel elements. Structural changes in channel components remote from the selectivity filter have recently been shown to be correlated to selectivity filter ion occupancy changes in inward rectifiers (Clarke *et al*, 2010). Importantly, such changes do not require large movements of the transmembrane helices. Thus, even though in some channels the action of the inner and outer gates may be coupled (Panyi and Deutsch, 2006, 2007; Ben-Abu *et al*, 2009), an 'inner gate' is not an essential element for controlling a C-type, selectivity filter-based gate (Clarke *et al*, 2010). It seems plausible that conformational changes in diverse channel elements, such as the extracellular loops or intracellular domains, could independently affect the conformation of the channel at the selectivity filter. In this regard, it is striking that a number of mutations have been identified in topologically similar regions that affect the temperature gating of TRPV1 and TRPV3 (Grandl *et al*, 2008, 2010; Myers *et al*, 2008). This coincidence is particularly remarkable because the two channel families share very little homology and raises the possibility that a selectivity filter-based gating mechanism is the principal mechanism for gating of other channels within the voltage-gated superfamily to which both K_{2P}s and TRPs are members (Yu *et al*, 2005).

It is possible that, in addition to the outer C-type-like gate, K_{2P}2.1 (TREK-1) possesses an intracellular gate formed by the M2 and M4 segments (Treptow and Klein, 2010). Mutations in the intracellular region of the putative pore-lining helices that could contribute to an inner gate, M2 and M4, have been shown to affect activity of K_{2P} channels from the TASK group (Barel *et al*, 2008; Ashmole *et al*, 2009), and the fly K_{2P} KCNK0 (Ben-Abu *et al*, 2009). Of the three modalities affected by our GOF mutants, the pressure response was the least blunted, a result that suggests that other parts of the channel may be important for this response. The general role of a K_{2P} inner gate and other questions, such as whether an inner gate involves the cytoplasmic C-terminus, and how such a gate might couple to the C-type gate, remain open for further study.

The diversity of amino-acid sequences and gating input modalities sets K_{2p} channels apart from other well-studied potassium channel classes such as inward rectifiers and voltage-gated channels (Enyedi and Czirjak, 2010). Our data strongly suggest that despite this exceptional diversity, the various sensing mechanisms act on a conserved core gating machinery that involves the extracellular elements of the pore-forming segments and the extracellular proximal part of the putative fourth transmembrane helix, M4. Although high-resolution structural data for K_{2P} channels is presently lacking, experimentally constrained K_{2P} channel models suggest that the Trp275 position should be intimately involved in supporting the structure of the selectivity filter (Kollewe *et al*, 2009). Such placement is consistent with the key role of the Trp275 in gating.

Because of their four transmembrane segment/two pore-forming region topology, K_{2P} channels have non-identical pore-lining segments, M2 and M4, that must interact with the selectivity filter elements in different ways. Further, this unusual topology dictates that each of our GOF channels will

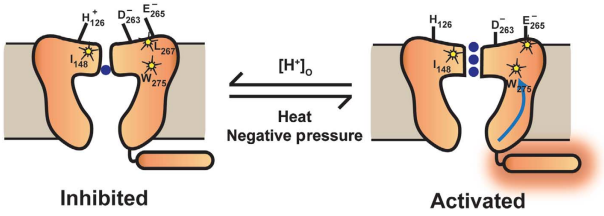

Figure 8 Cartoon model of K_{2P}2.1 (TREK-1) C-type-like gating by extracellular acidosis, heat, and pressure. Cartoon model of how extracellular protons ($[H^+]_O$), heat, and pressure affect the transition of K_{2P}2.1 (TREK-1) between a low-activity (inhibited) state and a high-activity (activated) state that involves a C-type-like gate. As suggested earlier (Sandoz *et al*, 2009), external acidification causes structural rearrangements in the pore triggered by electrostatic interactions between the protonated extracellular pH-sensing His126, and the negatively charged region in the P2-loop, which includes Asp263 and Glu265. We propose that Ile148, Leu267, and Trp275 (indicated with stars) are crucial elements of the C-type-like gate. As suggested elsewhere (Honore, 2007), temperature and mechanical stress have their sensing elements located in the intracellular C-terminal domain. We further propose that activated C-terminal domain (indicated with the orange halo) induces movements of the M4 transmembrane segment and affects channel activity through the C-type gate. The blue arrow indicates the putative pathway. For clarity, only one of the two cytoplasmic C-termini is depicted.

contain only two copies of the GOF mutations, not four as would be the case in a homomeric potassium channel from the other potassium channel classes. Hence, the unusual K_{2P} architecture may be the reason why, in contrast to homomeric potassium channels classes (Collins *et al*, 1997; Minor *et al*, 1999; Irizarry *et al*, 2002), we find that the upper portion of M4 tolerates changes in diverse K_{2P} family members (Figure 6). Thus, our data raise the interesting question of whether M2 and M4 have different functional roles and set a framework for future analysis of this issue.

Our results strongly suggest that the movement of the upper part of M4 is a central part of the core gating mechanism by which different sensor regions that respond to separate inputs couple to K_{2P} channel gating and that the changes in M4 affect the selectivity filter conformation (Figure 8). This key role for the upper part of M4 is in line with prior studies of other potassium channel classes that have identified gating-sensitive residues in the analogous region of the voltage-gated potassium channel *Shaker* (Ogielska and Aldrich, 1999; Yifrach and MacKinnon, 2002), demonstrated that changes in the conformation the KcsA pore-lining helix affect selectivity filter ion occupancy (Cuello *et al*, 2010), and that have shown gating induced changes in the inner part of the selectivity filter in KirBac3.1 (Gupta *et al*, 2010). Our data suggest that inputs such as pH_O, temperature, and pressure act by affecting structural rearrangements in the channel selectivity filter. These rearrangements are coupled to changes in the ion occupancy of the selectivity filter and the conformation of the outer pore and extracellular proximal portion of M4 that involve Ile148, Leu267, and Trp275 (Figure 8). Temperature and mechanical stress have sensing elements located in the intracellular C-terminal domain (Honore, 2007). The impact of the GOF mutations at Ile148, Leu267, and Trp275 on these modalities leads us to propose that changes in the C-terminal domain couple to the C-type gate through movements of the M4 transmembrane segment. These findings set the stage for future studies of the precise details by which the C-terminal tail couples to the C-type-like gate.

Because of the important role of the M4 region in K_{2P} gating, this channel element may prove to be a key site that can be targeted by both natural and synthetic compounds aimed at controlling K_{2P} function. In this regard, demonstration that K_{2P} channels can function in yeast should facilitate development of yeast-based assays for small molecules that overcome the intrinsic challenge posed by 'background' or 'leak' nature (Enyedi and Czirjak, 2010) of K_{2P} function.

Materials and methods

Molecular biology

Murine K_{2P} channels were cloned into pGEMHE/pMO, IRES-GFP (Invitrogen), or pYES2-MET25 (Minor *et al*, 1999) for expression in oocytes, COS7 cells, and yeast, respectively. For expression in COS7 cells, the haemagglutinin (HA) tag (YPYDVPDYA) was added to the N terminus of TREK-1. Cloning was performed using standard molecular biology procedures and verified by DNA sequencing.

Library construction and TREK-1 GOF selection in yeast

Standard techniques were used for yeast manipulation (Sherman, 2002). For full-length library construction, the K_{2P}2.1 (TREK-1) coding region along with 45 flanking nucleotides 5′ and 3′ to the gene was amplified from a pYES2-MET25-K_{2P}2.1 (*URA3*) plasmid using *Taq* DNA polymerase. Amplification used 29 cycles and conditions conducive to copying errors (100 mM Tris pH 8.3, 500 mM KCl, 70 mM $MgCl_2$, 0.1% gelatin). The PCR product was mixed with empty pYES-MET25 vector linearized at the points of K_{2P}2.1 (TREK-1) insertion (*Hin*dIII and *Xho*I), and transformed into the *ura*$^-$ SGY1528 yeast strain (Tang *et al*, 1995). Insertion of mutagenized K_{2P}2.1 (TREK-1) into the vector occurred via homologous recombination of the DNA regions flanking the K_{2P}2.1 (TREK-1) gene. Sequence analysis of 25 randomly picked clones from the library showed that 60% of plasmids carried one to three single nucleotide substitutions that were evenly distributed throughout the K_{2P}2.1 (TREK-1) gene. Yeast selection was performed as described previously (Minor *et al*, 1999). Briefly, yeast transformants (~50 000 total) were selected on synthetic medium without uracil and methionine (–Ura –Met), containing 100 mM KCl and replica plated onto –Ura –Met with 1.0 mM KCl. Following 3 days of growth at 30°C, colonies were replica plated onto –Ura –Met with 0.5 mM KCl. Plasmids were isolated, retested, and sequenced.

Electrophysiology

Unless indicated otherwise, all electrophysiological recordings were performed at room temperature. For two-electrode voltage-clamp measurements in *Xenopus laevis* oocytes, defolliculated stages V–VI oocytes were injected with 0.08–5.0 ng mRNA and assayed 24–72 h later. Each oocyte was impaled with two standard microelectrodes (0.3–3.0 MΩ) filled with 3 M KCl, and assayed using the GeneClamp 500B (MDS Analytical Technologies) amplifier controlled by the pClamp software (Molecular Devices). Data were digitized at 1 kHz using Digidata 1332A (MDS Analytical Technologies). For pH_O and temperature experiments, currents were evoked from a −80 mV holding potential with a 1-s long ramp from −150 to +50 mV in 2 K (standard ND96, containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2.0 mM $MgCl_2$) or 90 K (90 mM KCl, 8 mM NaCl, 1.8 mM $CaCl_2$, 2.0 mM $MgCl_2$) solutions buffered with 10 mM Tris (pH 9.0, 8.1), 5 mM HEPES (pH 7.8, 7.4, 7.1), or 5 mM MES (pH 6.5, 5.9). The solutions were exchanged consecutively from pH 9.0 to pH 5.9 (22.5°C), or from 12.5 to 32.5°C (pH 7.4). The effects of pH or temperature were reversible in all K_{2P}2.1 (TREK-1) mutants. Dose-response data were fitted with the equations: $I = I_{min} + (I_{max} - I_{min})/(1 + ([H^+]_O/K_{1/2})^H)$ where I_{max} and I_{min} are maximal and minimal current values, respectively, $K_{1/2}$ is a half-maximal effective concentration of extracellular protons ($[H^+]_O$), and H is the Hill coefficient or $I = I_{min} + (I_{max} - I_{min})/(1 + e^{(T_{1/2} - T)/S})$, where $T_{1/2}$ and S are the temperature of half-maximal activation and slope factor, respectively. For oocyte batches exhibiting high background chloride currents at pH ⩽ 6.5, NaCl and KCl were substituted with equimolar amounts of Na- or K-gluconate. Permeability ratios were determined under bionic conditions using the modified Goldman–Hodgkin–Katz equation, $pNa/pK = e^{F\Delta Erev/RT}$, where pNa and pK

are relative permeabilities for sodium and potassium, respectively, ΔE_{rev} is the difference between reversal potentials measured in 100 mM Na and 100 mM K solutions ($\Delta E_{rev} = E_{rev,Na} - E_{rev,K}$) and F, R, and T have their usual meanings (Hille, 2001). Currents were evoked by 60 ms step pulses from −150 to −50 mV in 10 mV increments from a holding potential of −80 mV in solutions containing 100 mM NaCl or KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, buffered with 10 mM Tris (pH 9.0), or 5 mM HEPES (pH 6.9–7.9). All reversal potential measurements were corrected for junction potential (2.5 mV). For most figures, 2–6 independent experiments were performed and 8–42 cells were analysed per construct.

Patch-clamp measurements were performed on African green monkey kidney COS7 cells. Cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL Life Technologies) supplemented with 10% fetal calf serum (Hyclone). Cells were transfected 1 day after plating, using a modified DEAE/Dextran protocol as previously described (Chemin *et al*, 2005). For single channel activity, 0.01 μg of plasmid DNA was transfected per 35 mm dish containing 20 000 cells per dish; for other experiments 0.1 μg was transfected. Experiments were performed between 2 and 4 days after transfection.

Stretch-response experiments were performed in cell-attached mode the pipette solution contained (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, and 10 HEPES at pH 7.4 with NaOH, and the bath solution contained (in mM): 155 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES at pH 7.2 with KOH. Patch pipettes of about 1.5 MΩ were used. Membrane patches were stimulated with negative pressure pulses, from 0 to −80 mm Hg in −10 mm Hg increments during 300 ms each 3 s, through the recording electrode using a pressure-clamp device (ALA High Speed Pressure-Clamp-1 system; ALA-Scientific). Single channel recordings were obtained in cell-attached mode using patch pipettes of about 4 MΩ and symmetrical solutions (in mM): 150 KCl, 5 EGTA-K, 1 EDTA-K, 10 HEPES, pH 7.2 with KOH. For all experiments, currents were filtered at 1 kHz, digitized at 20 kHz, and analysed with pCLAMP9.2 and ORIGIN6.0 software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: SNB and DLM conceived the study. SNB, RP, and KAC performed the experiments and analysed the data. EH and DLM analysed the data and provided guidance and support throughout. SNB, RP, EH, and DLM wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Alloui A, Zimmermann K, Mamet J, Duprat F, Noel J, Chemin J, Guy N, Blondeau N, Voilley N, Rubat-Coudert C, Borsotto M, Romey G, Heurteaux C, Reeh P, Eschalier A, Lazdunski M (2006) TREK-1, a K+ channel involved in polymodal pain perception. *EMBO J* **25:** 2368–2376

Ashmole I, Vavoulis DV, Stansfeld PJ, Mehta PR, Feng JF, Sutcliffe MJ, Stanfield PR (2009) The response of the tandem pore potassium channel TASK-3 (K(2P)9.1) to voltage: gating at the cytoplasmic mouth. *J Physiol* **587**(Part 20)**:** 4769–4783

Barel O, Shalev SA, Ofir R, Cohen A, Zlotogora J, Shorer Z, Mazor G, Finer G, Khateeb S, Zilberberg N, Birk OS (2008) Maternally inherited Birk Barel mental retardation dysmorphism syndrome caused by a mutation in the genomically imprinted potassium channel KCNK9. *Am J Hum Genet* **83:** 193–199

Bayliss DA, Barrett PQ (2008) Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. *Trends Pharmacol Sci* **29:** 566–575

Ben-Abu Y, Zhou Y, Zilberberg N, Yifrach O (2009) Inverse coupling in leak and voltage-activated K+ channel gates underlies distinct roles in electrical signaling. *Nat Struct Mol Biol* **16:** 71–79

Bichet D, Lin YF, Ibarra CA, Huang CS, Yi BA, Jan YN, Jan LY (2004) Evolving potassium channels by means of yeast selection reveals structural elements important for selectivity. *Proc Natl Acad Sci USA* **101:** 4441–4446

Blondeau N, Petrault O, Manta S, Giordanengo V, Gounon P, Bordet R, Lazdunski M, Heurteaux C (2007) Polyunsaturated fatty acids are cerebral vasodilators via the TREK-1 potassium channel. *Circ Res* **101:** 176–184

Bryan Jr RM, Joseph BK, Lloyd E, Rusch NJ (2007) Starring TREK-1: the next generation of vascular K+ channels. *Circ Res* **101:** 119–121

Bryan Jr RM, You J, Phillips SC, Andresen JJ, Lloyd EE, Rogers PA, Dryer SE, Marrelli SP (2006) Evidence for two-pore domain potassium channels in rat cerebral arteries. *Am J Physiol Heart Circ Physiol* **291:** H770–H780

Chatelain FC, Alagem N, Xu Q, Pancaroglu R, Reuveny E, Minor Jr DL (2005) The pore helix dipole has a minor role in inward rectifier channel function. *Neuron* **47:** 833–843

Chatelain FC, Gazzarrini S, Fujiwara Y, Arrigoni C, Domigan C, Ferrara G, Pantoja C, Thiel G, Moroni A, Minor Jr DL (2009) Selection of inhibitor-resistant viral potassium channels identifies a selectivity filter site that affects barium and amantadine block. *PloS ONE* **4:** e7496

Chemin J, Patel AJ, Duprat F, Lauritzen I, Lazdunski M, Honore E (2005) A phospholipid sensor controls mechanogating of the K+ channel TREK-1. *EMBO J* **24:** 44–53

Clarke OB, Caputo AT, Hill AP, Vandenberg JI, Smith BJ, Gulbis JM (2010) Domain reorientation and rotation of an intracellular assembly regulate conduction in Kir potassium channels. *Cell* **141:** 1018–1029

Cohen A, Ben-Abu Y, Hen S, Zilberberg N (2008) A novel mechanism for human K2P2.1 channel gating. Facilitation of C-type gating by protonation of extracellular histidine residues. *J Biol Chem* **283:** 19448–19455

Cohen A, Ben-Abu Y, Zilberberg N (2009) Gating the pore of potassium leak channels. *Eur Biophys J* **39:** 61–73

Collins A, Chuang H, Jan YN, Jan LY (1997) Scanning mutagenesis of the putative transmembrane segments of Kir2.1, an inward rectifier potassium channel. *Proc Natl Acad Sci USA* **94:** 5456–5460

Cordero-Morales JF, Cuello LG, Zhao Y, Jogini V, Cortes DM, Roux B, Perozo E (2006) Molecular determinants of gating at the potassium-channel selectivity filter. *Nat Struct Mol Biol* **13:** 311–318

Cuello LG, Jogini V, Cortes DM, Perozo E (2010) Structural mechanism of C-type inactivation in K(+) channels. *Nature* **466:** 203–208

Dillon DG, Bogdan R, Fagerness J, Holmes AJ, Perlis RH, Pizzagalli DA (2010) Variation in TREK1 gene linked to depression-resistant phenotype is associated with potentiated neural responses to rewards in humans. *Hum Brain Mapp* **31:** 210–221

Duprat F, Lauritzen I, Patel A, Honore E (2007) The TASK background K2P channels: chemo- and nutrient sensors. *Trends Neurosci* **30:** 573–580

Enyedi P, Czirjak G (2010) Molecular background of leak K+ currents: two-pore domain potassium channels. *Physiol Rev* **90:** 559–605

Fink M, Duprat F, Lesage F, Reyes R, Romey G, Heurteaux C, Lazdunski M (1996) Cloning, functional expression and brain localization of a novel unconventional outward rectifier K+ channel. *EMBO J* **15:** 6854–6862

Folgering JH, Sharif-Naeini R, Dedman A, Patel A, Delmas P, Honore E (2008) Molecular basis of the mammalian pressure-sensitive ion channels: focus on vascular mechanotransduction. *Prog Biophys Mol Biol* **97:** 180–195

Garry A, Fromy B, Blondeau N, Henrion D, Brau F, Gounon P, Guy N, Heurteaux C, Lazdunski M, Saumet JL (2007) Altered acetylcholine, bradykinin and cutaneous pressure-induced vasodilation in mice lacking the TREK1 potassium channel: the endothelial link. *EMBO Rep* **8:** 354–359

Goldstein SA, Bayliss DA, Kim D, Lesage F, Plant LD, Rajan S (2005) International Union of Pharmacology. LV. Nomenclature and molecular relationships of two-P potassium channels. *Pharmacol Rev* **57:** 527–540

Gordon JA, Hen R (2006) TREKing toward new antidepressants. *Nat Neurosci* **9:** 1081–1083

Grandl J, Hu H, Bandell M, Bursulaya B, Schmidt M, Petrus M, Patapoutian A (2008) Pore region of TRPV3 ion channel is specifically required for heat activation. *Nat Neurosci* **11:** 1007–1013

Grandl J, Kim SE, Uzzell V, Bursulaya B, Petrus M, Bandell M, Patapoutian A (2010) Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. *Nat Neurosci* **13:** 708–714

Gupta S, Bavro VN, D'Mello R, Tucker SJ, Venien-Bryan C, Chance MR (2010) Conformational changes during the gating of a potassium channel revealed by structural mass spectrometry. *Structure* **18:** 839–846

Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, Lang-Lazdunski L, Widmann C, Zanzouri M, Romey G, Lazdunski M (2004) TREK-1, a K+ channel involved in neuroprotection and general anesthesia. *EMBO J* **23:** 2684–2695

Heurteaux C, Lucas G, Guy N, El Yacoubi M, Thummler S, Peng XD, Noble F, Blondeau N, Widmann C, Borsotto M, Gobbi G, Vaugeois JM, Debonnel G, Lazdunski M (2006) Deletion of the background potassium channel TREK-1 results in a depression-resistant phenotype. *Nat Neurosci* **9:** 1134–1141

Hille B (2001) *Ion Channels of Excitable Membranes*, 3rd edn Sunderland, MA: Sinauer Associates, Inc

Honore E (2007) The neuronal background K2P channels: focus on TREK1. *Nat Rev Neurosci* **8:** 251–261

Honore E, Maingret F, Lazdunski M, Patel AJ (2002) An intracellular proton sensor commands lipid- and mechano-gating of the K(+) channel TREK-1. *EMBO J* **21:** 2968–2976

Hu H, Grandl J, Bandell M, Petrus M, Patapoutian A (2009) Two amino acid residues determine 2-APB sensitivity of the ion channels TRPV3 and TRPV4. *Proc Natl Acad Sci USA* **106:** 1626–1631

Irizarry SN, Kutluay E, Drews G, Hart SJ, Heginbotham L (2002) Opening the KcsA K+ channel: tryptophan scanning and complementation analysis lead to mutants with altered gating. *Biochemistry* **41:** 13653–13662

Kang D, Choe C, Kim D (2005) Thermosensitivity of the two-pore domain K+ channels TREK-2 and TRAAK. *J Physiol* **564** (Part 1)**:** 103–116

Kim Y, Bang H, Kim D (2000) TASK-3, a new member of the tandem pore K(+) channel family. *J Biol Chem* **275:** 9340–9347

Kollewe A, Lau AY, Sullivan A, Benoit R, Goldstein SA (2009) A structural model for K2P potassium channels based on 23 pairs of interacting sites and continuum electrostatics. *J Gen Physiol* **134:** 53–68

Lafreniere RG, Cader MZ, Poulin JF, Andres-Enguix I, Simoneau M, Gupta N, Boisvert K, Lafreniere F, McLaughlan S, Dube MP, Marcinkiewicz MM, Ramagopalan S, Ansorge O, Brais B, Sequeiros J, Pereira-Monteiro JM, Griffiths LR, Tucker SJ, Ebers G, Rouleau GA (2010) A dominant-negative mutation in the TRESK potassium channel is linked to familial migraine with aura. *Nat Med* **16:** 1157–1160

Lai HC, Grabe M, Jan YN, Jan LY (2005) The S4 voltage sensor packs against the pore domain in the KAT1 voltage-gated potassium channel. *Neuron* **47:** 395–406

Lopes CM, Zilberberg N, Goldstein SA (2001) Block of Kcnk3 by protons. Evidence that 2-P-domain potassium channel subunits function as homodimers. *J Biol Chem* **276:** 24449–24452

Lopez-Barneo J, Hoshi T, Heinemann SH, Aldrich RW (1993) Effects of external cations and mutations in the pore region on C-type inactivation of Shaker potassium channels. *Receptors Channels* **1:** 61–71

Maingret F, Fosset M, Lesage F, Lazdunski M, Honoré E (1999a) TRAAK is a mammalian neuronal mechano-gated K^+ channel. *J Biol Chem* **274:** 1381–1387

Maingret F, Honore E, Lazdunski M, Patel AJ (2002) Molecular basis of the voltage-dependent gating of TREK-1, a mechano-sensitive K(+) channel. *Biochem Biophys Res Commun* **292:** 339–346

Maingret F, Lauritzen I, Patel AJ, Heurteaux C, Reyes R, Lesage F, Lazdunski M, Honore E (2000) TREK-1 is a heat-activated background K(+) channel. *EMBO J* **19:** 2483–2491

Maingret F, Patel AJ, Lesage F, Lazdunski M, Honore E (1999b) Mechano- or acid stimulation, two interactive modes of activation of the TREK-1 potassium channel. *J Biol Chem* **274:** 26691–26696

Mathie A, Al-Moubarak E, Veale EL (2010) Gating of two pore domain potassium channels. *J Physiol* **588**(Part 17)**:** 3149–3156

Minor Jr DL (2009) Searching for interesting channels: pairing selection and molecular evolution methods to study ion channel structure and function. *Mol Biosyst* **5:** 802–810

Minor Jr DL, Masseling SJ, Jan YN, Jan LY (1999) Transmembrane structure of an inwardly rectifying potassium channel. *Cell* **96:** 879–891

Morton MJ, Abohamed A, Sivaprasadarao A, Hunter M (2005) pH sensing in the two-pore domain K+ channel, TASK2. *Proc Natl Acad Sci USA* **102:** 16102–16106

Murbartian J, Lei Q, Sando JJ, Bayliss DA (2005) Sequential phosphorylation mediates receptor- and kinase-induced inhibition of TREK-1 background potassium channels. *J Biol Chem* **280:** 30175–30184

Myers BR, Bohlen CJ, Julius D (2008) A yeast genetic screen reveals a critical role for the pore helix domain in TRP channel gating. *Neuron* **58:** 362–373

Noel J, Zimmermann K, Busserolles J, Deval E, Alloui A, Diochot S, Guy N, Borsotto M, Reeh P, Eschalier A, Lazdunski M (2009) The mechano-activated K+ channels TRAAK and TREK-1 control both warm and cold perception. *EMBO J* **28:** 1308–1318

Ogielska EM, Aldrich RW (1999) Functional consequences of a decreased potassium affinity in a potassium channel pore. Ion interactions and C-type inactivation. *J Gen Physiol* **113:** 347–358

Panyi G, Deutsch C (2006) Cross talk between activation and slow inactivation gates of Shaker potassium channels. *J Gen Physiol* **128:** 547–559

Panyi G, Deutsch C (2007) Probing the cavity of the slow inactivated conformation of shaker potassium channels. *J Gen Physiol* **129:** 403–418

Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M (1999) Inhalational anesthetics activate two-pore-domain background K^+ channels. *Nature Neurosci* **2:** 422–426

Patel AJ, Honore E, Maingret F, Lesage F, Fink M, Duprat F, Lazdunski M (1998) A mammalian two pore domain mechano-gated S-like K+ channel. *EMBO J* **17:** 4283–4290

Perlis RH, Moorjani P, Fagerness J, Purcell S, Trivedi MH, Fava M, Rush AJ, Smoller JW (2008) Pharmacogenetic analysis of genes implicated in rodent models of antidepressant response: association of TREK1 and treatment resistance in the STAR(*)D study. *Neuropsychopharmacology* **33:** 2810–2819

Rajan S, Wischmeyer E, Xin Liu G, Preisig-Muller R, Daut J, Karschin A, Derst C (2000) TASK-3, a novel tandem pore domain acid-sensitive K+ channel. An extracellular histiding as pH sensor. *J Biol Chem* **275:** 16650–16657

Sabbadini M, Yost CS (2009) Molecular biology of background K channels: insights from K(2P) knockout mice. *J Mol Biol* **385:** 1331–1344

Sadja R, Smadja K, Alagem N, Reuveny E (2001) Coupling Gbetagamma-dependent activation to channel opening via pore elements in inwardly rectifying potassium channels. *Neuron* **29:** 669–680

Sandoz G, Douguet D, Chatelain F, Lazdunski M, Lesage F (2009) Extracellular acidification exerts opposite actions on TREK1 and TREK2 potassium channels via a single conserved histidine residue. *Proc Natl Acad Sci USA* **106:** 14628–14633

Sherman F (2002) Getting started with yeast. *Methods Enzymol* **350:** 3–41

Su Z, Zhou X, Haynes WJ, Loukin SH, Anishkin A, Saimi Y, Kung C (2007) Yeast gain-of-function mutations reveal structure-function relationships conserved among different subfamilies of transient receptor potential channels. *Proc Natl Acad Sci USA* **104:** 19607–19612

Tang W, Ruknudin A, Yang W, Shaw S, Knickerbocker A, Kurtz S (1995) Functional expression of a vertebrate inwardly rectifying K^+ channel in yeast. *Mol Biol Cell* **6:** 1231–1240

Treptow W, Klein ML (2010) The membrane-bound state of K2P potassium channels. *J Am Chem Soc* **132:** 8145–8151

Yellen G (2002) The voltage-gated potassium channels and their relatives. *Nature* **419:** 35–42

Yi BA, Lin YF, Jan YN, Jan LY (2001) Yeast screen for constitutively active mutant G protein-activated potassium channels. *Neuron* **29:** 657–667

Yifrach O, MacKinnon R (2002) Energetics of pore opening in a voltage-gated K(+) channel. *Cell* **111:** 231–239

Yu FH, Yarov-Yarovoy V, Gutman GA, Catterall WA (2005) Overview of molecular relationships in the voltage-gated ion channel superfamily. *Pharmacol Rev* **57:** 387–395

Yuill KH, Stansfeld PJ, Ashmole I, Sutcliffe MJ, Stanfield PR (2007) The selectivity, voltage-dependence and acid sensitivity of the tandem pore potassium channel TASK-1: contributions of the pore domains. *Pflugers Arch* **455:** 333–348

Zhou X, Su Z, Anishkin A, Haynes WJ, Friske EM, Loukin SH, Kung C, Saimi Y (2007) Yeast screens show aromatic residues at the end of the sixth helix anchor transient receptor potential channel gate. *Proc Natl Acad Sci USA* **104:** 15555–15559

Zilberberg N, Ilan N, Goldstein SA (2001) KCNKO: opening and closing the 2-P-domain potassium leak channel entails 'C-type' gating of the outer pore. *Neuron* **32:** 635–648

