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REVIEW

Gating the pore of potassium leak channels

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Abstract A key feature of potassium channel function is the ability to switch between conducting and non-conducting states by undergoing conformational changes in response to cellular or extracellular signals. Such switching is facilitated by the mechanical coupling of gating domain movements to pore opening and closing. Two-pore domain potassium channels (K_{2P}) conduct leak or background potassium-selective currents that are mostly time- and voltage-independent. These channels play a significant role in setting the cell resting membrane potential and, therefore modulate cell responsiveness and excitability. Thus, K_{2P} channels are key players in numerous physiological processes and were recently shown to also be involved in human pathologies. It is well established that K_{2P} channel conductance, open probability and cell surface expression are significantly modulated by various physical and chemical stimuli. However, in understanding how such signals are translated into conformational changes that open or close the channels gate, there remain more open questions than answers. A growing line of evidence suggests that the outer pore area assumes a critical role in gating K_{2P} channels, in a manner reminiscent of C-type inactivation of voltage-gated potassium channels. In some K_{2P} channels, this gating mechanism is facilitated in response to external pH levels. Recently, it was suggested that K_{2P} channels also possess a lower activation gate that is positively coupled to the outer pore gate. The purpose of this review is to present an up-to-date summary of research describing the conformational changes and gating events that take place at the K_{2P} channel ion-conducting pathway during the channel regulation.

Keywords Gating · Two-pore domain potassium channels · C-type inactivation · Activation gate · TREK-1 · KCNK0

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Introduction

Potassium channels are the membrane proteins that enable the rapid (10^6–10^8 ions per second) and highly selective flux of K^+ ions across biological membranes, down the electrochemical K^+ gradient. By influencing and modulating the membrane potential, K^+ channels play a major role in various cellular processes, such as muscular excitability, neuronal integration, volume regulation, hormone secretion and many more (Hille 2001). Two structural elements endow potassium channels with the means to effectively control K^+ flux. A selectivity filter, located in the extracellular third of the ion pathway, enables rapid and selective conductance of potassium ions at a rate approaching the diffusion limit (Bezanilla and Armstrong 1972; Doyle et al. 1998). Secondly, there exist mechanisms to manipulate permeation pathway of K^+ channels, acting by moving designated protein segments in a process referred to as gating. The nature of the structural rearrangements that facilitate gating was deciphered with the determination of the

three-dimensional structure of two bacterial potassium channels, i.e., KcsA (Doyle et al. 1998) and MthK (Jiang et al. 2002), crystallized under conditions in which each is predicted to assume either the closed or open conformation, respectively (Fig. 1). In the closed conformation, the pore inner helices are straight and form a helix bundle near the intracellular side of the pore, to restrict the movement of K^+ ions (Fig. 1, left). In the KcsA structure, the bundle crossing narrows to about 3.5 Å in diameter, and is lined with hydrophobic amino acids to form the so-called 'hydrophobic seal' (Armstrong 2003). In MthK, the inner helices are bent at a hinge point (Jiang et al. 2002; Magidovich and Yifrach 2004), so that the cytoplasmic entrance to the ion conduction pathway is wide open (~12 Å; Fig. 1, right). A second gate, referred to as the slow or C-type inactivation gate (Hoshi et al. 1990), is located at the extracellular entrance to the ion conduction pathway. During the C-type inactivation, structural rearrangements at the selectivity filter promotes the collapse of this channel region (Cordero-Morales et al. 2006, 2007).

In the voltage-gated (K_v) channel family, the activation and slow inactivation pore gates were shown to be coupled (Baukrowitz and Yellen 1995, 1996; Panyi and Deutsch 2006, 2007). In *Shaker* channels, coupling was demonstrated in both directions. Whereas previous work had shown that opening of the activation gate induced closure of the slow inactivation gate (Baukrowitz and Yellen 1995, 1996), more recent work has shown that upper gate closure promotes lower gate opening (Panyi and Deutsch 2006, 2007).

Members of the potassium leak channels family, discovered over a decade ago, are of particular interest in terms of both structure and function. They are structurally unique in that each subunit possesses four transmembrane segments and two-pore-forming domains (2P/4TM), and hence, are often referred to as two-pore domain K^+ channels or K_{2P} channels (Choe 2002; Goldstein et al. 2001). The existence of two different pore-forming sequences (P1 and P2) on one subunit enforces the formation of an asymmetrical pore region in the dimeric functional channel. Such twofold symmetry might explain the relative resistance of K_{2P} channels to the classical K^+ channels blockers, quaternary ammonium ions (Fink et al. 1996; Lesage et al. 2000; Reyes et al. 1998), known to affect many fourfold symmetry-K^+ channels. K_{2P} channels remain active across the entire physiological voltage range and, thus, carry leak or background potassium currents that set the membrane resting potential near the potassium reversal potential. As such, altering potassium background currents can modulate cellular membrane potential and resistance and thus, cellular responsiveness and excitability. K_{2P} channels are molecular entities that provide a dedicated pathway for the establishment of potassium background currents, and serve various physiological roles, as discussed below. The mammalian $K_{2P}2.1$ channel was shown to be involved in depression (Heurteaux et al. 2006), pain perception (Alloui et al. 2006), general anesthesia, neuroprotection (Franks and Honore 2004) and more (Honore 2007). Several K_{2P} channels such as $K_{2P}3.1$ (KCNK3,

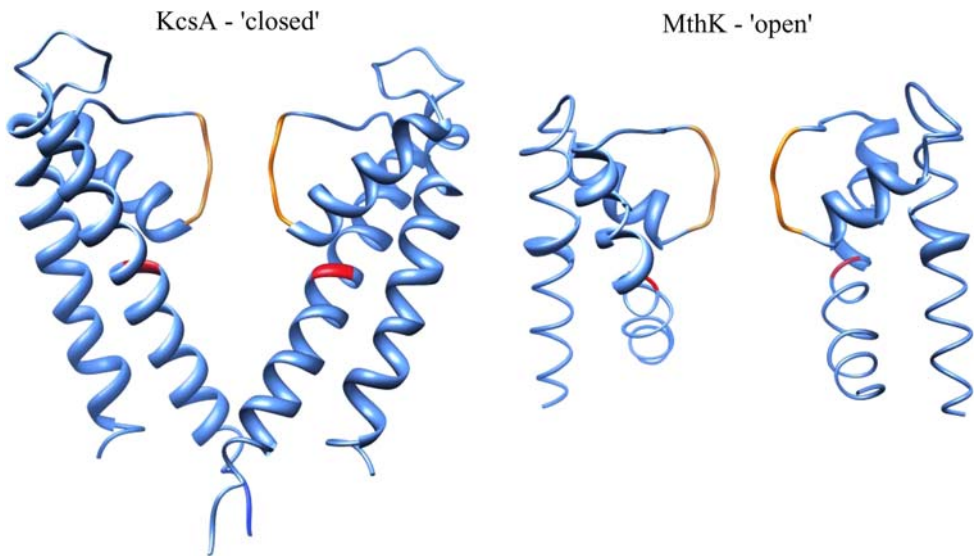

Fig. 1 Closed and opened conformations of the K^+ channel pore, as represented by the KcsA and MthK structures (Doyle et al. 1998; Jiang et al. 2002). Ribbon representations of two subunits of the closed pore conformation of KcsA (PDB accession number 1BL8; *left*) and the opened pore conformation of MthK (PDB accession number 1LNQ; *right*) are shown. In the closed conformation, the inner helices form a 'bundle crossing' which serves as a constriction at the cytoplasmatic entrance to the channel. In the open conformation, the inner helices are splayed open around a conserved glycine residue, the 'gating hinge' (*colored red*) (Jiang et al. 2002). The selectivity filter is *orange*

TASK-1) and KCNK0 (d-ORK1) were shown to modulate cardiac action potential and rhythms (Backx and Marban 1993; Gurney and Manoury 2009; Lalevee et al. 2006). K_{2P}3.1 and K_{2P}9.1 (TASK-3, KCNK9) channels contribute to changes in the activity mode of thalamocortical networks observed during the sleep–wake cycle (Meuth et al. 2003). K_{2P}2.1 and K_{2P}9.1 were found to be over-expressed in different types of human carcinomas where they may lead to a significant increase in cell proliferation (Mu et al. 2003; Pei et al. 2003; Voloshyna et al. 2008). It was also shown that K_{2P}9.1 channels can also increase the excitability by supporting high-frequency firing once an action potential threshold is reached (Brickley et al. 2007). A different mode by which K_{2P} channels can increase excitability was recently demonstrated by a new isoform of rat K_{2P}2.1, namely a protein lacking the first 56 residues, formed by alternative translation initiation, that allow the passage of sodium ions under physiological conditions (Thomas et al. 2008). This was proposed as a natural mechanism by which to alter excitability in the central nervous system, as the two K_{2P}2.1 isoforms are differentially expressed in a regional and developmental manner (Thomas et al. 2008). In a closely related channel, K_{2P}10.1 (TREK-2), a similar alternative translation initiation mechanism controls channel unitary conductance without altering selectivity (Simkin et al. 2008). The crucial physiological significance of K_{2P} channels was recently recognized when it was shown that a mutation in the maternal copy of *KCNK9,* encoding K_{2P}9.1, causes a novel syndrome of mental retardation, hypotonia and unique dysmorphism with elongated face [i.e., Birk-Barel syndrome (Barel et al. 2008)].

A key feature of K_{2P} channels is their ability to serve as a hub for a wide variety of chemical and physical stimuli that set the membrane resting potential and enable plasticity of excitable tissues. Among the signals known to affect K_{2P} channels are temperature (Maingret et al. 2000a), mechanical stretch (Maingret et al. 1999), external and internal pH (Cohen et al. 2008, 2009; Kim et al. 2000; Lopes et al. 2001; Maingret et al. 1999), phospholipids (Chemin et al. 2007; Lopes et al. 2005; Maingret et al. 2000b), cyclic nucleotides (Wagner and Dekin 1997), inhalational anesthetics (Patel et al. 1999), protein kinases and phosphatases (Cohen and Zilberberg 2006; Lopes et al. 2007; Murbartian et al. 2005; Veale et al. 2007; Zilberberg et al. 2000), molecular oxygen (Buckler et al. 2000), neuroprotective agents (Duprat et al. 2000), G-proteins (Chen et al. 2006; Lesage et al. 2000; Mathie 2007) and neurotransmitters (Talley et al. 2000) [this subject was thoroughly reviewed by (Dedman et al. 2009; Honore 2007)]. Although much is known about the regulation of K_{2P} channels, only little is known about the actual conformational changes that occur in response to cellular cues.

Here, we review recent findings on the different mechanisms gating K_{2P} channels. We discuss the role of the selectivity filter area as the primary gate, its function in pH sensing, the significance of conserved pore-adjacent residues and the carboxyl-terminal role in mediating regulatory stimuli. Finally, we consider the presence of a second, lower gate and its possible physiological implications.

Regulation affects K_{2P} channels gating

Several recent studies support the concept that both intra- and extracellular signals modify K_{2P} channels gating rather than conductance or surface expression. Phosphorylation of carboxyl-terminal residues is a common regulation mechanism of K_{2P} channels. Single KCNK0 channels move in a regulated fashion between a long-lived open-burst configuration (containing one open and three closed states) and a long-lasting closed state, C_{long}. Application of the protein kinase C activator, phorbol-12-myristate-13-acetate, leads wild-type KCNK0 channels to spend more time in the open-burst state due to a decrease in the frequency and duration of visits to C_{long} (Fig. 2a) (Zilberberg et al. 2000). Bockenhauer et al. (2001) found that phosphorylation of the K_{2P}2.1 channel carboxyl-terminal residue, Ser-348, by the catalytic subunit of protein kinase A (PKA) not only decreased the open probability (P_o) of the channel, but also imposed a voltage-dependent phenotype (Bockenhauer et al. 2001). K_{2P}10.1 (TREK-2) channels respond to carboxyl-terminal residue phosphorylation in an even more intricate manner, with changes in both P_o and the distribution of the channel conduction levels (Kang et al. 2007). Both phenotypes were strongly activated by arachidonic acid, membrane stretch and intracellular acidification. In addition, external signals such as acidic pH dramatically reduced the open probability of K_{2P}16.1 (TALK-1), K_{2P}17.1 (TALK-2), K_{2P}5.1 (TASK-2), K_{2P}9.1 and human K_{2P}2.1 channels without significantly altering their single-channel conductance (Cohen et al. 2008; Kang and Kim 2004; Lotshaw 2006; Reyes et al. 1998). Moreover, inhalational anesthetics were shown to significantly increase the open probability of both K_{2P}2.1 and K_{2P}3.1 channels (Patel et al. 1999).

What would be the mechanism by which K_{2P} channels gate their ion-conducting pathway? Evidently, the cytoplasmic carboxyl-terminal of K_{2P} channels is crucial for regulation by many environmental cues. The amino terminal end of the carboxyl-terminal was argued to interact with membrane phospholipids and to control the lipid- and mechano-sensitivity of members of this family (Chemin et al. 2005; Honore et al. 2002; Kim et al. 2001; Lopes et al. 2005). However, it was shown that the carboxyl-terminal region is not a channel gate by itself (Zilberberg et al. 2000). Deleting this region of the KCNK0 channel (KCNKΔ299-1001) did

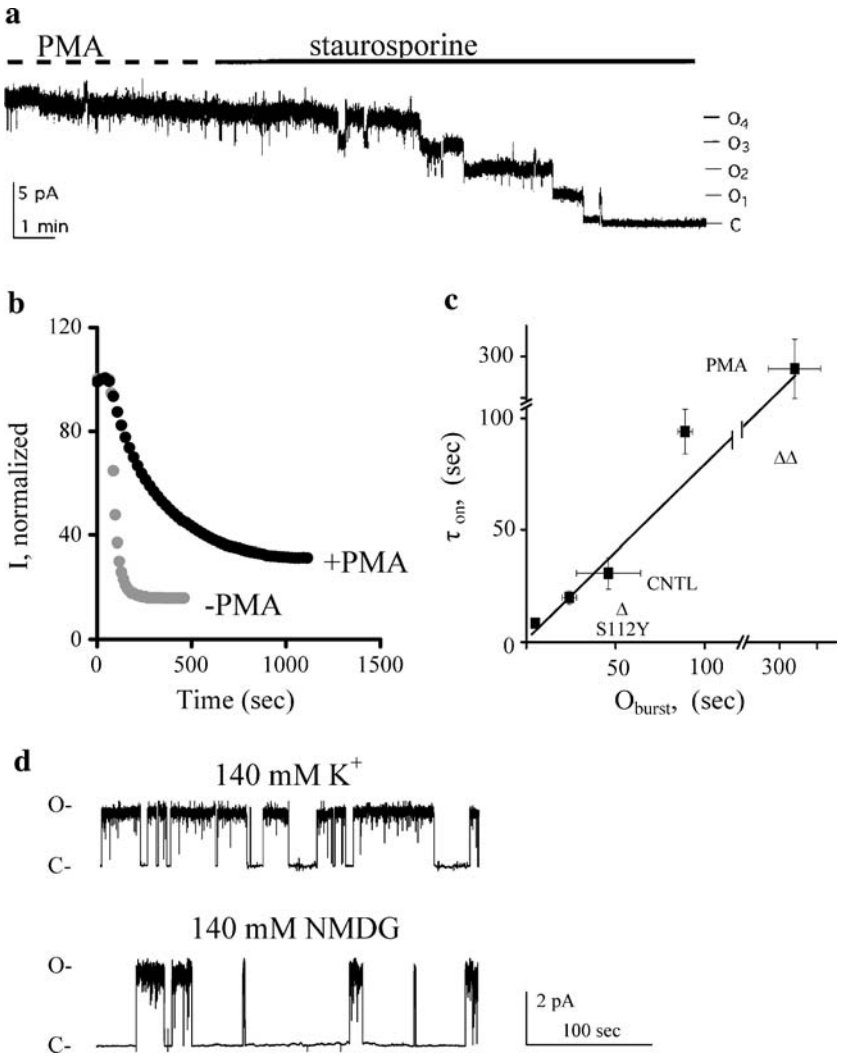

Fig. 2 Regulatory signals affect the K_{2P} channels upper gate. **a** Regulation alters occupancy of the long-lived closed state of the *Drosophila melanogaster* KCNK0 channel. Four KCNK0 channels were recorded in an on-cell patch at 60 mV at symmetrical (140 mM) potassium concentrations with the protein kinase C activator, phorbol-12-myristate-13-acetate (PMA), in the bath and then in the protein kinase inhibitor, staurosporine, as indicated. Data were sampled at 940 Hz and filtered at 20 Hz. Open (O) and closed (C) states levels are indicated. Adapted from Zilberberg et al. (2000). **b** Phosphorylation affects external the zinc blockade rate. Macroscopic currents were measured by two-electrode voltage clamp. Representative KCNK0 current magnitude during application of 100 μM zinc under control conditions and after a 15 min treatment with PMA (50 nM), as indicated. An order of magnitude increase in the apparent time constant (τ_{on}) for inhibition was observed after incubation with PMA, based on Zilberberg et al. (2001). **c** Zinc does not block during open bursts (O_{burst}). Macroscopic time constant for zinc inhibition (τ_{on}) versus single channel O_{burst} duration is shown. Whole-cell measurements were collected in 20 mM potassium solution with 100 μM zinc using a holding voltage of −40 mV and 200 ms pulses to 25 mV (mean ± SEM, n = 6–8 cells). Single channels were studied on-cell at 60 mV with 140 mM potassium solution in the bath and pipette, sampled at 940 Hz. Channels and conditions: KCNK0 under control conditions (CNTL); KCNK0 treated with 50 nM PMA (PMA); KCNK0Δ299-1001 channels (Δ); KCNK0-S112Y (S112Y); a deletion mutant lacking residues 619–1001 (ΔΔ), based on Zilberberg et al. (2001). **d** External potassium inhibits long closures. Single KCNK0Δ299-1001 channels were measured in on-cell patches at 60 mV with 140 mM potassium in the bath and 140 mM potassium or *N*-methyl-D-glucosamine (NMDG) solution in the pipette. Representative single channel traces were sampled at 500 Hz and filtered at 20 Hz. Open (O) and closed (C) states levels are indicated, based on Zilberberg et al. (2001)

not affect channel selectivity, unitary current amplitude or the number of closed states, but rather abolishes channel sensitivity to kinase modulators. Single channel recordings demonstrated that the truncated channel visited one open and three brief closed states within open bursts and one long-lived closed state, just like the wild-type channel. The main change induced by carboxyl-terminal deletion is the stabilization of movement between the open-burst and C_{long} phases. These findings support the idea that the carboxyl-terminal does not form a channel gate or act as a blocking particle, but rather operates to modify the stability of states achievable by the pore-forming segment alone. Therefore, a

different protein segment within the ion conduction pathway must serve as the channel gate that translates modulation of the carboxyl-terminal into ion pathway restriction.

Evidences for outer pore gating in K_{2P} channels

Direct evidence for the existence of a functional gate, located at the external side of the pore, came from the study of KCNK0 channels. As mentioned previously, KCNK0 channels gate by moving between two long-lived states, one open burst and one closed, in a non-voltage-dependent fashion. Occupancy of C_{long} is tightly regulated by kinase activity (P_o 0.99 to under 0.05) (Zilberberg et al. 2000). To explore the mechanism of regulated gating, single KCNK0 channels were studied following expression in *Xenopus* oocytes. A first clue was afforded by blocking studies. External zinc was found to inhibit KCNK0 current by selectively increasing the duration of C_{long} in a manner dependant on the presence of a histidine in the first pore loop (His-29). Indeed, zinc did not alter channel behavior during the open bursts thus, the apparent macroscopic forward rate constants for zinc blockade (τ_{on}) correlated with time spent in the open-burst state (Fig. 2b, c). Two important conclusions were drawn from this correlation. First, gating events are manifested as conformational changes at the outer side of the pore. Second, manipulation of the carboxyl-terminal via phosphorylation concurrently affects both the gating properties (P_o) and the outer pore conformation (Zilberberg et al. 2001).

K_{2P} channel outer pore gating resembles C-type inactivation of K_v channels

The state-dependent blockade of KCNK0 by zinc ions is reminiscent of the action of cadmium in stabilizing the C-type inactivated state of a cysteine-substituted *Shaker* channel (Yellen et al. 1994). Further experiments demonstrated that the transition between KCNK0 open and closed states is similar to C-type inactivation of voltage-gated channels (Zilberberg et al. 2001). Several lines of evidences support this assumption. External permeating ions or tetraethylammonium (TEA) ions are known to slow K_v channel C-type inactivation by preventing the collapse of the selectivity filter according to the 'foot-in-the-door' model (Baukrowitz and Yellen 1995; Choi et al. 1991; Grissmer and Cahalan 1989; Kurata and Fedida 2006; Lopez-Barneo et al. 1993; Ogielska and Aldrich 1999). Similarly, replacing pipette potassium in on-cell patches with sodium or NMDG reduced KCNK0 open-burst probability from 0.73 to 0.32 and 0.24, respectively, due to a decreased open-burst time and increased C_{long} duration (Fig. 2d). Furthermore, while TEA reduced apparent KCNK0 single channel conductance, it also produced an increase in open-burst probability and suppressed visits to C_{long}.

Atypical of K_{2P} channels, K_{2P}6.1 (TWIK-2) channels inactivate (Patel et al. 2000). Although inactivation was accelerated by increased temperatures, the same process was inhibited by external K^+ ions. Although the exact molecular mechanism of K_{2P}6.1 inactivation was not determined, its potassium sensitivity is, again, reminiscent of the C-type inactivation observed with *Shaker* channels (Patel et al. 2000).

Next, the role of specific residues, known to influence K_v C-type inactivation, in KCNK0 gating was evaluated. An external threonine (Thr-449), located three residues away from the selectivity filter signature sequence, was shown to play an important role in the regulation of the inactivation rate of the *Shaker* channel. Several substitutions at this site dramatically altered the C-type gating kinetics (Lopez-Barneo et al. 1993). It should be noted that in some mammalian K_v channels, mutation of *Shaker* Thr-449 homologous residues displayed weaker effects on the time course of C-type inactivation (Kurata and Fedida 2006). Nonetheless, mutation of the homologues site in KCNK0 (S112Y) increased C_{long} 4.5-fold, while intraburst open and closed state dwell times were unaffected. As expected from a mutant with altered C-type gating, the rate of zinc inhibition was faster in the S112Y mutant, as compared to the wild-type channel (~threefold decrease in τ_{on}). The second residue studied was an extracellular conserved glutamate located at the extracellular extreme of the outer helix of the pore in both K_v and K_{2P} channels. The significance of this conserved residue was revealed by two groups who reported an acceleration of C-type inactivation following the substitution of *Shaker* Glu-418 with either glutamine or cysteine (Larsson and Elinder 2000; Ortega-Saenz et al. 2000). The negative charge of Glu-418 was suggested to stabilize the open conformation of the selectivity filter and to reduce entry into the C-type state. Similarly, a homologues substitution in KCNK0 (E28C) resulted in a sevenfold decrease in unitary P_o, an effect that was reversed upon modification by the negatively charged thiol-specific reagent, methanethiosulfonate ethylsulfonate (MTSES). MTSES application also restored wild-type zinc blockage kinetics. Larsson and Elinder further demonstrated that disulfide linkage between cysteine residues substituting Glu-418 and Val-451 stabilized C-type inactivation. In contrast, disulfide linkage of E418C to G452C appeared to slow inactivation and favored the open state of the channel (Larsson and Elinder 2000). These results are in accordance with the crystal structure of the bacterial KcsA channel in which Glu-51 is predicted to form hydrogen bonds with the backbone amides of Thr-85 and Val-84 and the side chain hydroxyl of Thr-85 (positions analogous to *Shaker* Glu-418, Gly-452 and Val-451,

respectively; Doyle et al. 1998). Functional studies revealed that gating of KCNK0 can be manipulated in the same manner (Zilberberg et al. 2001). Like *Shaker* residues Glu-418 and Gly-452, KCNK0 residues Glu-28 and Thr-115 appear to be in close proximity, based on their ability to crosslink when altered to cysteines (Zilberberg et al. 2001).

Taken together, these findings confirm the existence of a functional upper gate in a member of the K_{2P} family where, the transition between open and closed states is correlated with conformational changes in the external side of the pore.

External pH facilitates C-type gating

The activity of several K_{2P} channels is sensitive to changes in extracellular pH. Among these are $K_{2P}3.1$, $K_{2P}9.1$ and the human variant of $K_{2P}2.1$ (Cohen et al. 2008; Duprat et al. 1997; Kim et al. 2000; Lopes et al. 2001; Rajan et al. 2000). On the other hand, the TALK subfamily of channels [$K_{2P}5.1$, $K_{2P}15.1$ (TALK-1) and $K_{2P}16.1$ (TALK-2)] are activated by extracellular alkalinization (Girard et al. 2001; Han et al. 2003; Kang and Kim 2004; Reyes et al. 1998). Transient pH variations occur in all three compartments of nervous tissue, i.e., neurons, glial cells and extracellular spaces, in response to neuronal stimulation, to neurotransmitters and hormones, as well as in a secondary manner to metabolic activity and ion transport (Deitmer and Rose 1996). Shifts in blood flow, synchronous activation of nerve cells, seizure or spreading depression have been associated with interstitial alkaline shift or acidosis that could persist for minutes in different segments of the central nervous system [reviewed by Chesler (2003)]. Such changes are known to modulate neuronal tissue excitability, partly by affecting different K_{2P} targets. Extracellular protons were suggested to promote C/P-type inactivation of the *Shaker* channel, presumably by means of interaction with negative charges in the extracellular entrance of the channel (Starkus et al. 2003). External pH was suggested to regulate K_{2P} channel activity by facilitation of C-type inactivation through several mechanisms. We will now discuss the three main proposed mechanisms.

The TALK subfamily

Modulation of the $K_{2P}5.1$ (TASK-2) channel by extracellular pH involves changes in P_o without affecting single channel conductance (Kang and Kim 2004; Reyes et al. 1998). Several extracellular charged residues had been implicated as mediating channel response to alkalization (Morton et al. 2005). This concept was, however, later disputed and an alternative mechanism, involving a single arginine residue, located near the second pore domain (Arg-224), was proposed as being responsible for the pH-dependent activity of $K_{2P}5.1$ as well as of the TALK channels (Niemeyer et al. 2007). Based on molecular simulations, this study revealed a hydrophobic environment around Arg-224, located at the outermost portion of the fourth transmembrane helix and close to the pore region (Fig. 3a, b). Neutralization of this arginine residue was shown to abolish the pH dependence of $K_{2P}5.1$, whereas its replacement by other basic amino acids shifted its pH sensitivity in correlation with the relative pK_a values of their side chains. It was suggested, therefore, that, in the protonated form, Arg-224 prevents occupancy of the selectivity filter by K^+, thereby promoting a blocked state, similar to C-type inactivation (Niemeyer et al. 2007).

The TASK subfamily

The $K_{2P}3.1$ and $K_{2P}9.1$ channels share a histidine residue at position 98, just following the GYG motif, shown to be fundamental to the sensitivity of these channels to extracellular acidification (Clarke et al. 2008; Kim et al. 2000; Lopes et al. 2001; Morton et al. 2003; Rajan et al. 2000). Models of the $K_{2P}3.1$ structure indicate that the His-98 side chain lies behind the selectivity filter of the channel, rather than directly in the ion conduction pathway (Yuill et al. 2007). It was, therefore, suggested that protonation of His-98 induces conformational changes at the selectivity filter, rather than simply causing channel blockage. In accordance with this hypothesis, mutations at the selectivity filter of $K_{2P}3.1$ altered not only channel selectivity, but also pH sensitivity as well (O'Connell et al. 2005; Yuill et al. 2004, 2007). In addition, inhibition of $K_{2P}3.1$ current by protons was shown to be potassium-dependent (Lopes et al. 2000), implying the involvement of an upper gate in pH sensing. Structural models and molecular dynamic simulations have highlighted the importance of a water molecule behind the selectivity filter for pH-induced gating of $K_{2P}3.1$ (Stansfeld et al. 2008; Yuill et al. 2007). This water molecule was suggested to interact with the backbone amides of Tyr-96 and Gly-97 and with the side chains of Thr-89 and the suggested proton sensor, His-98 (Fig. 3c). According to the suggested mechanism, protonation of His-98 prevents this residue from forming the hydrogen bond with the water molecule. Destabilization of the water molecule weakens its other interactions, leading to rotation of the Tyr-96-Gly-97 peptide bond that removes the carbonyl oxygens from the conduction axis (Stansfeld et al. 2008; Yuill et al. 2007). These movements destabilize the K^+ ion at position S_0. In addition, the protonated His-98 residue rotates upwards to create an electropositive barrier for K^+ ions traversing the outer mouth of the channel. This proposal is supported by the existence of a water molecule stabilizing

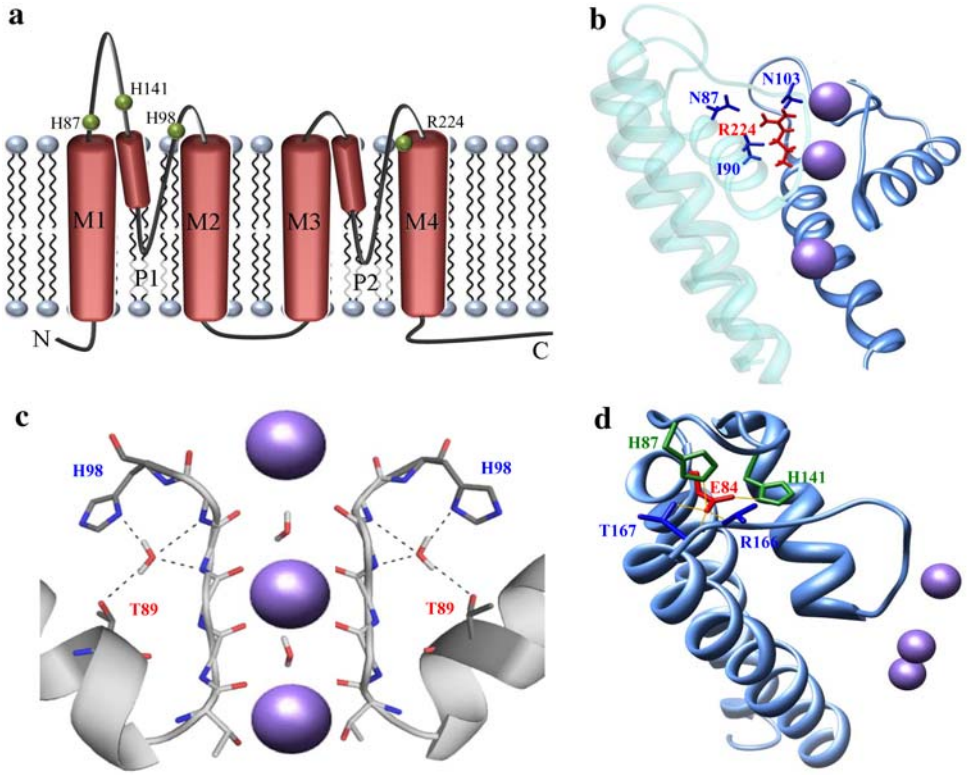

Fig. 3 External pH facilitates C-type gating of various K_{2P} channels. **a** Predicted membrane topology of a K_{2P} subunit. *Circles* indicate pH-sensing residues and their predicted location in the first and second turret loops. **b** A $K_{2P}5.1$ model, based on the structure of the $K_v1.2$ channel (Long et al. 2005). The model was generated as a homodimer with a single monomer being shown for clarity. The Arg-224 sensor (*red*) is predicted to project toward the selectivity filter region. Asn-87, Asn-103 and Ile-90 are shown in *blue*. Helices are identified and the permeation pathway is shown by K^+ ions (*violet*) at cation binding sites S1, S2 and the internal cavity, based on the model presented in Niemeyer et al. (2007). **c**. A $K_{2P}3.1$ model, illustrating the interactions of a water molecule with the backbone of Tyr-96 and Gly-97 and the side chains of Thr-89 and His-98 in the unprotonated form of His-98, according to molecular dynamic simulations, based on Yuill et al. (2007). **d** pH sensing mechanism of human $K_{2P}2.1$. Ribbon representation of one subunit of the bacterial KcsA potassium channel, based on the published structure (Doyle et al. 1998). Predicted hydrogen bonds between KcsA residues are presented as *orange lines*. The side chain of Glu-51 is predicted to form hydrogen bonds with the backbone amide groups of Val-84 and Thr-85 and the side chain hydroxyl group of Thr-85. The homologous $K_{2P}2.1$ residues are Glu-84 (*red*), Arg-166 and Thr-167 (*blue*), respectively. KcsA Ala-54 and Leu-59 were replaced in this presentation by histidines, as present at the homologous positions in $K_{2P}2.1$ [i.e., His-87 and His-141 (*green*), respectively] based on Cohen et al. (2008)

the selectivity filter of KcsA (Cordero-Morales et al. 2006) and the discovery of a similar mechanism in human ether-a-go-go (HERG) potassium channels (Stansfeld et al. 2008).

Interestingly, although they both possess a histidine residue at position 98, $K_{2P}3.1$ channels are more sensitive to extracellular pH than $K_{2P}9.1$ channels. A possible explanation for this difference was recently provided by the elegant work by Clarke and colleagues who generated chimeric channels in which the M1P1 loop (the loop between the first transmembrane domain and the pore region) was swapped between $K_{2P}3.1$ and $K_{2P}9.1$ channels and visa versa (Clarke et al. 2008). The $K_{2P}3.1$ M1P1 loop increased the pH sensitivity of $K_{2P}9.1$ channels ($K_{2P}9.1_{K2P3.1M1P1}$, pKa = 6.7 compared with $K_{2P}9.1$, pKa = 6.4), whereas the $K_{2P}9.1$ M1P1 loop decreased the pH sensitivity of $K_{2P}3.1$ channels ($K_{2P}3.1_{\ K2P9.1M1P1}$, pKa = 7.0, as compared with $K_{2P}3.1$, pKa = 7.5). These findings suggest that residues in the first turret loop might influence the pH sensor at the selectivity filter. Indeed, $K_{2P}9.1$ Glu-70 from the extracellular loop was postulated to appose His-98, as the two residues were suggested to form a binding site for zinc (Clarke et al. 2004). In addition, paired cysteine mutation experiments have provided strong evidence that Glu-70 and His-98 are found in close apposition (Clarke et al. 2008).

Human TREK-1

It was recently reported that external acidification, within the physiological range, strongly inhibits the human $K_{2P}2.1$ channel on two distinct time scales (Cohen et al. 2008, 2009). Single channel analysis showed that at low external pH values, channel open probability but not single channel conductance was decreased, suggesting an effect

on channel gating. None of the previously mentioned pH-sensing mechanisms applies to $K_{2P}2.1$. $K_{2P}2.1$ channels neither possess a histidine residue at the selectivity filter, like $K_{2P}3.1$/$K_{2P}9.1$, nor do they sense external pH by Lys-286 (homologous to Arg-224 in $K_{2P}5.1$), as mutating Lys-286 to alanine failed to abolish $K_{2P}2.1$ inhibition by protons (Cohen et al. 2008). Alternatively, two histidine residues (i.e., His-87 and His-141), located at the long pre-pore extracellular linker (the M1P1 loop), were found to govern the fast response of the channel to changes in external pH (Cohen et al. 2008). Mutating His-87 to glutamine, as found at position 87 of the murine $K_{2P}2.1$ channel, resulted in a reduced pH sensitivity profile, similar to that of the murine variant. Furthermore, mutating His-141 to an alanine dramatically decreased channel responsiveness to external acidification, shifting the sensitivity curve away from the physiological range. Neither His-87 nor His-141 are predicted, according to the known K^+ channel structures (Doyle et al. 1998), to lie along the ion conduction pathway (Fig. 3a). This, therefore, rules out the possibility that protons act through pore blockade. It was next tested whether His-87 and His-141 mediate pH-induced closure of the external gate by a mechanism similar to C-type inactivation. Previous findings support the notion that $K_{2P}2.1$ utilizes the C-type gating mechanism as it shows an increase in single channel P_o (due to suppression of a long-closed state) when potassium is exchanged for rubidium (Bockenhauer et al. 2001) and macroscopic $K_{2P}2.1$ currents increase when external NMDG is replaced by potassium (Fink et al. 1996). Indeed, elevating external potassium concentrations dramatically interfered with pH inhibition of channel activity. As conformational modification of the pore area alters the selectivity filter arrangement, thereby increasing sodium permeability (Starkus et al. 1997; Yuill et al. 2007), the ionic selectivity of $K_{2P}2.1$ at different external pH values was measured. Lowering the pH of a 100 mM sodium solution from 9.0 to 7.0 caused a significant right-shift of 20 mV in the membrane resting potential of oocytes expressing wild-type $K_{2P}2.1$ channels, but not the pH-insensitive H141A mutant. Accordingly, the K^+/Na^+ permeability ratio was considerably decreased (close to a threefold change) in wild-type, but not in the mutated channels. These results implied that $K_{2P}2.1$ channel utilizes an upper gate, similar to KCNK0, which could be closed by acidic pH. Next, the effects of two mutations, homologous to those previously shown to accelerate KCNK0 C-type gating (Zilberberg et al. 2001), were evaluated. Ser-164, the $K_{2P}2.1$ homolog of KCNK0 Ser-112, was mutated to tyrosine (S164Y) and Glu-84, homologous to KCNK0 Glu-28, was mutated to alanine (E84A). The $K_{2P}2.1$-S164Y mutant demonstrated increased pH sensitivity, with a pK of 8.1, while the E84A mutation, expected to similarly affect C-type gating, presented currents that were significantly less sensitive to external pH. The decreased pH sensitivity of the E84A mutant suggested that it may be an integral part of the pH sensing mechanism. Therefore, a mechanism was suggested for proton-induced gating of $K_{2P}2.1$ in which positively charged histidine residues (His-87 and His-141) form salt bridges with Glu-84 and draw it away from its natural interactions with Arg-166 and Thr-167, thus causing a collapse of the selectivity filter region (Fig. 3d). Indeed, the physical proximity of Glu-84 and the two histidine residues have been demonstrated. Replacing Glu-84 with a cysteine residue (E84C) created a novel Cd^{2+}-binding site in which both histidine residues participated (Cohen et al. 2008).

Interactions of turret residues with the pore

Interactions between M1P1 loop residues and pore-adjacent residues that affected pH sensitivity or C-type inactivation were demonstrated in other potassium channels as well. For example, the first 20 of the 48 amino acids that form the M1P1 loop of the recently discovered *Drosophila* TASK channel, dTASK6, was shown to participate in the proton sensitivity of the channel (Doring et al. 2006). Recent work suggested that the M1P1 loop of $K_{2P}9.1$ lies in close apposition to the pore and can regulate channel function by influencing sensitivity to both zinc and pH (Clarke et al. 2008). The 42-amino acid-long pre-pore extracellular loop of the HERG potassium channel, was suggested to interact with the mouth of the pore to affect HERG rapid C-type-like inactivation (Clarke et al. 2006; Liu et al. 2002; Torres et al. 2003). It was previously suggested that in $K_v1.4$ channels, protonation of an extracellular histidine residue (His-508) induces C-type inactivation by decreasing K^+ occupancy of the coordination site at the selectivity filter. Destabilization is achieved by either long-range electrostatic interactions or in an indirect manner via the positively charged residue, Lys-532, found adjacent to the GYG motif (+3 position) (Claydon et al. 2004). Finally, several residues, located at the distal region of the turret (Thr-462-Pro-468), were shown to dominate pH sensitivity and P/C-type inactivation regulation of $K_v1.5$ channels (Eduljee et al. 2007; Kehl et al. 2002).

It is well established that extracellular pH modulates gating of members of the K_{2P} family. Although various sensing mechanisms were recently proposed for different channels, they all employ conformational changes at the selectivity filter area. Thus, the upper gate is seemingly an important component in the proper physiological function of K_{2P} channels. Furthermore, the exceptionally long M1P1 loop of K_{2P} channels might play a pivotal role in transferring extracellular signals to gating changes by interacting with the outer pore area.

The discovery of a lower gate in K_{2P} channels

In K_v channels, two functional restrictions are formed along the ion conduction pathway, namely the activation and the slow inactivation (C-type) gates. Moreover, these two-pore gates were shown to be functionally negatively coupled, such that the opening of one causes the other to close (Baukrowitz and Yellen 1995, 1996; Panyi and Deutsch 2006, 2007).

The distinct physiological roles of K_v and K_{2P} channels in electrical signaling must reflect fundamental differences in the intrinsic thermodynamic properties of their respective pore domains, in particular in the mode of operation of the pore gates that control the flow of K^+ ions across the membrane. Until recently, no evidence has been found for the existence of a lower activation gate in K_{2P} channels. We will now discuss recent findings that imply the presence of a lower activation gate in K_{2P} channels and consider its interaction with the upper C-type gate.

Ben-Abu et al. (2009) employed several complementary approaches to test the existence of a lower activation gate in KCNK0. First, a chimeric protein containing the first pore domain of KCNK0 and the voltage sensor domain of the *Shaker* voltage-activated channel was constructed. The chimera showed voltage-dependent potassium currents in a pattern typical of a voltage-activated potassium channel. This chimera showed pore openings and closures on a time scale of milliseconds, which is typical for lower activation gate movement rather than the upper one. In addition, a slowing down of the closure kinetics was observed, with the activation curve showing a 23 mV shift toward a more negative activation midpoint value, compared with the wild-type *Shaker* channel (Fig. 4a, b). These two observations not only suggested the existence of a lower activation gate in the KCNK0 channel, but also reflected the tendency of this gate toward the open state. Sequence differences in the activation gate region of the K_v and K_{2P} channel families might offer an explanation for this assertion. Although the activation gate region of K_v channel family members is mostly spanned by hydrophobic residues, the corresponding region of K_{2P} channel family members is largely lined by glycine residues (Fig. 4c). It was previously shown that, whereas in *Shaker* channels the closed state of the activation gate is stable (Yifrach and MacKinnon 2002), in KCNK0, the open pore conformation is favored (Zilberberg et al. 2001). Sequence differences at the activation gate may be responsible for the inverted conformational stability of the pore domain in the K_v (closed) and K_{2P} (open) channels. Replacement of each hydrophobic residue in the *Shaker* channel activation gate pore domain with glycine stabilized the activation gate in the open state (Ben-Abu et al. 2009). Similarly, replacing glycine residues with hydrophobic residues in the activation gate pore domain of KCNK0 lowered the probability for channel opening. Finally, the direct proof of the existence of a lower activation gate in the K_{2P} channel was obtained by blocking experiments using intracellularly applied Ba^{2+} ions. Because these ions gain access to their binding sites via the lower activation gate, mutated K_{2P} channels whose lower activation gate is always open will be blocked faster by intracellular injection of Ba^{2+} ions, while K_{2P} channels carrying a different mutation (where the activation gate is mostly closed) will be slower to block. It was indeed shown that a permanently open KCNK0 channel mutant (G134D) was blocked faster than mostly closed, double-mutated channels (G134D-G139 V and G134D-G141 V), thus providing clear, direct evidence for the existence of an activation gate in the KCNK0 channel.

Next, it was tested whether the function of the two (upper and lower) gates is coupled, in a manner similar to what occurs in K_v channels. To evaluate gating events at the outer pore, zinc blockage rate was examined. It was found that replacement of glycine residues with hydrophobic amino acids in the activation gate region of the K_{2P} channel (G134A, G139 V, G141 V) affected not only closure of the lower gate but also that of the upper gate. For all mutants, the observed apparent time constant for zinc blockage decreased several fold. Therefore, not only did the mutated channels show a stabilized closed state of the activation gate, they also caused the upper inactivation gate to close. This qualitative finding received quantitative reinforcement from analysis of transition amplitude in a thermodynamic gating cycle, which describes the four possible states of a double-gated channel in a simplified, reductionist manner. This double-mutation cycle enabled the calculation of the character (positive or negative) and magnitude of the two gates' coupling values. This calculation showed that mutations in the K_{2P} channel activation gate gave rise to coupling values which were much higher than 1, suggesting positive coupling between the gates. Taken together, these findings suggest that K_{2P} channels can form a lower activation gate which is stabilized in the open conformation due to glycine residues found at the inner helix bundle crossing. Furthermore, unlike K_v channels (Panyi and Deutsch 2006, 2007), the lower activation and upper inactivation gates of K_{2P} channels are positively coupled, i.e., the opening of the lower activation gate stimulates the opening of the upper slow inactivation gate. This mechanism ensures a constant, time-independent, flow of potassium ions, allowing K_{2P} channels to fulfill their role in regulating the membrane resting potential.

Conclusion

To maintain the membrane resting potential close to the potassium Nernst potential, K_{2P} channels respond to

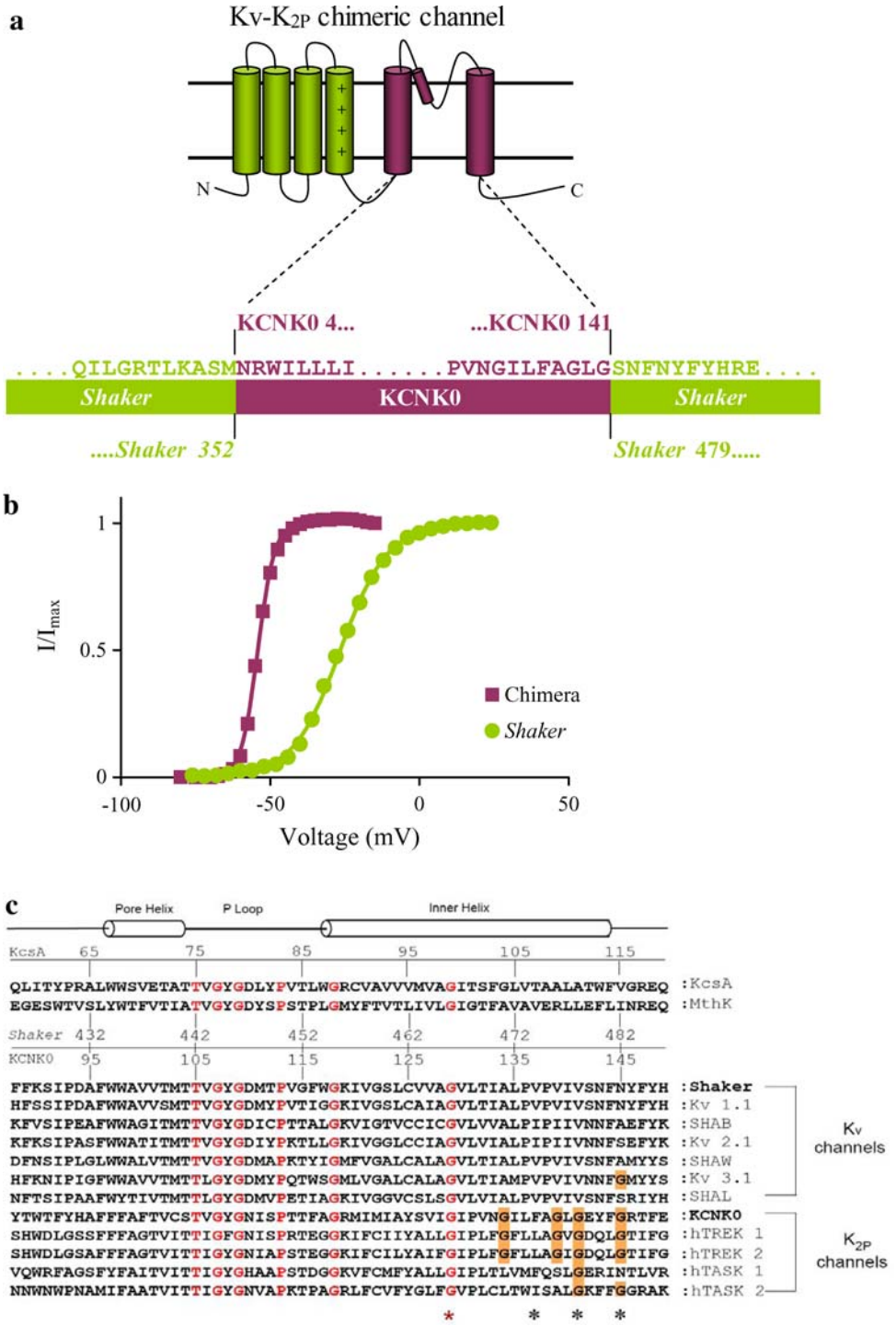

Fig. 4 K_{2P} channels contain a lower activation gate. **a** Sequence boundaries of a K_v–K_{2P} chimeric channel protein. **b** Voltage-activation curves for currents recorded from *Xenopus* oocytes expressing wild-type or chimeric K_v–K_{2P} channel proteins, under two-electrode voltage clamp. Smooth curves correspond to a two-state Boltzmann function, based on Ben-Abu et al. (2009). **c** Multiple sequence alignment of the inner helix region of several voltage-gated and K_{2P} (first pore domain) K^+ channels. *Black asterisks* indicate 'hydrophobic seal' positions, as previously defined by Armstrong (2003). The *red asterisk* indicates the gating hinge position. The four glycine residue positions discussed in the text are highlighted in *yellow*

various intra- and extracellular signals by undergoing several conformational changes. The selectivity filter area of K_{2P} channels can clearly function as an outer pore gate, presenting behavior similar to the slow inactivation mechanisms of other potassium channels. This gate can be manipulated by both extracellular signals, such as external pH, and by cytoplasmic signals, such as phosphorylation of the carboxyl-terminal of the channel. In some K_{2P} channels, it was shown that the closing of the upper gate in response to changes in external pH requires interactions with specific residues in the M1P1 loop. The mechanism by which the cytoplasmic carboxyl-terminal modulates the upper gate remains to be determined. Recent work suggests that K_{2P} channels possess a lower activation gate, that is, positively coupled to the upper, slow inactivation gate, as opposed to the negative coupling present in K_v channels gates.

Differences in the operation of the activation and slow inactivation pore gates of K_v and K_{2P} channels may underlie their distinct roles in electrical signaling. Positive coupling of the lower and upper gates ensures that at all membrane voltages, the two-pore gates are open at the same time, ensuring the maintained leak of K^+ current across the membrane, thereby enabling K_{2P} channels to fulfill their role in setting the resting membrane potential close to the *Nernst* potential for K^+ ions. It has yet to be determined to what extent the gating of the upper inactivation gate modulates the thermodynamic stability of the inner activation gate. There is no doubt that deciphering the crystal structure of a K_{2P} channel in various environmental conditions will be a major leap forward in this direction.

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