Voltage-dependent activation in EAG channels follows a ligand-receptor rather than a mechanical-lever mechanism

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Ether-a-go-go family (EAG) channels play a major role in many physiological processes in humans, including cardiac repolarization and cell proliferation. Cryo-EM structures of two of them, Kv10.1 and human ether-a-go-go-related gene (hERG or Kv11.1), have revealed an original nondomain-swapped structure, suggesting that the mechanism of voltage-dependent gating of these two channels is quite different from the classical mechanical-lever model. Molecular aspects of hERG voltage-gating have been extensively studied, indicating that the S4-S5 linker (S4-S5L) acts as a ligand binding to the S6 gate (S6 C-terminal part, S6T) and stabilizes it in a closed state. Moreover, the N-terminal extremity of the channel, called N-Cap, has been suggested to interact with S4-S5L to modulate channel voltage-dependent gating, as N-Cap deletion drastically accelerates hERG channel deactivation.

In this study, using COS-7 cells, site-directed mutagenesis, electrophysiological measurements, and immunofluorescence confocal microscopy, we addressed whether these two major mechanisms of voltage-dependent gating are conserved in Kv10.2 channels. Using cysteine bridges and S4-S5L–mimicking peptides, we show that the ligand/receptor model is conserved in Kv10.2, suggesting that this model is a hallmark of EAG channels. Truncation of the N-Cap domain, Per-Arnt-Sim (PAS) domain, or both in Kv10.2 abolished the current and altered channel trafficking to the membrane, unlike for the hERG channel in which N-Cap and PAS domain truncations mainly affected channel deactivation. Our results suggest that EAG channels function via a conserved ligand/receptor model of voltage gating, but that the N-Cap and PAS domains have different roles in these channels.

Voltage-gated potassium (Kv) channels regulate a variety of cellular processes, including membrane polarization (1, 2), apoptosis (3), cell proliferation (4), and cell volume (5). In connection with such a variety of functions of Kv channels, mutations in these channels cause a variety of pathological conditions in humans: neurological disorders (6, 7), cardiac arrhythmias (2), multiple sclerosis (8), and pain syndrome (9). It has also been shown that Kv channels are associated with the development of malignant tumors cancer (10). Kv10 channels belong to the ether-a-go-go family (EAG),5 as hERG channels. Two isoforms of Kv10 channels are expressed in mammals: Kv10.1 (eag1) and Kv10.2 (eag2), which show 70% amino acid sequence identity. Kv10.1 has been detected mainly in the brain, whereas Kv10.2 is also expressed in other tissues such as the skeletal muscles, heart, placenta, lungs, and liver (11).

Atomic structures of rat Kv10.1 (12) and human hERG (Kv11.1) channels (13) highlighted many structural similarities: nonswapped pore and voltage domains, i.e. facing voltage-sensor and pore domains are from the same subunit, short S4-S5 linkers (S4-S5L) and similar N-terminal structures such as Per-Arnt-Sim (PAS) and N-Cap domains. We hypothesized that molecular mechanisms of gating identified in hERG may apply to the closely related Kv10 channels.

In the heart, deactivation of hERG channels (closing of the activation gate) is slow, allowing a major role for this channel in the late repolarization phase of the action potential. In this channel, two mechanisms have been shown to play a major role in the deactivation process.

First, hERG deactivation is due to a voltage-dependent interaction between the S4–S5 region, including the S4–S5 linker and a part of S5 (named S4–S5L, Fig. 1A) and the C-terminal part of the S6 segment (named here S6T), which is the activation gate (14, 15). In other words, at resting potentials, S4–S5L (the ligand) is bound to the S6T gate (the receptor) and locks it in a closed state (as shown in Fig. 1B, left). Upon membrane depolarization, S4 drags S4–S5L out of the S6T gate, and the channel...

5 The abbreviations used are: EAG, ether-a-go-go; PAS, Per-Arnt-Sim; WGA, wheat germ agglutinin; DAPI, 4′,6-diamidino-2-phenylindole; tbHO2, tert-butylhydroperoxide; EGFP, enhanced green fluorescent protein.
opens (Fig. 1B, middle). Upon repolarization S4-S5L binds to the S6T gate and channel deactivates (back to Fig. 1B, left). Here, using two distinct approaches, we observe that this ligand/receptor mechanism, which we originally proposed for hERG, is conserved in KV10.2 (15).

Second, hERG deactivation is modulated by the channel N terminus. In the N-terminal eag domain, deletion in the N-Cap and/or the PAS domains, profoundly accelerated deactivation with no major effect on maximal current amplitude (16–19). Moreover, covalent binding of the N-Cap and the S4-S5L closed the channel (20). These observations suggested that the N-Cap and/or the PAS domains regulate channel deactivation by modulating S4-S5L and S6T interaction. In Kv10.2, we show that the eag domain presents quite different functions. Deletions of the N-Cap and PAS domains, separately or altogether, completely abolish channel activity, at least partially due to a defect in membrane trafficking.

In conclusion, we show that slowing activating channels follow an allosteric model, in which the voltage sensor and pore domains are weakly coupled, via a ligand, S4-S5L, and a receptor, S6T. In Kv10.2, such coupling is not modulated by the eag domain, as proposed for hERG (20).

**Results**

**As in hERG, covalent binding of S4-S5L to S6T locks the Kv10.2 channel closed**

The similar structures of hERG and Kv10.1 (12, 13) showing a nonswapped arrangement of the pore and voltage sensor domains suggest that voltage-gating of these channels does not follow the classical mechanical-lever model in which S4-S5L constricts the S6T gate (21). Also, functional evidence obtained by several groups including ours, strongly suggests that hERG channels do not follow the mechanical-lever model (14, 15).

First, Ferrer and collaborators, observed that introduction of a cysteine in S4-S5L (D540C) and another cysteine in S6T (L666C) locks the channel in a closed state in oxidative conditions. This was associated to a restricted movement of the voltage sensor in oxidative condition, as measured by gating currents, suggesting the formation of a disulfide bridge (14). Also, mutagenesis experiments on hERG suggested electrostatic interaction between Asp-540 and Leu-666 playing a major role in hERG voltage-dependent gating (22). Such observations suggest that in the WT hERG channel, specific interactions between S4-S5L and the activation gate (S6T) stabilize the closed channel. In transfected COS-7 cells, we could reproduce the experiments originally done by Ferrer and collaborators (14) in the Xenopus oocyte model. Moreover we could observe that a S4-S5L mimicking peptide inhibits the hERG channel, suggesting a ligand/receptor model, in which S4-S5L, directly under the control of the voltage sensor S4, binds to S6T to lock the channel in a closed state (see Fig. 1B, right).

To check whether Kv10.2 channels follow the same voltage-gating mechanism as hERG channels, we aligned the amino acid sequences of the two channels, and mutated to a cysteine Asp-339 and Met-474 in Kv10.2, corresponding to hERG positions in which cysteines were previously introduced: Asp-540 in

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**Figure 1. Alignment used to design the cysteine mutants and the S4-S5L peptide, hypothetical ligand/receptor model.** A, alignment between hERG and Kv10.2. This alignment was used to (i) introduce the cysteines in S4-S5L and S6T, and (ii) design the S4-S5L peptide from the previously identified hERG S4-S5L inhibiting peptide. S4-S5L refers to the S4-S5 region, including the S4-S5 linker and a part of S5. S6T refers to the C-terminal part of the S6 segment, which is the activation gate. The alignment was obtained using Cobalt (43). In red are represented the basic residues, in yellow acidic residues, and in purple the position of the narrowest part of the bundle crossing, also the gating residue (44). The color boxes represent the transmembrane segments. Introduced cysteines in hERG (15) and Kv10.2 in the present study are indicated. Gray line represents the inhibiting S4-S5L peptide in hERG (15). Red line represents the peptide engineered in Kv10.2 for the present study.

B, left: scheme of the hypothetical ligand/receptor model in which S4-S5L (deep blue) binds to S6T (light blue) to stabilize the channel in a closed state. Middle, upon membrane depolarization, S4 pulls S4-S5L out of the S6T receptor, allowing channel opening. Right, the Kv10.2 S4-S5L peptide (red) mimics endogenous S4-S5L, locking the channel in its closed conformation.
Voltage-dependent gating mechanism of EAG channels

S4–S5₁ and Leu-666 in S6₁ (14, 15) (black arrows in Fig. 1A). As for the hERG double cysteine mutant, a 2-h application of 0.2 mM tert-butyldihydroperoxide (tbHO₂) led to an almost complete inhibition of the D339C/M474C Kᵥ₁₀.₂ channel current (Fig. 2, A and B). Knowing the high homology between hERG and Kᵥ₁₀.₂ (65%), we supposed that D339C and M474C in Kᵥ₁₀.₂ also form a disulfide bridge in oxidative conditions, as a cause of the drastic current reduction. As control experiments, 0.2 mM tbHO₂, application had no effect on tail current amplitude when only one (D339C or M474C Kᵥ₁₀.₂) or none (WT Kᵥ₁₀.₂) of the two cysteines was introduced (Fig. 2, A–H) and activation curves showed a similar shift in all conditions (Fig. S1). Because a 2-h tbHO₂ application represents a slow time course for a putative disulfide bridge formation, we also applied a higher concentration of tbHO₂ (2 mM) for a shorter time. As in the Ferrer study (14) on hERG, this concentration led, in around 12 min, to an almost complete inhibition of the D339C/M474C Kᵥ₁₀.₂ channel current but not the WT channel current (Fig. 3). This inhibition was reversed by 10 mM DTT, as in the Ferrer study on hERG (14).

To strengthen the hypothesis of the interaction between S4–S5₁ and S6₁ in Kᵥ₁₀.₂, we also tested the tbHO₂ effect on another double mutant of the Kᵥ₁₀.₂ channel. We chose the E343C/M474C Kᵥ₁₀.₂ channel, because Glu-343, in S4–S5₁, is aligned with Glu-544 in hERG, which when mutated to a cysteine together with L666C in hERG S6₁ (Fig. 1A), also lead to channel inhibition, suggesting the formation of a disulfide bridge (15). We observed an inhibition of the current after 15 min incubation of 2 mM tbHO₂, which is reversed by 10 mM DTT (Fig. 4, A and B). Control experiments showed no effect either on tail current amplitude or on the activation curve when only one (E343C or M474C Kᵥ₁₀.₂) or none (WT Kᵥ₁₀.₂) of the two cysteines was introduced (Fig. 4, C–H, Fig. S2). Altogether, these observations confirmed that two different disulfide bridges created between the two introduced cysteines in S4–S5₁ and S6₁ locks Kᵥ₁₀.₂ in a closed state, as in hERG (14, 15).

As in hERG, a Kᵥ₁₀.₂ S4–S5₁ mimicking peptide inhibits Kᵥ₁₀.₂ channels

Recently, we observed that (i) co-expressing a hERG S4–S5₁ mimicking peptide with hERG channel partially inhibited the generated current and (ii) covalently binding this peptide to the hERG channel completely inhibited it (15). These observations suggest that in hERG channels, S4–S5₁ rather acts as a voltage-controlled ligand that binds to the S6₁ gate and stabilizes it in the closed state (Fig. 1B, left, for the endogenous interaction and B, right, for the mimicking peptide). This mechanism of gating is consistent with Ferrer et al. (14) observation that covalent binding of S4–S5₁ and S6₁ channel regions locks the channel in a closed state. Using the alignment between hERG and Kᵥ₁₀.₂ (Fig. 1A), we designed the Kᵥ₁₀.₂ S4–S5₁ peptide from the position of the S4–S5₁ peptide sequence that was inhibiting the hERG channel (15). Co-expressing the Kᵥ₁₀.₂ channel with its specific S4–S5₁ peptide led to a profound decrease in current density (more than 70%), with no shift in the activation curve, as observed for hERG (Fig. 5, A–E). As an additional control, co-expressing Kᵥ₁₀.₂ with a scramble S4–S5₁ peptide led to current density similar to the one observed in the absence of peptide (Fig. 5, A and B).

Because the S4–S5₁ peptide is supposed to interfere with the gating, it may be intriguing that activating and tail currents are inhibited to the same extent, and that neither the activation curve, nor the activation kinetics are modified (Fig. 5, C–F). To address this issue, we used a kinetic model mimicking channel activity in the presence/absence of the peptide (Fig. 6), based on a previous model of KCNE1–KCNQ1 and S4–S5₁ peptide interaction (23). In the KCNE1–KCNQ1 model, the presence of the peptides was affecting the channel activation curve and activation kinetics. However, when constrained to Kᵥ₁₀.₂ kinetics, the model did not show any alteration of activation kinetics or steady-state activation (Fig. 6, B and C). This is because peptide binding and unbinding rates (0.02 and 0.04 s⁻¹, respectively) are lower than channel opening/closing rates, because increasing these rates leads to alterations in channel activation kinetics and steady-state activation (Fig. 6, B and C). A peptide with similar binding/unbinding rates impact KCNE1–KCNQ1 channel activation curve and activation kinetics (23) because gating kinetics of this channel are slower than Kᵥ₁₀.₂ kinetics. Altogether, these observations support the ligand/receptor model of voltage dependence in Kᵥ₁₀.2 channels.

As opposed to hERG, N-Cap and PAS domain-deleted Kᵥ₁₀.₂ channels are not functional

The results described above suggest that in both Kᵥ₁₀.₂ and hERG channels, deactivation is due to S4–S5₁ binding to S6₁ and consequent stabilization of the closed state. It has been shown by several works that intracellular N-cap and PAS domains of hERG (shown in Fig. 7A for Kᵥ₁₀.₂, cf. alignment in Fig. S3) modulate channel deactivation kinetics. Most importantly truncated hERG channels missing N-Cap or the whole eag domain (N-Cap + PAS), when expressed in Xenopus oocytes, showed robust currents but a more than 5-fold acceleration in deactivation (16–19). Also in mammalian cells, it has been observed that the eag domain is not necessary for hERG channel trafficking, consistent with the observation of robust currents in this model (24, 25). Another work showed that N-Cap is close to S4–S5₁ (20). Thus, N-Cap may modulate channel deactivation through a direct interaction with this linker. Based on all these observations, we supposed that deletion of both N-Cap and PAS domains in Kᵥ₁₀.₂ should give rise to functional channels with accelerated deactivation, as in the study on hERG in mammalian cells (25). Transfection of WT Kᵥ₁₀.₂ tagged with 1D4 at the C terminus gave rise to a current similar to a previous description (6) and immunofluorescence experiments using this tag showed plasma membrane enrichment of the channel, compared with the intracellular compartment (Fig. 7, B–F). Surprisingly, deletion of N-Cap domain, PAS domain, but also of both domains all resulted in non-functional Kᵥ₁₀.₂ channels (Figs. 8B, 9B, and 10B). Immunofluorescence experiments on N-Cap and/or PAS domains truncated channels showed no membrane enrichment of the channels (Figs. 8, C–F, 9, C–F, and 10, C–F). These findings
suggest a trafficking defect of the N-Cap and/or PAS domains truncated Kv10.2 channels.

As opposed to hERG, coexpressing a Kv10.2 N-Cap mimicking peptide with truncated Kv10.2 does not counteract the effect of channel truncation

For the hERG channel, it has been shown that a peptide corresponding to the first 16 amino acids of the channel is sufficient to reconstitute slow deactivation to hERG lacking this region (19). Similarly, another study has shown that injection of the purified eag domain, corresponding to the first 135 amino acids of hERG, into oocytes expressing eag-truncated hERG, restores the deactivation kinetics to WT-like in more than 24 h (17). Based on these previous observations on hERG, we proposed that co-expression of specific Kv10.2 N-Cap mimicking peptides with the N-Cap–truncated Kv10.2 channel should recover its expression and activity at the plasma membrane. Surprisingly again, Kv10.2 channel activity was not recovered in the presence of N-Cap mimicking peptide (n/H11005). This observation further suggests that N-Cap and PAS domains play distinct roles in hERG and Kv10.2 function.

Co-expression of WT and truncated Kv10.2 channels gives rise to a right shift in the activation curve as compared with homomeric WT channels, but no change in deactivation kinetics

To evaluate the potential effects of N-Cap truncation on channel activity, we co-expressed Kv10.2 missing the N-Cap with the WT channel, in an attempt to generate heteromers. We observed robust voltage-dependent currents, showing a 30-mV shift in the activation curve toward depolarized potential, demonstrating the generation of such heteromers (Fig. 11, C–F). This shift in the activation curve suggests that N-Cap deletion leads not only to a Kv10.2 trafficking defect but also to a gating defect. In the hERG channel, deletion of N-Cap did not lead to a shift of the activation curve, but an acceleration of deactivation (26). In the present experiments on Kv10.2, no change in deactivation kinetics was observed when the N-Cap–truncated channel was co-expressed with the WT channel. Again, we observed a 30-mV shift in the activation curve toward depolarized potentials, but no modification in deactivation (Figs. 11 and 12). Thus, although we are likely recording the
combined activities of tetrameric channels containing different ratios of the WT and truncated subunits, it appears that N-terminal deletion of KV10.2 impacts the steady-state activation curve rather than deactivation kinetics.

Discussion

From the present and previous works, we suggest that among voltage-gated channels, coupling between voltage sensor movement and pore gating falls into two categories: (i) the mechanical-lever model: an obligatory coupling in which the S4 resting state directly translates into S6 gate-closed state. This mechanical-lever model, inferred from structural data in Shaker-like channels (27), also applies to eukaryotic sodium channels, as suggested by recent structural studies (28, 29); (ii) the ligand/receptor model: the obligatory coupling cannot hold if the S6 gate is able to open, even if S4 segments are in the resting state, as shown for hERG and KCNQ1 channels (30–32), and, vice versa, if the S6 gate is able to close, even if S4

Figure 4. Introduction of 2 cysteines in the S4-S5 and S6 regions of KV10.2 (E343C/M474C KV10.2) locks the channel closed in oxidative conditions. A, representative, superimposed recordings of the E343C/M474C KV10.2 current after 15 min incubation in Tyrode without (control) or with 2 mM tbHO₂ (tbHO₂), or after a subsequent 5-min incubation in 10 mM DTT. Left inset, activation voltage protocol used (one sweep every 8 s). Middle and right insets, schemes of S4-S5/S6, with introduced cysteines in the presence of tbHO₂ or DTT, respectively (stars). B, mean ± S.E. E343C/M474C KV10.2 maximum tail-current density, in control, tbHO₂, or tbHO₂ followed by DTT. ***, p < 0.001, Mann-Whitney test. C and D, same as A and B for WT KV10.2. E and F, same as A and B for E343C KV10.2. G and H, same as A and B for M474C KV10.2.
segments are in the activated state (33). We recently demonstrated this ligand/receptor model in hERG channels by using several approaches. Here, we obtained similar results on KV10.2 using similar approaches.

First, introduction of cysteines in S4-S5L and S6T lock the channel in a closed state in oxidative conditions, suggesting the formation of a disulfide bridge, as in hERG. This suggests that the same gating mechanism applies to KV10.2. Introduction of cysteines in both S4-S5L and S6T may lead to a nonnative conformation that favors an S4-S5L interaction with S6T, which would not be met in the WT channel. But in the second set of experiments, a S4-S5L mimicking peptide, without any introduced cysteine, inhibits KV10.2 channel, also without any introduced cysteine, further suggesting the capability of S4-S5L to stabilize the channel closed state. Noteworthy, similar channel-specific peptides have the same effect in KCNQ1 and hERG channels (15, 23). Complementary experiments in hERG revealed that S4-S5L peptide effects was on channel gating, and not channel trafficking (15).

Altogether these experiments suggest that KV10.2 follows the ligand/receptor mechanism observed in hERG. In both channels, S4-S5 linkers are short (12, 13), thus it is likely that the part of S5 that is present in the peptide also plays a role in closed channel stabilization. Further structural data of hERG and KV10.2 channels in the closed state should clarify the residues involved in the S4-S5L and S6T interaction. Noteworthy, this ligand/receptor model is consistent with the observation that the voltage-dependent closure of the related Kv10.1 channel, but also of the hERG channel, requires at least a part of the inhibiting S4-S5L to be covalently linked to the voltage sensor S4 (34, 35).

As opposed to similar ligand/receptor gating mechanisms in the two channels, the role of the eag domain (N-Cap + PAS) is quite different between hERG and KV10.2. In hERG channels, the eag domain (N-Cap + PAS) is not necessary for channel activity and mainly modulates the current deactivation rate (17–19). Here, we observed that truncation of the KV10.2 eag domain renders the channel nonfunctional, at least partly due to trafficking defects. In rescue experiments with the WT channel, we observed that eag domain deletion or even only N-Cap deletion are associated with a shift in the activation curve, but no change in channel deactivation. The contrary is observed in hERG channel: an altered deactivation, no shift in the activation curve (26, 36). Our results suggest major differences in functional roles for the N-Cap and PAS domains between KV10.2 and hERG. Altogether, this work suggests a conserved ligand/receptor (allosteric) model of voltage gating, but divergent roles in eag domains among channels of the EAG family.

In combination with previous work on hERG, this study highlights the voltage-gated channels superfamily, divergent gating mechanisms (obligatory versus allosteric) that matches divergent structures (swapped versus nonswapped domain, respectively) and divergent kinetics (fast versus slow activating channels, respectively). Nonswapped domains (hERG, KV10) may provide less contact between S4-S5L and S6T (12, 13). We
propose that this weak coupling between S4-S5L and S6T provides a framework for a two-step channel activation: first, the fast S4 movement drags the ligand S4-S5L out of its receptor on the S6T gate, followed by slow gate opening. This allosteric regulation of the S6T gate by S4-S5L may explain how in slowly activating channels, movement of S4 is not concomitant to pore opening (37).

Experimental procedures

**Plasmid constructs**

pCDNA6 hKv10.2 was subcloned into pMT3 vector using the standard PCR overlap extension method (38). A 1D4 immunoaffinity tag (derived from the C terminus of bovine rhodopsin) was added to the C terminus of all constructs (39). The D339C, E343C, M474C, E343C/M474C, and D339C/M474C

**Figure 6.** Kinetic model of Kv10.2 and its interaction with the S4-S5L peptide. A, kinetic model schemes. This model is based on a previous work on KCNE1-KCNQ1 (23). a, kinetic model in the absence of peptide, on which optimization has been performed (see "Experimental procedures"). Optimized transition rates are presented in Table 1. b, binding of exogenous S4-S5L locks the channel and prevents its opening. Peptides are supposed to interact with each monomer in the unlocked states. B, simulated currents during step protocols (same as in Fig. 5), in the absence (Ctrl) or presence of S4-S5L peptide at the indicated S4-S5L on/off rates. C, gray filled circles: experimental activation curves and half-activation times in control condition. Other symbols: simulated values in the absence of peptide (control, open circles), or in the presence of peptides, at the indicated rates of peptide binding/unbinding.
mutations were inserted into the pMT3-Kv.10.2 construct using the QuikChange™ site-directed mutagenesis-based technique using Accuprime Pfx polymerase (ThermoFisher Scientific) according to the standard protocol recommended by the manufacturer. Truncation mutants were constructed by deleting residues 2 to 24 (ΔN-CAP), 2 to 134 (Δeag), and 25 to 134 (ΔPAS) using the standard PCR overlap extension method. PCR products were digested with HindIII and XbaI and ligated into pCDNA6 and PMT3 vectors. All constructs were confirmed by sequencing. Oligonucleotides encoding Kv.10.2 peptides were synthesized by TOP Gene Technologies and contained a Xhol restriction enzyme, followed by a methionine (ATG) for translation initiation, a glycine (GGA) to protect the ribosome-binding site during translation, and the nascent pep-
A BamHI restriction enzyme site was synthesized at the 3’ end immediately following the translational stop codon (TGA). These oligonucleotides were then ligated into pIRES2-EGFP (Clontech) and sequenced.

Cell culture and transfection

The African green monkey kidney-derived cell line COS-7 was obtained from the American Type Culture Collection (CRL-1651) and cultured in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml of streptomycin) at 5% CO₂ and 95% air, maintained at 37 °C in a humidified incubator. Cells were transfected in 35-mm Petri dishes when the culture reached 50–60% confluence, with 4 μg of total DNA complexed with 12 μl of FuGENE-6 (Roche Molecular Biochemical) according to the standard protocol recommended by the manufacturer. In different experiments, plas-
mid quantities were optimized to keep current amplitudes in a range that undetectable currents were rare, and large currents inducing incorrect voltage-clamp were also rare. Immunofluorescence and confocal microscopy experiments were done with pCDNA6-Kv10.2 for which channel expression was lower than with pMT3-KV10.2, to limit intracellular accumulation of the protein. For disulfide bridge experiments, COS-7 cells were co-transfected with 3.6 μg of pMT3-WT, D339C/M474C, E343C/M474C, pMT3-WT, D339C, E343C, M474C, D339C, E343C, M474C, Kv10.2, and 0.4 μg of pEGFP.

For S4-S5L peptide experiments, COS-7 cells were co-transfected with 2 μg of pMT3-WT Kv10.2 and 2 μg of pIRES2-EGFP plasmids encoding or not the S4-S5L peptide. As an additional control, a pIRES2-EGFP plasmid encoding a scramble S4-S5L peptide was used. In pIRES2-EGFP plasmids, the second cassette (EGFP) is less expressed than the first cassette, guaranteeing high levels of peptide expression in fluorescent cells.

Figure 9. Δeag Kv10.2 characterization in transfected COS-7 cells. A, domains organization of the channel, showing the eag domain deletion. B, representative, superimposed recordings of the Δeag Kv10.2–1D4 current. Inset: voltage protocol used (one sweep every 8 s). C, representative confocal immunostainings of Δeag Kv10.2–1D4 in transfected COS-7 cells (in green). WGA is used as a membrane marker (in red). Nuclei are stained with DAPI (in blue). Scale bar = 15 μm. D, mean ± S.E. fluorescence of Kv10.2 signal in plasma membrane (M1 and M2) and cytosol, as measured in E and F, normalized by the average Kv10.2 fluorescence. E and F, left: expanded view of two selected cells, showing the Golgi (stars) and the line used for the line plots shown at the right. Lines have been placed as far as possible from the Golgi to generate accurate plasma membrane plots. Right: line plots of WGA (red) and Kv10.2 (green) at the level of the drawn lines at the left. Higher Kv10.2 fluorescence densities are not observed in the region of the plasma membrane, as opposed to the WT condition.
For N-terminal deletion experiments, COS-7 cells were co-transfected with 2 μg of pC6DNA6-N-Cap truncated Kv10.2 and 2 μg of pIRES2-EGFP plasmids encoding or not the N-Cap mimicking peptide. For immunofluorescence and confocal microscopy experiments, COS-7 cells were transfected with 4 μg of pC6DNA6-WT/truncated Kv10.2. For WT/truncated heteromeric Kv10.2 experiments, COS-7 cells were co-transfected with 1.8 μg of pMT3-WT Kv10.2, 1.8 μg of pMT3-truncated Kv10.2, and 0.4 μg of pEGFP. Cells were re-plated onto 35-mm Petri dishes the day after transfection for patch clamp experiments.

**Electrophysiology**

One day after splitting, COS-7 cells were mounted on the stage of an inverted microscope and constantly perfused by a Tyrode solution (cf. below) at a rate of 1–3 ml/min. The bath solution contained (in mM): NaCl 137, KCl 5, CaCl2 1, MgCl2 1, HEPES 5, and glucose 5. After a 20-min equilibration period, cells were perfused with a solution containing (in mM): NaCl 100, KCl 5, CaCl2 1, MgCl2 1, HEPES 5, and glucose 5, and 100 μM tetrodotoxin. Membrane potential was clamped to potentials of −100, −140, and −150 mV.

**Figure 10. ΔPAS Kv10.2 characterization in transfected COS-7 cells.**

A, domains organization of the channel, showing the PAS domain deletion. B, representative, superimposed recordings of the ΔPAS Kv10.2–1D4 current. Inset: voltage protocol used (one sweep every 8 s). C, representative confocal immunostainings of ΔPAS Kv10.2–1D4 in transfected COS-7 cells (in green). WGA is used as a membrane marker (in red). Nuclei are stained with DAPI (in blue). Scale bar = 15 μm. D, mean ± S.E. fluorescence of Kv10.2 signal in plasma membrane (M1 and M2) and cytosol, as measured in E and F, normalized by the average Kv10.2 fluorescence. E and F, left: expanded view of two selected cells, showing the Golgi (stars) and the line used for the line plots shown at the right. Lines have been placed as far as possible from the Golgi to generate accurate plasma membrane plots. Right: line plots of WGA (red) and Kv10.2 (green) at the level of the drawn lines at the left. Higher Kv10.2 fluorescence densities are not observed in the region of the plasma membrane, as opposed to the WT condition.
temperature was maintained at 22.0 ± 2.0 °C. Stimulation and data recording were performed with Axon pClamp 10, an A/D converter (Digidata 1440A), and an Axopatch 200B amplifier (all Molecular Devices). Patch pipettes (tip resistance: 2–3 megohms) were pulled from soda lime glass capillaries (Kimble-Chase) and coated with wax. Currents were recorded in the whole-cell configuration, pipette capacitance and series resistance were electronically compensated (by around 75%). Activation protocols were adjusted to the voltage-dependence of the construct as in the previous study on hERG (15). Activation curves were obtained from the tail currents and fitted by Boltzmann equations.

**Confocal microscopy**

Immunohistological analyses were performed to study cell localization of transfected WT/truncated Kv10.2–1D4 in COS-7 cells. Twenty-four hours after transfection, cells were plated on IBIDI plates for 24 h. Cells were then fixed with 4% formaldehyde, stained for 10 min at room temperature with Alexa Fluor™ 647 conjugated wheat germ agglutinin (WGA; ThermoFisher), a plasma membrane marker, permeabilized with 0.5% saponin and blocked with 1% PBS/BSA. Cells were then incubated with a mouse mAb directed against the 1D4 tag diluted in PBS (Abcam). Secondary antibody staining was per-
formed using Alexa 488-conjugated anti-mouse antibody. DAPI was used for nuclear staining. Conventional imaging was performed using a LSM710-Confocor3 (Zeiss) and a Nikon Confocal A1RSi microscope system equipped with a SR Apo 100×/1.49 N.A objective. Images were analyzed with ImageJ software. In figures, but not for analyses, Enhance Local Contrast adjustment was performed on WGA staining to highlight plasma membrane staining. To quantify fluorescence, the line plot was arbitrarily segmented in 3 different regions: the first and last 5–15% of the line plot, corresponding to plasma membrane (M1 and M2), and the remaining intermediate 70% of the line plot, corresponding to the intracellular compartment. For each of these regions in each cell, Kᵥ10.2 fluorescence intensity values were normalized by the average cell Kᵥ10.2 fluorescence intensity signal (41).

### Solutions

Cells were continuously superfused with a HEPES-buffered Tyrode solution containing (in mmol/liter): NaCl 145, KCl 4, MgCl₂ 1, CaCl₂ 1, HEPES 5, glucose 5, pH adjusted to 7.4 with NaOH. Patch pipettes were filled with the following solution (in mmol/liter): KCl 100, K-gluconate 45, MgCl₂ 1, EGTA 5, HEPES 10, pH adjusted to 7.2 with KOH. For experiments in Fig. 3, 1 mM K₂ATP was added to limit current rundown. The membrane-permeable oxidizing agent tbHO₂ was obtained from Sigma. Incubation of COS-7 cells with 0.2 or 2 mM tbHO₂ was realized at room temperature.

### Kinetic model

The Kᵥ10.2 kinetic model (Fig. 6A, top) contains two voltage sensor transitions, and two open states, as in the model of Iᵥsₖ (42). This model was optimized using IchMASCOT (J.A. De Santiago-Castillo and M. Covarrubias) to fit traces of the representative control of Fig. 5. Optimized transition rates are presented in Table 1. Next, another model was designed (Fig. 6A, bottom), with an additional state in which S4-S5L mimicking peptide binds to the pre-open state and stabilizes it, as in Ref. 23. Various S4-S5L binding/unbinding transition rates were applied, and the effects on the biophysical parameters were studied.

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**Table 1**

Optimized transition rates used in the model presented in Fig. 6

The abbreviations use are: \( F = 96485 \) C mol⁻¹ (Faraday constant); \( R = 8.314 \) J mol⁻¹ K⁻¹ (gas constant); \( T = 297 \) K; V (membrane potential) in V.

| Transition description | Transition rate (s⁻¹) |
|-----------------------|----------------------|
| Voltage sensor transition rates | |
| Closed Locked 2 (CL2) → Closed Locked 1 (CL1) | \( \alpha \) 8.50 - \( \frac{V - F}{R \cdot T} \) |
| CL1 → CL2 | \( \beta \) 13.35 - \( \frac{V - F}{R \cdot T} \) |
| CL1 → Closed unlock (CU) | \( \gamma \) 15.95 - \( \frac{V - F}{R \cdot T} \) |
| CU → CL1 | \( \delta \) 4.12 × 10⁻² - \( \frac{V - F}{R \cdot T} \) |
| Concerted opening transition rates | |
| 4 monomers CU or CUP6 → Open state 1 (O1) | \( \theta \) 260.7 - \( \frac{V - F}{R \cdot T} \) |
| O1 → 4 monomers CU or CUP6 | \( \eta \) 64.66 - \( \frac{V - F}{R \cdot T} \) |
| O1 → Open state 2 (O2) | \( \nu \) 6.20 × 10⁻² - \( \frac{V - F}{R \cdot T} \) |
| O2 → O1 | \( \omega \) 12.74 - \( \frac{V - F}{R \cdot T} \) |

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**Figure 12.** Co-expression of WT and truncated Kᵥ10.2 channels is not associated with changes in deactivation kinetics, as compared with WT channel. A, representative, superimposed recordings of COS-7 cells transfected with WT Kᵥ10.2 channel (left), WT and ΔN-Cap Kᵥ10.2 (middle), and WT and Δeag Kᵥ10.2 (right). Upper inset: deactivation tail voltage protocol used (prepulse duration, 3 s, one sweep every 7 s). \( \beta \), mean ± S.E. Kᵥ10.2 deactivation time constant, obtained from a monoexponential fit of the deactivating current.

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Statistics

All data are expressed as mean ± S.E. Statistical differences between current densities (data points are not normally distributed) were determined using nonparametric Mann-Whitney test. Statistical differences between activation parameters, V_0.5\(K\) (data points are normally distributed) were determined using unpaired Student’s t tests. A value of \(p < 0.05\) was considered significant.

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