hERG S4-S5 linker acts as a voltage-dependent ligand that binds to the activation gate and locks it in a closed state

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Delayed-rectifier potassium channels (hERG and KCNQ1) play a major role in cardiac repolarization. These channels are formed by a tetrameric pore (S5–S6) surrounded by four voltage sensor domains (S1–S4). Coupling between voltage sensor domains and the pore activation gate is critical for channel voltage-dependence. However, molecular mechanisms remain elusive. Herein, we demonstrate that covalently binding, through a disulfide bridge, a peptide mimicking the S4-S5 linker (S4-S5L) to the channel S6 C-terminus (S6C) completely inhibits hERG. This shows that channel S4-S5L is sufficient to stabilize the pore activation gate in its closed state. Conversely, covalently binding a peptide mimicking S6C to the channel S4-S5L prevents its inhibiting effect and renders the channel almost completely voltage-independent. This shows that the channel S4-S5L is necessary to stabilize the activation gate in its closed state. Altogether, our results provide chemical evidence that S4-S5L acts as a voltage-controlled ligand that binds S6C to lock the channel in a closed state, elucidating the coupling between voltage sensors and the gate in delayed rectifier potassium channels and potentially other voltage-gated channels.

Voltage-dependent ion channels are ubiquitously expressed in human tissues. They perform a plethora of physiological functions such as generation and modulation of the electrical activity in excitable tissues, modulation of neurotransmitter and hormone release, and electrolyte transport in epithelia. Canonical voltage-gated ion channels are tetramers of subunits containing six transmembrane segments (S1 to S6). Each of the four subunits is composed of one voltage sensor domain (S1 to S4) and a pore domain (S5-S6). The four pore domains tetramerize to generate a single pore module, which is regulated by the four voltage sensor domains1,2. One key question remains: how do the voltage sensor domains regulate pore gating? Several independent approaches support the idea that interaction between the S4-S5 linker (S4-S5L) and the S6 C-terminal (S6C) part plays a critical role in that regulation2-5. First, Lu and collaborators pinpointed the sequence complementarity between S4-S5L and S6C in the Shaker channel4, 5. Second, structural data indicated the close proximity between S4-S5L and S6C and suggested a mechanical lever model to explain the coupling between the voltage sensor and the gate of the related Kv1.2 channel2. However, the mechanism may prove to be more complex. Since 2006, several works on other eukaryotic Kv channels suggested state-dependent S4-S5L and S6C interactions3, 6-10, but the precise mechanism remains elusive and may vary from one channel to another.

In a previous study, we suggested that the voltage dependence of the cardiac voltage-gated KCNQ1 channel (Kv7.1) follows a ligand/receptor model. In this model, S4-S5L is a ligand whose interaction with S6C is only possible at resting potentials, and this interaction locks the channel in a closed state. Upon depolarization of the plasma membrane, S4 pulls the S4-S5L out of its binding site and unlocks the channel (Fig. 1A). We validated this ligand/receptor model by using peptides mimicking S4-S5L and S6C and evaluating their effects on the channel activity. However the peptides effects were moderate, probably due to the low affinity between native S4-S5L and S6C, which is necessary for S4-S5L ligand unbinding and channel opening during membrane depolarization11. In the present study, we started with a similar strategy of peptide native-sequence mimicry to evaluate the ligand/receptor model in hERG (Kv11.1), a key channel in cardiac and neuronal electrical activity. As in the KCNQ1 channel.
results, hERG-mimicking peptide effects were moderate. In a previous study on this channel, a disulfide bond was created between S4-S5L and S6T, stabilizing it in a closed state. Here, after the first step of peptide identification, we used the same cysteine approach to increase the peptide-channel interaction and to target the peptide to its hypothetical receptor (S6T for S4-S5L and vice-versa). We observed that covalently binding a peptide mimicking S4-S5L to the endogenous S6T could completely inhibit hERG. On the other hand, covalently binding a peptide mimicking S6T to the endogenous S4-S5L rendered the channel almost voltage-independent, most likely by preventing the inhibitory effect of S4-S5L on S6T in the channel.

Altogether, these results suggest that the voltage dependence of these two delayed rectifier channels, KCNQ1 and hERG, follows a ligand/receptor model in which S4-S5 linker acts as an inhibitor, locking the activation gate under the control of the voltage sensor S4.

Results

One S4-S5L peptide inhibits hERG channels. To identify a potential inhibitory S4-S5L peptide, three S4-S5L encoding plasmids were designed based on sequence alignment with KCNQ1 (Fig. 1B), in which the ligand/receptor model was first suggested, the S4-S5 linker (S4-S5L) being the inhibiting ligand and the S6 C-terminus (S6T) acting as the receptor. We observed that covalently binding a peptide mimicking S4-S5L to the endogenous S6T could completely inhibit hERG. On the other hand, covalently binding a peptide mimicking S6T to the endogenous S4-S5L rendered the channel almost voltage-independent, most likely by preventing the inhibitory effect of S4-S5L on S6T in the channel.

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lower expression of hERG protein in the presence of this peptide, as compared to control conditions (Fig. 3A and C). Thus, these results are consistent with the ligand/receptor model. However, the S4-S5L (−3) peptide did not show any effect on current density and led to a 5-mV negative shift in the activation curve (Fig. 2A–C, Supplemental Table 1). The S4-S5L (−3) peptide presents 5 charged amino-acids, 3 positively charged on one face of the helix and 2 negatively charged on the other face. The absence of any effect on current density suggests a non-specific effect such as membrane charge screening, locally changing the potential detected by the voltage-sensor.

The S4-S5L (0) peptide induced a paradoxical increase in hERG current density without any effect on the activation curve (Fig. 2A–C, Supplemental Table 1). Western blot experiments revealed that this peptide led to a doubling of hERG protein as compared to control conditions (Fig. 3A and C). Surprisingly, this peptide also induced an increase in GFP expression (by a factor of 7.1, p < 0.01), as shown in Fig. 3A. However expression of endogenous control GAPDH was not altered by S4-S5L (0) (Fig. 3A and C).

This set of experiments shows specific effects of only one of the S4-S5L. The S4-S5L (+3) peptide decreased the current density without modifying the activation curve, similar to the effects of the S4-S5L inhibiting peptides on KCNQ1 channel[1]. This is consistent with the ligand/receptor model.
One $S_6$ peptide activates hERG channels. If $S_6$ is the receptor for $S_4$-$S_5$, then a peptide mimicking endogenous $S_6$ should act as a decoy to the endogenous $S_4$-$S_5$, preventing its binding to the endogenous $S_6$, and increasing the channel activity. Indeed, the $S_6$ ($-3$) peptide up-regulated the WT channel and shifted the activation curve (and inactivation curve) by 5 mV toward negative potentials (Fig. 2A,E–G, Supplemental Table 1) without any change in the hERG expression level (Fig. 3B and C), which is similar to the effects of the $S_6$-activating peptides on KCNQ1 channel 11 and therefore also consistent with the ligand/receptor model. The other peptides, namely $S_6$ ($0$) and $S_6$ ($+3$) did not produce any effect.

Altogether, the effects of one inhibiting and one activating peptide were similar for both hERG and KCNQ1 channels 11. Moreover, both hERG inhibiting and activating peptides are aligned with those of the KCNQ1 channel (Fig. 1). In conclusion, these two similarities suggest that the ligand/receptor model applies to both channels.

A cysteine disulfide bond reinforces the effect of the $S_4$-$S_5$ ($+3$) peptide, leading to full inhibition of hERG channel. Although consistent with the ligand/receptor model, the $S_4$-$S_5$ ($+3$) effect is moderate, as in KCNQ1, likely because the $S_4$-$S_5$/$S_6$ interaction has to be loose, as explained in the introduction. In Xenopus oocytes, introduction of a cysteine pair in the hERG channel (D540C in $S_4$-$S_5$ with L666C in $S_6$) has been previously shown to stabilize the channel in a closed conformation through the formation of a disulfide bond 8. We reasoned that this disulfide bond could be exploited to covalently bind the mimicking $S_4$-$S_5$ ($+3$) peptide to the $S_6$ channel, which should enhance its inhibitory effect.

First, we evaluated the effects of oxidizing the native cysteines on WT hERG activity. We observed that in COS-7 cells, as previously shown in Xenopus Oocytes, WT currents were unaffected after 2 hours of incubation with 0.2 mM tert-butylhydroperoxide (tbHO$_2$; Fig. 4A,C and D, Supplemental Table 2), showing that oxidizing the native cysteines does not have any effect on channel gating and trafficking. Such a tbHO$_2$ treatment led to a permanent closure of the D540C-L666C hERG mutant and this inhibition was reversed by subsequent incubation with 10 mM DTT (Fig. 4B and E, Supplemental Table 2). Finally, the current density of the single mutants D540C and L666C was not altered by incubation with tbHO$_2$ (Supplemental Fig. 2), confirming that the D540C-L666C mutant inhibition by tbHO$_2$ is specifically due to the disulfide bond formed between D540C and L666C.
The inhibiting S4-S5, (+3) peptide does not contain the D540 residue (Fig. 1), but the E544 residue, located on the same side of the S4-S5 helix as D540, is negatively charged as well. Both D540 and E544 may interact electrostatically with R665 (next to L666) in S6 T when the channel is in the closed state12, 13. Therefore, we tested if a cysteine introduced in E544 hERG instead of D540 and another cysteine introduced in L666 hERG could also form a disulfide bridge, which would lead to a permanent closure of the channel. COS-7 cells were transfected with the E544C-L666C double mutant hERG, and the generated current was indeed inhibited by incubation with 0.2 mM tbHO2, while the E544C and L666C single mutants were not (Fig. 5A, Supplemental Fig. 2). Since E544C/L666C can form a disulfide bond within the channel, we tested if this same cysteine pair could also covalently link the E544C mutated S4-S5L (+3) peptide onto the S6T of L666C single mutant hERG channel. Cells were co-transfected with the E544C S4-S5L (+3) peptide and the L666C channel. Two hours of incubation with 0.2 mM tbHO2 almost completely inhibited the current (Fig. 5B, Supplemental Table 3), and this effect was specifically due to the presence of both cysteines (Fig. 5C and D, Supplemental Table 3). In conclusion, covalently binding S4-S5L to S6T inhibited the channel, strongly suggesting that S4-S5L acts as a ligand binding to S6T and locking the channel in a closed state.

hERG inhibition provoked by E544C S4-S5, (+3) peptide covalent binding to L666C mutant channel is reversible. Cells co-transfected with the E544C S4-S5, (+3) peptide and the L666C channel were incubated for 2 hours with tbHO2, followed by 2 hours incubation with 10 mM DTT. This double treatment reversed the effect induced by tbHO2 incubation, as current densities were comparable to the control condition (Fig. 6). To exclude that the current recovery is due to de novo arrival of hERG at the plasma membrane during the 2 hours DTT incubation, we tested two additional conditions: 1) 10 mM DTT was applied for only 10 minutes or 2) we measured the current in the presence of 10 mM DTT in the pipette tip. In both conditions, current densities were similar to control, consistent with DTT application leading to peptide unbinding.

Figure 4. Introduction of 2 cysteines in the S4-S51 and S67 regions of hERG (D540C-L666C hERG) locks the channel closed in oxidative conditions. (A) Representative, superimposed recordings of the WT hERG current (3.6 µg plasmid plus 0.4 µg GFP plasmids) after 2 h incubation in Tyrode without (control) or with 0.2 mM tbHO2 (tbHO2). (B) D540C-L666C hERG channels, same as in A (3.6 µg plasmid plus 0.4 µg GFP plasmids). Cartoons: introduction of a cysteine is symbolized by a star. (C) Mean WT hERG tail-current density at −40 mV after a prepulse at +100 mV. (D) Activation curve obtained from the tail currents, using the protocol shown in (B). (E) Mean D540C-L666C hERG tail-current density at −40 mV after a prepulse at +100 mV, after 2 h incubation in Tyrode without (control) or with 0.2 mM tbHO2 (tbHO2), or after 2 h incubation in 10 mM DTT following tbHO2 incubation (2 h DTT). ***p < 0.001 versus control and ###p < 0.001 versus tbHO2, Mann-Whitney test.
hERG activation by S4-S5L (0) peptide is not reinforced by its covalent binding through a disulfide bond. S4-S5L (0) peptide increased WT hERG current density (Fig. 2A and B), but this effect is due to an increased expression of hERG protein, suggesting no effect of S4-S5L (0) on channel gating. Consistent with this hypothesis, the current generated by COS-7 cells co-transfected with E544C-L666C hERG plasmid plus 0.4 µg GFP plasmids and right, mean tail-current density at −40 mV after a prepulse at +100 mV after 2 h incubation in Tyrode without (control) or with 0.2 mM tbHO₂ (tbHO₂), both using the voltage protocol shown in inset. (B) left, representative, superimposed recordings of the single mutant L666C hERG current in the presence of E544C S4-S5 (-3) peptide (1 µg L666C hERG plus 3 µg peptide plasmids) and right, mean tail-current density measured in the same conditions as A. (C,D) Mean tail-current density in the presence of only one cysteine, i.e. L666C hERG + S4-S5L (+3) peptide (C) or WT hERG + E544C S4-S5L (+3) peptide (D). ***p < 0.001 versus control, Mann-Whitney test.

A cysteine disulfide bond reinforces the effect of the activating S6₇ peptide, rendering the channel almost voltage independent. Since the S6₇ peptide contains the L666 residue, we mutated this residue to a cysteine and tested the effect of this mutated peptide on the D540S single mutant hERG channel.
**Figure 6.** hERG inhibition provoked by E544C S4-S5L (+3) peptide covalent binding to L666C mutant channel is reversible. Mean hERG tail-current density at −40 mV after a prepulse at +100 mV of L666C hERG channels in the presence of E544C S4-S5L (+3) peptide (1 μg L666C hERG plus 3 μg peptide plasmids) after 2 h incubation in Tyrode without (control) or with 0.2 mM tbHO2 (control and tbHO2, respectively, same results as in Fig. 5B), and after 2 h or 10 min incubation in 10 mM DTT following tbHO2 incubation, or after direct application of 10 mM DTT in the pipette tip, following tbHO2 incubation. ***p < 0.001 versus control, **p < 0.01 and ***p < 0.001 versus tbHO2, Mann-Whitney test.

**Figure 7.** Unexpected hERG activation by S4-S5L (0) peptide is not reinforced by its covalent binding though a disulfide bond. (A) Top, representative, superimposed recordings of the L666C hERG current in the presence of E544C S4-S5L (0) peptide (1 μg L666C hERG plus 3 μg peptide plasmids) after 2 h incubation in Tyrode without (control) or with 0.2 mM tbHO2 (tbHO2), using the protocol shown in (B). (B) D540C S4-S5L (0) peptide same as in A.
both in the absence and in the presence of tbHO₂. As shown in Fig. 8C, the D540C channel presents a atypical activation curve, with a double Boltzmann and a voltage-independent component, even at very negative potentials, unlike the WT channel. This suggests that the mutation D540C by itself alters the role of endogenous S4-S5L as an inhibiting ligand. This voltage-independent component is similar to the one induced by some mutations in KCNQ1 that were interpreted as decreasing the interaction between S4-S5L and S6T\(^{11,14}\). Covalent binding of the L666C S6T\((-3)\) peptide to the D540C hERG channel dramatically increased the voltage-independent component (Fig. 8B and D). Such a tbHO₂-dependent increase in the voltage-independent component was not observed in absence of the peptide (Fig. 8A and C). The voltage-independent component is potassium selective (Fig. 8E). These observations further confirm the ligand/receptor mechanism by demonstrating that S4-S5L is necessary to lock the channel gate in a closed state.

Covalent binding of the L666C S6T\((-3)\) peptide to the channel D540C also leads to a 12-mV negative shift in the inactivation curve (Fig. 8G and Supplemental Table 5). In the hERG channel, inactivation is not directly coupled to activation\(^{15,16}\), suggesting that the observed effect of the L666C S6T\((-3)\) peptide on the inactivation curve is not a consequence of a modification of activation. Of note, S6 peptides bind to hERG S4-S5L (Y542-F557) which is surrounded by regions that are involved in inactivation: double mutant cycle analysis suggests that interaction between L529, L532, V535 in S4 and I560, L564, I567 in S5 play a role in inactivation gating\(^{17}\). Thus, covalent binding of S6T to S4-S5L may alter this interaction leading to the observed effect on inactivation.
Discussion

In a previous study on KCNQ1, and in the present study on hERG, we proposed a ligand/receptor model of voltage dependence. In this model, S4-S5L would act as a ligand locking the S6T gate in the closed state. To test this, we used S4-S5L and S6T mimicking peptides. Our results are in favor of this hypothesis. However, the effects were moderate. Here, we used another approach to reinforce the peptide effects on the hERG channel by introducing a cysteine pair covalently linking the mutated peptide to the single mutant channel. We observed that covalently linking the E544C S4-S5L (+3) peptide to the L666C hERG almost completely inhibits the channel (Fig. 5B), by presumably preventing the outward splaying of the S6 helices. Covalently linking the L666C S6T (−3) peptide to D540C hERG renders the channel almost completely voltage independent (Fig. 8D). We postulate that the covalently bound S6T (−3) peptide blocks the normal interaction between endogenous S4-S5L and S6T which stabilizes the closed state, and thus the channel remains locked in the open state. For both peptides, either S4-S5L or S6T, covalent link to the hERG channel clearly reinforces the respective effects of these two peptides when first tested on WT hERG channel without any introduced cysteine (as in Fig. 2). Altogether, these observations further reinforce the ligand/receptor model of voltage dependence. hERG S4-S5L and S6T peptides that produce the most potent effects are aligned with the most potent peptides of KCNQ1, strengthening the notion of a similar model for KCNQ1 and hERG activation gating (Fig. 9).

Cysteine introduction either in S4-S5L or S6T endogenous segments leads to alteration of the single mutant channel activity, as shown in Supplemental Fig. 3. This alteration is indicative of a change in the channel conformation. This observation may give rise to an alternative hypothesis: an artificial S4-S5L/S6T interaction is only due to the cysteine introductions and does not exist in WT conditions. However, (i) S4-S5L (+3) and S6T (−3) peptides altered WT channel current density, suggesting that the ligand/receptor model is relevant for the WT situation even without the disulfide bond, (ii) despite the alteration of the channel voltage dependence due to the cysteines introduction, covalently linking the peptide to its target leads to effects which are consistent with the ligand/receptor model. Indeed, we observed, on one hand, a complete inhibition by E544C S4-S5L (+3) on L666C hERG, and, on the other hand, a loss of voltage dependence by L666C S6T (−3) on D540C hERG. In conclusion, two independent sets of experiments (with or without cysteines) are consistent with the ligand/receptor model. Interestingly, the same combination of cysteine introduction in S4-S5L (0) peptide (E544C) and hERG (L666C), led to a complete loss of the current increase which was demonstrated to be nonspecific to the channel activity change but rather due to the channel expression increase when probed by western blot experiments. Altogether, our results suggest that cysteine introduction in the channel preserved the ligand/receptor mechanism that was originally observed in WT hERG (and also KCNQ1).

In the absence of structural data of Kv channels in the closed state, molecular mechanisms underlying the coupling between voltage-sensor movement and pore opening are still under debate. This coupling interaction has been suggested to be electromechanical and categorized as "attractive" or "repulsive", depending on the force exerted on the gate by the voltage sensors via the S4-S5L and S6T interaction. In the "attractive" coupling, the S4 voltage sensors pull the gate to open it, requiring tight binding between S4-S5L and S6T. In the "repulsive" coupling, the voltage sensors push the gate to constrict it. In the present work on hERG channel, the ligand/receptor model corresponds to a third mechanism in which there is no need of a force directly linking the voltage-sensor and the gate, neither "attractive" nor "repulsive". Rather, S4-S5L seems to act as a ligand that binds S6T to lock the channel in a closed state. When S4 segments are in the "down" state at negative potential, S4-S5L binds to S6T, locking the channel in the closed state. When S4 segments are in the "up" state, they pull S4-S5L out of its binding pocket (S6T), unlocking the channel. This molecular mechanism is consistent with previous observations made on hERG channels and other K+ channels:

![Figure 9](image-url). hERG inhibiting (S4-S5L (+3)) and activating peptides (S6T (−3)) are aligned with KCNQ1 inhibiting and activating peptides. Same as in Fig. 1B, onto which the results obtained with hERG have been added. A check sign (✓) indicates that the S4-S5L peptide inhibits the channel and that the S6T peptide activates the channel.
Ferrer and collaborators showed that introduction of two cysteines, one in S4-S5, and one in S6, in the hERG channel, locks it in a closed state via a disulfide bond. This is consistent with our hypothesis that S4-S5 acts as an endogenous inhibitor, as confirmed by the exogenous peptide approach.

Split hERG channels expressed as two distinct proteins, one containing the voltage sensor domain and one containing the pore, keep the same voltage dependence as the full-length channel. In accordance with our model, where there is no mechanical force transduction from S4 movement to S6 opening, there is no need for physical continuity between the two parts of the channel for activation transduction.

The ligand/receptor model also helps in understanding why some hERG mutants, such as D540K, are open by both membrane depolarization and membrane hyperpolarization. Since specific interactions between S4-S5 and S6, are needed to keep the channel closed, a charge reversal mutation on S4-S5 may destabilize the interaction and lead to channel opening when S4-S5 is pulled out of its S6, binding pocket, either toward the cytosol (hyperpolarization) or toward the membrane (depolarization).

Of note, it has been recently shown that the structure of the K,10.1 channel, which is 65% homologous to hERG, is incompatible with the mechanical lever mechanism. In the open state structure of K,10.1, S4-S5, is a short linker that is not domain swapped and, thus, is not in a position to function as a mechanical lever, leaving the possibility that the K,10.1 follows the same ligand/receptor model as hERG and KCNQ1.

This ligand/receptor model implies that when S4-S5 is absent, the hERG activation gate should be in the open state, independent of the potential, as observed in one potassium and one sodium bacterial channels. In other words, this gate is intrinsically more stable in the open state. Surprisingly, hERG pore domain construct (S3-S6) alone, isolated from the voltage sensor domain, gives rise to a nonfunctional channel. One particularity for hERG, compared to the aforementioned bacterial channels, is that the cytosolic N-terminus deletion greatly accelerates deactivation. Thus, the N-terminus stabilizes the hERG voltage sensor domain, which also deletes the N-terminus, may render hERG nonfunctional.

Of note, this ligand/receptor model is completely in frame with the allosteric model of voltage-dependence suggested for KCNQ1 and hERG, in which voltage-dependent movement of S4 is allosterically coupled to pore-opening transitions. In such a model, the gating of KCNQ1 can be well described by an allosteric model where the channel can open before all S4 voltage sensors have been activated. Our ligand/receptor model suggests that allosteric coupling is realized through the interaction between S4-S5 and S6, (leading to channel closure), which is favored when S4 segments are in the “down” state at resting potential and not favored when S4 segments are in the “up” state.

In conclusion, the results that we obtained in the hERG channel with S4-S5 and S6 mimicking peptides, with or without cysteine introduction, reinforce the ligand/receptor model originally observed in KCNQ1 and suggest that this model applies to other voltage-gated channels. In Shaker channels, sequence complementarity between S4-S5 and S6, has also been shown to be critical to coupling voltage sensor movement and pore opening. Testing peptides mimicking S4-S5 and S6, in Shaker-like channels would confirm whether the ligand/receptor model applies to Shaker-like channels.

**Methods**

**Plasmid constructs.** Oligonucleotides encoding hERG peptides were synthesized by TOP Gene Genes and contained a Xhol restriction enzyme, a methionine (ATG) for translation initiation, and a glycine (GGA) to protect the ribosome binding site during translation and the nascent peptide against proteolytic degradation. A BamHI restriction enzyme site was synthesized at the 3′ end immediately following the translational stop codon (TGA). These oligonucleotides were then cloned into pIRES2-EGFP (Clontech) and sequenced.

Three different S4-S5 and S6, plasmids were designed with the same length (16 amino-acids for S4-S5 and 13 for S6,) but different starting positions in order to compensate for the insecurity of the alignment due to poor homology between hERG and KCNQ1 sequences. Multiple alignment was realized with Clustal Omega. This program aligned the predicted transmembrane domains (UniProt Q12809 for hERG, P51787 for KCNQ1) and the predicted position of the narrower part of the bundle crossing, also named gating residue, of hERG and KCNQ1. We first designed a hERG S4-S5, peptide based on the most potent KCNQ1 S4-S5, (L251-L266). Since mutagenesis studies suggested a close proximity of D540 in S4-S5, and R665 in S6, we chose two peptides shifted by 3 and 6 amino acids to the N-terminus to include the D540 residue. Names of the peptides were given according to their position along the sequence: S4-S5, (−3) is A356-F551, S4-S5, (0) is L539-M554, and S4-S5, (+3) is Y542-F557. For S6, peptides, we first selected the hERG sequence which aligned with the most potent peptide of KCNQ1 (I346-K358). Since in KCNQ1 the effects of the peptides were increased as the sequence was shifted to the C-terminus, we tried two other peptides position-shifted by 3 and 6 amino acids towards the C-terminus. S6, (−3) is G657-G669, S6, (0) is S660-R672, and S6, (+3) is I663-T675.

**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 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 DNA (2 to 4 µg total DNA as described below) complexed with Fugene-6 (Roche Molecular Biochemical) according to the standard protocol recommended by the manufacturer. In the screening experiments used to identify the most potent peptides (Fig. 2), COS-7 cells were co-transfected with 0.6 µg pSI-hERG and 1.4 µg pIRE2-EGFP (Clontech) plasmids either encoding or not encoding a peptide. In the experiments with cysteine introduction, some mutations are associated with a decrease in current amplitude (D540C, E544C) or an increase in current amplitude (L666C). Plasmid quantities...
were thus optimized (i) to maximize the quantity of peptides, as assessed by the amount of florescence, and (ii) to keep the average current amplitude in the same range, in order to avoid undetectable currents or an incorrect voltage clamp. As a result, experiments on channels containing a D540C, E544C or L666C mutation were done with 1 μg mutated pSI-hERG and 3 μg pEGFP. Experiments on double-mutant hERG channels (DS540C/L666C or ES540C/L666C) were performed with 3.6 μg pSI-hERG and 0.4 μg pEGFP. In pIRES2-EGFP plasmids, the second cassette (EGFP) is less expressed than the first cassette, guaranteeing high levels of peptides expression in fluorescent cells. Cells were re-plated onto 35-mm Petri dishes the day after transfection for patch-clamp experiments.

**Western blot.** 20 μg of protein lysate from transfected COS-7 cells were fractionated on one-dimensional polyacrylamide gels and analyzed using western blotting with a goat polyclonal antibody against hERG (Santa Cruz Biotechnology, ref: SC-15968), a rabbit polyclonal antibody against GFP (Invitrogen Molecular Probes, ref: A11122), and a mouse monoclonal antibody against GAPDH (Santa Cruz Biotechnology, ref: SC-32233). Bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-goat (Santa Cruz Biotechnology, ref: SC-2922), goat anti-rabbit (Santa Cruz Biotechnology, ref: SC-2054), and goat anti-mouse (Santa Cruz Biotechnology, ref: SC-2055) secondary antibodies. Stain free gel technology (Bio-Rad) was used as loading control for protein normalization: band intensities were first normalized to the intensity of the corresponding stain free membrane lane, and ratios were then normalized to control hERG condition.

**Electrophysiology.** The 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 temperature was maintained at 22.0 ± 2.0°C. Stimulation and data recording were performed with an Axon pClamp 10 through an A/D converter (Digidata 1440A) using an Axopatch 200B (all Molecular Devices). Patch pipettes (tip resistance: 2–3 MΩ) were pulled from soda lime glass capillaries (Kimble-Chase). Currents were recorded in the whole-cell configuration. hERG activation and inactivation curves were obtained from the tail currents and fitted by Boltzmann equations.

**Solutions.** The cells were continuously superfused with a HEPES-buffered Tyrode solution containing (in mmol/L): 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/L): KCl 100, Kgluconate 45, MgCl2 1, EGTA 5, HEPES 10, pH adjusted to 7.2 with KOH. The membrane-permeable oxidizing agent tert-butylhydroperoxide (tbHO2) and dithiothreitol (DTT) were obtained from Sigma. Incubations with tbHO2 and DTT were realized at room temperature.

**Statistics.** All data are expressed as means ± S.E.M. Statistical differences between samples were determined using Student’s t-tests, rank-sum tests (when data were not normally distributed), and two-way analysis of variance associated with a Bonferroni post-hoc test when needed. In the screening experiment (Fig. 2 and Supplemental Table 1), a value of p < 0.10 was considered significant. In the rest of the study, a value of p < 0.05 was considered significant.

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Author Contributions
G.L. and Z.L. designed the experiments. O.M. realized the experiments under the supervision of G.L. and Z.L. O.M. prepared the figures. G.L. wrote the manuscript.

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