The mutation A341V in the S6 transmembrane segment of KCNQ1, the α-subunit of the slowly activating delayed-rectifier K⁺ (Iₖₛ) channel, predisposes to a severe long-QT1 syndrome with sympathetic-triggered ventricular tachyarrhythmias and sudden cardiac death.

Rationale: The mutation A341V in the S6 transmembrane segment of KCNQ1, the α-subunit of the slowly activating delayed-rectifier K⁺ (Iₖₛ) channel, predisposes to a severe long-QT1 syndrome with sympathetic-triggered ventricular tachyarrhythmias and sudden cardiac death.

Objective: Several genetic risk modifiers have been identified in A341V patients, but the molecular mechanisms underlying the pronounced repolarization phenotype, particularly during β-adrenergic receptor stimulation, remain unclear. We aimed to elucidate these mechanisms and provide new insights into control of cAMP-dependent modulation of Iₖₛ.

Methods and Results: We characterized the effects of A341V on the Iₖₛ macromolecular channel complex in transfected Chinese hamster ovary cells and found a dominant-negative suppression of cAMP-dependent Yotiao-mediated Iₖₛ upregulation on top of a dominant-negative reduction in basal current. Phosphomimetic substitution of the N-terminal position S27 with aspartic acid rescued this loss of upregulation. Western blot analysis showed reduced phosphorylation of KCNQ1 at S27, even for heterozygous A341V, suggesting that phosphorylation defects in some (mutant) KCNQ1 subunits can completely suppress Iₖₛ upregulation. Functional analyses of heterozygous KCNQ1 WT:G589D and heterozygous KCNQ1 WT:S27A, a phosphorylation-inert substitution, also showed such suppression. Immunoprecipitation of Yotiao with KCNQ1-A341V (in the presence of KCNE1) was not different from wild-type.

Conclusions: Our results indicate the involvement of the KCNQ1-S6 region at/or around A341 in cAMP-dependent stimulation of Iₖₛ, a process that is under strong dominant-negative control, suggesting that tetrameric KCNQ1 phosphorylation is required. Specific long-QT1 mutations, including heterozygous A341V, disable this regulation. (Circ Res. 2012;110:211-219.)

Key Words: ion channels | long-QT syndrome | potassium | torsade de pointes
brane segment) was reported in a large South African founder population.11 This hot-spot LQT1 mutation, found globally, results in an unusually severe clinical phenotype compared with other LQT1 mutations, irrespective of their amino acid location (transmembrane versus N/C terminal) or dominant-negative characteristics.12 Brisk variations in autonomic response increase the arhythmic risk in A341V LQT1 patients.13 Also, polymorphisms in \( \alpha \)- and \( \beta \)-adrenergic receptors (enhancing the autonomic response) are found more often in symptomatic than nonsymptomatic \( \text{KCQ}_{-1} \)-A341V carriers.13

In the present study, we investigated the molecular control of \( I_{Ks} \) during \( \beta \)-AR stimulation, hypothesizing that A341V induces a useful case to advance understanding of the \( I_{Ks} \) macromolecular signaling complex, and to obtain novel insights into the determinants of phenotypic severity by A341V and other LQT1 mutations.

**Methods**

An extended overview of the Methods is provided in the Online Data Supplement accompanying this article. A summary of the main aspects is given here.

**Electrophysiological Characterization of Wild-Type and Mutant \( I_{Ks} \) in Chinese Hamster Ovary Cells**

Chinese hamster ovary (CHO) cells were transiently transfected with human \( \text{KCQ}_{-1} \) (WT, mutant or 1:1 WT plus mutant), human \( \text{KCQ}_{1} \), human Yotiao, and GFP. GFP-fluorescent cells were used for whole-cell patch-clamp analysis at room temperature. Cells were studied with standard pipette solution (containing in mmol/L K-aspartate 110, ATP-K_2 5, MgCl_2 1, CaCl_2 1, EGTA 1, HEPES 10; pH = 7.3 with KOH) or with cAMP and okadaic acid (OA) added to the pipette solution to mimic the effect of \( \text{cAMP} \)/OA, after correction for temperature and effects of the macromolecular signaling complex, and to obtain novel insights into the determinants of phenotypic severity by A341V and other LQT1 mutations.

**Computational Analysis of Mutation Effects on APD**

A computational model of the canine epicardial myocyte that includes a detailed localized model of \( \beta \)-AR stimulation14 was used to study the effects of the A341V mutation. Parameters of the nonphosphorylated and phosphorylated populations of \( I_{Ks} \) channels in this model were fitted to the experimental data obtained in the electrophysiological studies in CHO cells in the absence or presence of \( \text{cAMP} \)/OA, after correction for temperature and effects of the expression system. The cell model was used to simulate the effects of the A341V mutation on APD in the absence or presence of \( \beta \)-AR stimulation.

**Commmunoprecipitation of \( \text{KCQ}_{-1} \) and Yotiao**

For communoprecipitation studies, CHO cells were transfected with \( \text{KCQ}_{-1} \)-WT or \( \text{KCQ}_{-1} \)-A341V plus \( \text{KCQ}_{-2} \)-Yotiao. CHO cells were lysed and the \( \text{KCQ}_{-1} \) complex was precipitated with an anti-\( \text{KCQ}_{-1} \) antibody and protein G sepharose beads. Western blot analysis of whole-cell lysates or immunocomplexes was performed with anti-\( \text{KCQ}_{-1} \) and anti-Yotiao antibodies and the relative intensity of Yotiao signal (to \( \text{KCQ}_{-1} \) signal) was compared for WT and A341V samples.

**Analysis of \( \text{KCQ}_{-1} \) Phosphorylation**

For Western blot experiments, phosphorylation of \( \text{KCQ}_{-1} \) was induced by incubating the CHO cells (\( \text{KCQ}_{-1} \)-WT + \( \text{KCQ}_{-2} \)-Yotiao, \( \text{KCQ}_{-1} \)-A341V + \( \text{KCQ}_{-2} \)-Yotiao, or nontransfected CHO cells as negative controls) at \( 37 \)°C with 300 \( \mu \)mol/L CPT-cAMP plus 0.2 \( \mu \)mol/L OA or with control solution for 10 minutes. Western blot analysis was performed and membranes were probed with anti-\( \text{KCQ}_{-1} \)-phospho-S27 and anti-\( \text{KCQ}_{-1} \) antibodies. The \( \text{KCQ}_{-1} \)-monomer band of approximately 70 kDa was quantified in both S27 phospho and total \( \text{KCQ}_{-1} \) blots to calculate the fraction of phosphorylated \( \text{KCQ}_{-1} \).

**Colocalization Experiments**

CHO cells were grown on coverslips and transfected with \( \text{KCQ}_{-1} \)-myc, \( \text{KCQ}_{-1} \)-A341V-GFP, \( \text{KCQ}_{-2} \), and Yotiao cDNAs. Stainings were made with mouse monoclonal anti–c-Myc–conjugated and Texas red–conjugated goat anti-mouse antibodies. The antibody-induced patching technique was used for immunostaining. Coverslips were mounted in 75% glycerol, 0.02 mol/L TRIS-HCl (pH = 8.0), 0.8% NaN_3, 2% 1,4-diazabicyclo[2.2.2]octane (DABCO; pH = 8.0) and cells analyzed under a confocal microscope 24 hours after transfection.

**Data Analysis**

Voltage dependence of activation (evaluated from normalized tail-current amplitudes) was fitted with a Boltzmann equation: \( \text{I}_{\text{Ks}} = \frac{1}{1 + \text{exp}(V - V_{1/2})/k} \) to determine the membrane potential for half-maximal activation \( (V_{1/2}) \) and the slope factor \( (k) \). Time courses of activation and deactivation were fitted with single exponential functions. Data are expressed as mean±SEM. Unpaired Student \( t \) test or 1-way ANOVA with Tukey post hoc test were used to assess differences between groups. \( P<0.05 \) was considered statistically significant.

**Results**

\( \text{KCQ}_{-1} \)-A341V Results in a Dominant-Negative Suppression of \( \text{cAMP} \)-Dependent Upregulation of \( I_{Ks} \) on Top of a Dominant-Negative Reduction in Basal Current

The \( \text{KCQ}_{-1} \)-A341V residue is located in the middle of the S6-transmembrane domain, just before the PAG “hinge” motif15 and is extremely well conserved among the families of voltage-gated K⁺ channels (Online Figure I).

We found that, consistent with previous results,11 the \( \text{KCQ}_{-1} \)-A341V mutation resulted in a significant reduction of \( I_{Ks} \). The \( I_{Ks} \)-tail amplitude after 5-second depolarizing pulses at +60 mV was reduced by 96% and 46% in homozygous (\( \text{A341V}_{\text{HOM}} \)) and heterozygous (\( \text{A341V}_{\text{HET}} \)) conditions, respectively (Figure 1A and 1B). A significant rightward shift in channel activation was also observed (Figure 1C and 1D). In addition, there was a significant slowing of activation kinetics (time constants at +60 mV
were 1306±156 ms, 1947±265 ms, and 7303±1486 ms in WT, A341VHet and A341VHom (respectively), as well as faster deactivation (time constants at −80 mV: 501±61 ms, 205±14 ms, and 144±24 ms; P<0.05 versus WT for both A341VHet and A341VHom). Together, these altered kinetics contributed to the dominant-negative reduction in repolarizing current for depolarizations of physiological duration in A341VHet (74% reduction in peak IKs after 300-ms depolarizing pulses, not shown). No differences were found in the number of cells showing membrane expression or the fluorescence intensity at the membrane between WT and A341V (Online Figure II), indicating that the observed reduction in IKs is unlikely to be due to reduced expression. We also found no differences in the effect of WT and A341VHom on IK1 current, which has been reported for other trafficking-deficient KCNQ1 mutations (Online Figure III).

To study the effect of simulated βAR (as part of sympathetic) stimulation on WT and A341V IKs channels, transfected CHO cells were stimulated with cAMP/OA in the pipette solution. WT IKs was significantly upregulated in response to stimulation with cAMP/OA (Figure 1A, left panel; 63% increase in tail-current amplitude). In contrast, both A341VHet and A341VHom channels showed a negligible increase in current amplitude (Figure 1A and 1B). The leftward shift in I-V relationship that occurred during cAMP-dependent stimulation for WT IKs channels was also absent (Figure 1C and 1D). This cAMP desensitivity, even in A341VHet, suggested that IKs modulation is under dominant-negative control, whereby presence of one defect subunit may be sufficient to abolish IKs upregulation.

**Effect of Combined Dominant-Negative A341V Effects on Ventricular Repolarization**

Patients carrying the A341V mutation can show pronounced QTc prolongation (see Figure 2A; adapted from Shimizu16), and QTc is longer among symptomatic than among asymptomatic carriers.11 Moreover, QTc duration can prolong during exercise, despite an increase in heart rate (Figure 2A, right panel16). We examined the effect of A341V on ventricular repolarization using a computational model of βAR stimulation in the canine ventricular myocyte (Online Figure IV, V).14 The A341V mutation caused a major reduction in IKs amplitude during the action potential, even in heterozygous conditions (Figure 2B, bottom panels). Under basal conditions, IKs plays a minor role in ventricular repolarization, and, consistently, loss of IKs by A341V resulted only in minimal APD prolongation (Figure 2B, top left; ΔAPD=2.6 ms). In contrast, during βAR stimulation APD was significantly prolonged in A341VHet conditions during βAR stimulation compared with WT.
Mechanisms Underlying the Suppressed cAMP Responsiveness of A341V $I_{Ks}$

The A341V mutation could disrupt the βAR modulation of $I_{Ks}$ through various mechanisms, including (1) disruption of the conformational changes occurring after phosphorylation of KCNQ1-S27; (2) disruption of KCNQ1 interaction with Yotiao thereby reducing local PKA availability; or (3) disruption of phosphorylation of S27, even in the presence of βAR stimulation, in line with the ECG changes observed in A341V carriers.

Half-maximal activation potential, similar to that observed after the application of cAMP/OA (Figure 3C). In contrast to cAMP/OA, the S27D substitution resulted in a significant upregulation of A341V in both heterozygous and homozygous conditions (Figure 3A and 3B middle and right panels), as well as a significant leftward shift in half-maximal activation potential (Figure 3C). These data refuted hypothesized mechanism 2 to explain the disrupted cAMP responsiveness of A341V.

Yotiao serves a dual function: not only is it required for phosphorylation of S27 by PKA, it also actively participates in the conformational changes of the channel complex after phosphorylation of S27. We investigated the interaction between KCNQ1 and Yotiao through communoprecipitation experiments in CHO cells expressing KCNQ1-WT + KCNE1 + Yotiao (WT) or KCNQ1-A341V + KCNE1 + Yotiao (A341V). Bottom blots are probed with anti-Yotiao and anti-KCNQ1 antibodies after precipitation of the immunocomplex from lysates, using an anti-KCNQ1 antibody in the same conditions. The A341V mutation could disrupt the KCNQ1-Yotiao interaction in WT and A341VHomo. Intensity of Yotiao signal from immunocomplexes was corrected for KCNQ1 signal intensity in all conditions and results were normalized to KCNQ1-WT + KCNE1 + Yotiao. Figure 4. KCNQ1/Yotiao interaction remains intact in the presence of the A341V mutation. A, Representative Western blots of Yotiao and KCNQ1 in lysates (top) of nontransfected CHO cells, CHO cells transfected with KCNQ1-WT + KCNE1 + Yotiao (WT) or KCNQ1-A341V + KCNE1 + Yotiao (A341V). Bottom blots are probed with anti Yotiao and anti-KCNQ1 antibodies after precipitation of the immunocomplex from lysates, using an anti-KCNQ1 antibody in the same conditions. B, Quantification of KCNQ1/Yotiao interaction in WT and A341VHomo. Intensity of Yotiao signal from immunocomplexes was corrected for KCNQ1 signal intensity in all conditions and results were normalized to KCNQ1-WT + KCNE1 + Yotiao. C, I-V relationship for A341VHomo currents in the absence of cAMP/OA, presence of cAMP/OA, presence of the S27D phosphomimetic substitution, or S27D without cotransfection of Yotiao (empty vector). Voltage-clamp protocol is identical to Figure 1. Increase in tail-current amplitude by the S27D substitution occurs only in the presence of Yotiao.

Figure 3. The phosphomimetic substitution S27D “rescues” cAMP-dependent upregulation of the A341V mutation. A, Averaged current traces of WT (left), A341VHomo (middle), or A341VHomo(right) channels cotransfected with KCNE1 and Yotiao in the absence or presence of stimulation with intrapipette cAMP/OA, or in the presence of the phosphomimetic substitution S27D. Voltage-clamp protocol as in Figure 1. B, Tail I-V relationship for the conditions in A. Note different y-scales (indicated with arrows). C, Membrane potential resulting in half-maximal activation. cAMP responsiveness of WT IKs is characterized by a significant leftward shift in half-maximal activation (*P<0.05) that is not present in A341VHomo or A341VHeterozygous. In contrast, the S27D substitution results in significant shifts for all conditions.
were incubated with either 300 nM/Yotiao or 100 nM/KCNE1 WT KCNE1. Combined, our data and those by Chen et al. suggest that cAMP-dependent upregulation of heterozygous IKs, both for A341V Hom and A341V Het, is responsible, at least partly, for the loss of IKs phosphorylation at S27 in the presence of A341V.

Quantification of fractions of WT and heterozygous cotransfection of WT and A341V. The lower two blots show similar results for WT and heterozygous cotransfection of WT and A341V. A c-Myc epitope was inserted in the extracellular loop between transmembrane segments 1 and 2 of KCNQ1-WT and the KCNQ1-A341V was tagged with GFP at the C-terminus. Top left panel shows a confocal image of KCNQ1-WT obtained with an anti-c-Myc antibody in a representative CHO cell. Top right panel shows GFP signal in the same cell. When the 2 signals are overlaid (bottom left panel), a clear colocalization of WT and A341V KCNQ1 subunits can be observed at the cell membrane (yellow), confirming “heterozygous” channel expression. The bottom right panel shows the relative signal-intensity profiles of KCNQ1-WT and KCNQ1-A341V along the cross sections at the light green line. Both signals show increased activity at the cell boundaries. White scale bar indicates 10 μm.

Dominant-Negative Suppression of cAMP/PKA-Dependent $I_{Ks}$ Upregulation in LQT1 Syndrome

The reduction in KCNQ1-A341V phosphorylation is similar to that observed by Chen et al. for the Yotiao mutation S1570L. They also show a complete loss of $I_{Ks}$ enhancement (in this case due to reduced KCNQ1-Yotiao interaction). Combined, our data and those by Chen et al. suggest that cAMP-dependent $I_{Ks}$ upregulation is under strong dominant-negative control of KCNQ1-S27 phosphorylation, which is further supported by the functional finding of a striking loss of cAMP-dependent upregulation of heterozygous KCNQ1-A341V.

To further investigate the disruption of upregulation in heterozygous conditions, we first confirmed that KCNQ1-WT and KCNQ1-A341V are both expressed in the membrane by immunolocalization. Coexpression of KCNQ1-WT–c-Myc and KCNQ1-A341V-GFP constructs allowed simultaneous detection of WT (using an anti-c-Myc antibody) and A341V subunits. Figure 6 shows individual signals of KCNQ1-WT (top left panel) and KCNQ1-A341V (top right panel). The overlay of these signals (Figure 6, bottom left panel) indicates strong colocalization (yellow color) of WT and A341V subunits in the cell membrane. This is confirmed by the cross-section profile of both signal intensities (Figure 6, bottom right panel), which shows enhanced intensity at the membrane. Correlation between c-Myc and GFP signal intensity profiles was 0.81 ± 0.01 in 50 cells of 4 transfections.

We next compared the loss of cAMP-dependent upregulation by KCNQ1-A341V with the LQT1 mutations KCNQ1-A344V,19 KCNQ1-G589D,20 and KCNQ1-K557E. Suppression of $I_{Ks}$ upregulation has previously been reported for the KCNQ1-G589D (KCNQ1-Fin) mutation, which disrupts the binding of Yotiao to the KCNQ1 C-terminus.5 We have recently reported on the LQT1 mutation K557E found in a Dutch family with a relatively mild clinical phenotype in which cAMP-dependent $I_{Ks}$ enhancement is intact.21 Responsiveness to cAMP was quantified by an increased tail-current...
amplitude over the entire population of cells studied (Figure 7A) as well as an increase in current amplitude for each individual cell during a 5-minute cAMP/OA wash-in protocol (Figure 7B and 7C). WT, heterozygous and homozygous K557E, and heterozygous and homozygous A344V all showed a pronounced responsiveness to cAMP. In contrast, A341V and G589D IKs were unresponsive to stimulation, even when coexpressed with WT IKs (Figure 7). These results demonstrate that the loss of upregulation observed by A341V does not apply to the very similar mutation A344V (same amino acid substitution, only 3 residues apart and, based on our heterozygous G589D data).

To determine if the dominant-negative suppression of cAMP-dependent upregulation by A341V and G589D could be due to their common effects on KCNQ1-S27, and to investigate if alterations in KCNQ1-S27 alone can exert dominant-negative control of cAMP/PKA-dependent IKs upregulation, independent of other known pathogenic mutations, we analyzed a 1:1 coexpression of WT KCNQ1 with KCNQ1-S27A (together with KCNE1 and Yotiao). This heterozygous S27A condition will disable the PKA phosphorylation site in approximately half of the KCNQ1 subunits. In contrast to the pronounced increase observed with cAMP/OA treatment in WT IKs, there was no significant increase in IKs-tail amplitude in heterozygous KCNQ1-S27A cells stimulated with cAMP/OA (Figure 8). Although there was still a small (but nonsignificant) increase in IKs, in heterozygous S27A, this increase was also present in cells with homozygous S27A expression after treatment with cAMP/OA (19% increase in tail current after 5-second depolarizing pulses to +90 mV in both cases, not shown), and no significant differences were found between heterozygous S27A in the absence or presence of cAMP (Figure 8).

**Discussion**

In the present study, we provide novel mechanistic insights into cAMP-dependent upregulation of IKs and its alteration by the S6 transmembrane LQT1 mutation A341V. We show that A341V confers a dominant-negative suppression of current upregulation. This loss of cAMP sensitivity is not due to a restriction of the conformational changes that occur after S27 phosphorylation but instead is due to the inability of PKA to phosphorylate KCNQ1-S27 despite the presence of Yotiao. The combined dominant-negative reduction in basal current and loss of cAMP sensitivity result in a pronounced prolongation of repolarization during βAR stimulation in a computational model. Moreover, we show that dominant-negative suppression of upregulation is shared by other LQT1 mutations that show loss of βAR modulation in homozygous conditions (eg, KCNQ1-G589D) and by heterozygous substitutions at S27. Our results suggest that tetrameric phosphorylation of KCNQ1 is required. This finding has important implications for the clinical severity of KCNQ1 mutations similar to A341V, because genetically heterozygous carriers will exhibit a “homozygous phenotype” with respect to βAR modulation. The present data stress the importance of investigating molecular signaling besides basal function of IKs in the human congenital LQT syndrome.

**Requirement of a Macromolecular Signaling Complex for IKs Modulation**

Specific macromolecular signaling is required for the modulation of voltage-gated ion channels. Modulation of IKs by...
PKA requires a complex comprising KCNE1, Yotiao, and β-tubulin, besides the pore-forming α-subunit KCNQ1. In pathological conditions, the composition of the macromolecular complex is even further complicated by the fact that most LQT1 mutations (including A341V) are inherited in an autosomal-dominant mode. In these conditions, variable mixtures of normal and mutant KCNQ1 subunits interact in the membrane-expressed $I_{Ks}$ channels.

KCNQ1 and β-tubulin appear to operate downstream of channel phosphorylation in mediating the conformational changes that increase $I_{Ks}$, because no difference in KCNQ1 phosphorylation was found after disruption of KCNE1 or microtubular interaction, despite altered $I_{Ks}$ regulation. Interactions between KCNQ1 and KCNE1 have been reported for the extracellular, transmembrane, and intracellular parts of the subunits. Panaghi et al have shown that KCNE1 interacts with the KCNQ1 pore region through residues F339 and F340. On the basis of a homology model of the structure of KCNQ1, Smith et al determined that A341V does not face the lipid bilayer but instead interacts with S6-residues of other subunits (Online Figure I). This makes it unlikely that A341 interacts directly with KCNE1. However, the A341V mutation may induce conformational changes in the KCNQ1-S6 segment that indirectly alter KCNQ1/KCNE1 interactions. Our S27D data show a clear upregulation in the presence of A341V, indicating that the interaction between KCNQ1 and KCNE1 is preserved, at least partly. In agreement, a recent study showed that KCNQ1-A341V in the absence of KCNE1 or in the presence of the mutant KCNE1-T58A results in nonfunctional channels. However, the exact role of KCNE1 in influencing A341V-mutated $I_{Ks}$ and its upregulation requires further study.

Our S27D data also indicate that the Yotiao contribution downstream of phosphorylation is intact. Previously, Kurokawa et al showed that S27D is unable to upregulate WT $I_{Ks}$ when Yotiao is not included. We confirm these results for A341V-mutant $I_{Ks}$.

Analysis of KCNQ1 with a phospho-sensitive antibody indicated that despite the presence of Yotiao, phosphorylation of KCNQ1 is reduced in the presence of A341V. The exact molecular mechanism by which this occurs is currently unclear but may involve a reduced functional interaction between the KCNQ1 C-terminal–coupled Yotiao and the N-terminal phosphorylation site S27. Interestingly, however, the A341V mutation is located in the S6 transmembrane spanning domain of KCNQ1, a region that has not previously been reported to play a role in cAMP-dependent modulation of $I_{Ks}$. However, the intact upregulation in the presence of the very similar mutation A344V suggests that the role of S6 in cAMP-dependent $I_{Ks}$ upregulation involves specific residues. The fact that A341 is an extremely conserved residue located just prior to the S6 “hinge domain” (Online Figure I) indicates that even small perturbations of the amino-acid sequence at this position may cause pronounced alterations of channel structure.

Marx et al have shown that the KCNQ1-G589D mutation in homozygous conditions results in loss of cAMP-dependent $I_{Ks}$ upregulation due to disruption of the KCNQ1-Yotiao interaction. We report that this abolishment of upregulation occurs also in heterozygous conditions. This illustrates that at least two KCNQ1 mutations (G589D and A341V), in different parts of the channel, show such dominant-negative suppression.

Marx et al and Yang et al have demonstrated that the substitution KCNQ1-S27A disables the N-terminal phosphorylation site and reduces cAMP-dependent $I_{Ks}$ upregulation. Yang et al also observed a contribution of the “atypical” PKA sites S468/T470, whereas Marx et al demonstrate a complete loss of cAMP-dependent $I_{Ks}$ upregulation in the presence of S27A, consistent with our data. These differences may be related to the absence of Yotiao cotransfection in the experiments of Yang et al, but further studies are clearly required to explore this. We found that the phosphomimetic substitutions KCNQ1-S468D/T470D were unable to upregulate $I_{Ks}$ (not shown), further indicating that S27 is the main phosphorylation site involved in cAMP-dependent $I_{Ks}$ upregulation. Importantly, we show here that the lack of cAMP-dependent $I_{Ks}$ upregulation was also observed on heterozygous KCNQ1-S27A substitution (a condition in which half of the N-terminal phosphorylation sites are disabled). In heterozygous conditions, a binomial distribution of subunit composition is expected in which 9.75% of the channels will have at least 1 mutation in the KCNQ1 tetramer. The complete lack of upregulation in this case indicates that PKA-dependent channel upregulation is under dominant-negative control by alterations at S27 in which a single defective subunit is sufficient to abolish upregulation and suggests a requirement for tetrameric phosphorylation or a strong cooperation between the four subunits in cAMP-dependent channel modulation.

Mechanisms Underlying Phenotypic Severity of A341V and Role of Genetic Risk Modifiers

Schwartz et al found that increased autonomic sensitivity, for example, resulting from polymorphisms in adrenergic receptors, was an arrhythmia-risk modifier in A341V mutations. This observation is in line with our computational simulations that indicate the most pronounced APD prolongation during high levels of βAR stimulation. The data presented in the present report readily explain why genetic variants of adrenergic receptors that boost sympathetic responsiveness of the heart are risk modifiers in A341V carriers due to the combined loss of basal $I_{Ks}$ and its cAMP-dependent upregulation.

Both A341V and G589D show complete loss of cAMP-dependent upregulation in heterozygous conditions but have different clinical severities. Although there are important differences in peak basal currents at physiological potentials for 300-ms depolarizations (1.03 ± 0.27 pA/pF versus 3.07 ± 0.30 pA/pF at 20 mV for A341Vhet and G589Dhet, respectively; $P<0.05$), which may contribute to their phenotypic differences, it is clear that ionic mechanisms within $I_{Ks}$ itself do not provide the complete picture of repolarization instability in vivo. Variants in NOS1AP have been shown to affect QT duration in the normal population and have been reported as risk modifiers in A341V carriers. Chang et al have shown that NOS1AP overexpression affects repolarization through $I_{Kr}$ and $I_{CaL}$. Previously, we have shown that the
effect of reduced Ikᵣ is larger when repolarization is impaired by pharmacological blockade of Ikᵣ. Thus, NOS1AP variants probably function next to KCNQ1 mutations, affecting among other things Ikᵣ. The important role of Ikᵣ as a repolarization reserve³,²² may then partly explain their role as risk modifiers in A341V carriers.³³

Conclusions

Loss of cAMP-dependent upregulation of Ikᵣ, even under heterozygous conditions, on top of a dominant-negative reduction in basal current, is a biophysical characteristic of KCNQ1-A341V that may contribute to its phenotypic severity in patients. This loss of upregulation is related to reduced phosphorylation of KCNQ1 at S27. Moreover, we show in general that cAMP/PKA-dependent Ikᵣ upregulation is under strong dominant-negative control of KCNQ1 phosphorylation at S27. These data have important implications for future studies of LQT1 mutations because they highlight the importance of studying the effects of heterozygous expression of mutations, similar to their occurrence in most patients, and they indicate that baseline I-V characteristics alone are insufficient to determine the electrophysiological consequences of LQT1 mutations. Finally, our data point to an as-yet unknown involvement of the S6 region of KCNQ1 at and/or around A341 in mediating βAR modulation of Ikᵣ.

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Disclosures

None.

References


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Novelty and Significance

What Is Known?

- The slowly activating delayed-rectifier K⁺ current (I_Ks) plays a major role in ventricular repolarization, particularly during conditions of increased sympathetic tone when I_Ks is upregulated through cAMP-dependent signaling.
- Loss-of-function mutations in the pore-forming KCNQ1 subunit cause long-QT syndrome type 1 (LQT1), predisposing mutation carriers to ventricular tachyarrhythmias, particularly during exercise or arousal.
- The hot-spot mutation A341V in the S6 transmembrane segment of KCNQ1 (KCNQ1-A341V) results in an unusually severe clinical phenotype with adrenergic receptor polymorphisms as independent arrhythmia risk modifiers.

What New Information Does This Article Contribute?

- KCNQ1-A341V causes a dominant-negative suppression of cAMP-dependent I_Ks upregulation on top of a dominant-negative reduction in baseline current when the I_Ks macromolecular complex is expressed in Chinese hamster ovary cells.
- Loss of cAMP-dependent I_Ks upregulation is due to loss of phosphorylation at Serine 27 (S27) but not due to reduced KCNQ1/Yotiao interaction or hindrance of current upregulation after alterations at S27.
- Heterozygous expression of the C-terminal mutation KCNQ1-G589D (“Fin mutation”), as well as heterozygous expression of the phospho-inhibitory substitution KCNQ1-S27A (in the absence of other pathogenic mutations), also results in loss of cAMP-dependent I_Ks upregulation, indicating that cAMP/PKA-dependent I_Ks upregulation is under strong dominant-negative control of KCNQ1 phosphorylation at S27.
- The mutation KCNQ1-A341V results in an unusually severe clinical LQT1 phenotype. Although several arrhythmia risk modifiers, including adrenergic receptor polymorphisms and NOS1AP, have been identified, the mechanisms underlying the clinical severity of A341V remain incompletely understood. We show that A341V (but not the LQT1 mutation A344V) results in a loss of cAMP-dependent I_Ks upregulation due to impaired S27 phosphorylation, even in heterozygous conditions. This is the first time that a mutation in the transmembrane segment S6 is identified to be involved in cAMP-dependent I_Ks regulation and the first time that loss of upregulation is found in heterozygous conditions. Loss of upregulation on top of a dominant-negative reduction in baseline current may contribute to the severity of A341V. Previously, the C-terminal mutation G589D has been shown to result in loss of cAMP-dependent upregulation in homozygous conditions. We show that G589D also results in loss of upregulation in heterozygous conditions. Using heterozygous expression of S27A, we show that reduced S27 phosphorylation (without of other pathogenic mutations) results in a dominant-negative suppression of I_Ks upregulation. These data have important implications because a heterozygous genotype can result in a homozygous phenotype with respect to cAMP-dependent I_Ks upregulation in certain LQT1 patients.
Dominant-Negative Control of cAMP-Dependent \( I_{\text{KS}} \) Upregulation in Human Long-QT Syndrome Type 1


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Supplemental Material

Dominant-Negative Control of cAMP-Dependent $I_{Ks}$ Upregulation in Human Long-QT Syndrome Type 1

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1. Extended Methods

1.1 Molecular Biology

Wild-type (WT) human \( KCNQ1 \) (also known as KvLQT1 or Kv7.1) in a pIREs2-GFP vector and human \( KCNE1 \) (minK, IsK) in a pBK-CMV vector, as well as human \( KCNH2 \) were kindly provided by Prof. Dirk J. Snyders, University of Antwerp, Antwerp, Belgium. Yotiao was subcloned in a pGW1 vector (a gift from Prof. Robert S. Kass, Columbia University, New York, NY, USA). KCNH2 was subcloned in a pcDNA3 vector.

The \( KCNQ1 \) mutations A341V, A344V, K557E and G589D were generated by site-directed mutagenesis using the QuickChange® II XL system (Stratagene, La Jolla, CA, USA). Oligonucleotide design and reaction conditions (extension time of 2 minutes/kb) were according to the manufacturer’s instructions (Stratagene). All constructs were completely sequenced (BigDye® Terminator Ready kit v1.1, Applied Biosystems) and analyzed on an ABI PRISM® 3100 Genetic Analyzer. Plasmid DNA for mammalian expression was grown in DH5α cells (Invitrogen Co, Carlsbad, CA, USA) and then isolated from the bacterial cells using a miniprep spin kit (Qiagen). Similar procedures were used to combine the phosphomimetic substitutions S27D and S27A in WT \( KCNQ1 \) or A341V-\( KCNQ1 \).

The \( KCNQ1 \)-myc construct was generated by inserting the c-myc epitope (N-EQKLISEEDL-C) in the S1-S2 external loop of \( KCNQ1 \) after nucleotide position 438 (between glutamate 146 and glutamine 147). Two initial PCR reactions were performed to generate c-myc containing \( KCNQ1 \) products (PCR 1 and 2 in Online Table I). Once c-myc-epitope-containing \( KCNQ1 \) complementary cDNA strands were synthesized, the conditions were set for further PCR amplification: 95 °C for 2 min, 1 min at the corresponding annealing temperature (see Table 1), and 70 °C for 1 and 2 min, respectively. These settings were applied for 25 cycles. The amplified PCR products were subsequently hybridized using a fusion PCR (PCR 3 in Online Table I). After fusion-PCR product purification, \( KCNQ1 \)-myc construct was subcloned into PGEMT-easy cloning vector (Promega Corp. Madison, WI, USA) using Spe I and BamH1 restriction sites for ligation and was sequenced as described above. For expression in Chinese Hamster Ovary (CHO-K1)
cells (CHO cells), the KCNQ1-myc construct was subcloned into a pBK-CMV vector. In all PCR reactions, KOD Hot Start DNA polymerase Kit (Novagen, EMD Biosciences, La Jolla, CA, USA) was used according to the manufacturer’s instructions. Restriction enzymes were from Fermentas (Fermentas GmbH, Germany).

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primer</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR 1</td>
<td>hKCNQ1_mycF</td>
<td>F: 5’- GAGCAGAAGCTGATCTCAGAGGAGGA</td>
<td>534</td>
<td>57 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCGCCCTGGCC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR 2</td>
<td>hKCNQ1_mycR</td>
<td>R: 5’- CAGGTCCTCTCTGAGATCGCTTCT</td>
<td>1852</td>
<td>67 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTCCCTCTCGATGGGTGACAGCCACGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR3</td>
<td>hKCNQ1 Nterm_Fsp1</td>
<td>F: 5’-actaagtAACCGTCAGATCCGCTAG-3’</td>
<td>2386</td>
<td>72 °C</td>
</tr>
<tr>
<td></td>
<td>hKCNQ1_CtermR_BamH1</td>
<td>R: 5’- gatccCCAGACAGCATGGTCCAT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Online Table I. PCR settings for the generation of KCNQ1-myc construct. All PCRs were performed in the KCNQ1 gene with accession number NM_000218.

1.2 Cell Culture and Transfection for Electrophysiology Experiments

CHO cells were cultured at 37 °C/5% CO₂ in Ham’s F-12 medium (Gibco, Invitrogen, Co.) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. For patch-clamp recordings in WT or homozygous mutant experiments KCNQ1 (0.5 µg; WT or mutant of interest) and KCNE1 (1 µg) cDNA were transiently co-transfected in CHO cells using Fugene 6 (Roche Diagnostics Nederland B.V., Almere, The Netherlands). In coexpression studies (examining heterozygous conditions) 0.25 µg WT KCNQ1 and 0.25 µg mutant KCNQ1 were co-transfected with 1.0 µg KCNE1. In addition, Yotiao (2 µg) was co-transfected in all patch-clamp experiments in which WT or mutant Iₖs was studied, unless specified otherwise. In experiments assessing the α-α-subunit interactions between KCNQ1 and KCNH2, 0.5 µg WT or A341V KCNQ1 was co-transfected with 0.5 µg KCNH2. Cells were used for analysis 24 hrs after transfection.

1.3 Electrophysiology

For electrophysiological studies, CHO cells were harvested from the 35-mm culture dish by brief trypsinization, washed twice with culture medium (Ham’s F-12 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin) and placed in a perfusion chamber on an inverted
microscope that was continuously perfused with external Tyrode solution containing (in mmol/L): NaCl 132, KCl 4.8, MgCl₂ 1.2, CaCl₂ 2, HEPES 10, and glucose 5 (pH = 7.4 with NaOH). Pipettes were filled with pipette solution containing (in mmol/L): K-aspartate 110, ATP-K₂ 5, MgCl₂ 1, CaCl₂ 1, EGTA 11, HEPES 10 (pH = 7.3 with KOH). In some experiments, 0.2 μmol/L okadaic acid (OA; EMD Biosciences, La Jolla, CA, USA) and 200 μmol/L cAMP (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands), indicated as +cAMP/OA throughout, were added to the pipette solution to activate PKA and thereby mimic stimulation of β-adrenergic receptors. In KCNQ1 (WT or A341V) / KCNH2 co-transfections HMR1556 (500 nmol/L in DMSO; a kind gift of Dr. H. Gögelein, Sanofi-Aventis, Frankfurt am Main, Germany) was used to selectively inhibit the current carried by KCNQ1. Experiments were performed by the whole-cell patch-clamp technique at room temperature (23±1 °C) using an Axopatch 200B amplifier and pCLAMP software (Axon Instruments, Union City, CA, USA). Patch pipettes were pulled using a DMZ-Universal-Puller (Zeitz-Instruments Vertriebs GmbH, Martinsried, Germany), using 2 mm borosilicate glass capillaries (World Precision Instruments Germany GmbH, Berlin, Germany). Pipette resistance ranged between 1.5 and 3 MΩ. Access resistance was accepted when below 8 MΩ and was electronically compensated at 60-80% series resistance. Currents were sampled at 2 kHz after low-pass filtering at 1 kHz through a Digidata 1322A data acquisition system (Axon Instruments). Current densities (pA/pF) were obtained after normalization to cell capacitance determined with Clampex 9.

For WT and mutant Iks studies, the following voltage-clamp protocols were used: (i) a cAMP/OA-wash-in protocol started after membrane rupture and correction for cell capacitance and consisted of 20 pulses (given at 15-second intervals for a total of 5 minutes) to +60 mV for 2 seconds (from a holding potential of -70 mV) followed by 2 seconds at a holding potential of -40 mV to measure tail currents during wash in of cAMP and OA; (ii) an activation protocol consisting of a 5-s depolarizing pulse (between -50 mV and +90 mV, 10 mV increments) to determine peak currents and activation kinetics followed by 6 s at -40 mV to measure tail currents, and (iii) a deactivation protocol of 4 s at +60 mV followed by repolarization to a potential between -120 mV and -30 mV (in steps of 10 mV). For KCNH2/HERG studies, 1-s depolarizing pulses from -50 mV to +50 mV were used, followed by a 2-s step to -50 mV (Online Figure III).
1.4 Phosphorylation Assays, Protein Extracts, Immunoprecipitation and Western Blots

Phosphorylation of KCNQ1 channels in CHO cells was induced by incubating cells at 37 °C under 5% CO₂ atmosphere with 300 μmol/L CPT-cAMP (Biaffin GmbH & Co, Kassel, Germany) plus 0.2 μmol/L OA (EMD Biosciences, La Jolla, CA, USA), or with vehicle solution, diluted in F12 medium without serum and without antibiotics during 10 min. For total protein extraction, cells were washed twice in chilled phosphate-buffered saline (PBS) and centrifuged at 3,000 × g for 10 min to separate cells from PBS. The pellet was then lysed on ice with HES buffer (20 mmol/L HEPES pH 7.4, 1 mmol/L EDTA, 255 mmol/L sucrose), supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics Nederland B.V., Almere, The Netherlands) and lysates were homogenized by repeated passing (10 times) through a 25G (0.45 x 16 mm) needle.

Total protein extracts (20 μg) were resuspended in SDS 4× (40% glycerol, 5% β-mercaptoethanol, 8% SDS, 0.04% bromophenol blue, 240 mmol/L Tris/Cl pH 6.8) and heated at 55 °C during 5 min. Samples were then centrifuged during 5 min at 13,000 rpm at room temperature and 20 μl of the protein extract was loaded and run in SDS-PAGE (6% acrylamide/bisacrylamide) with Mini-Protean Tetra-Cell (Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane (Amersham, GE Healthcare, Fairfield, CT, USA) using Mini Trans-Blot cell (Bio-Rad).

For co-immunoprecipitation studies, CHO cells were transfected with KCNQ1-WT or KCNQ1-A341V (in pBK vector) + KCNE1 + Yotiao. CHO cells were lysed in 150 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 5 mmol/L EDTA and 1% Triton X-100 supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Cell extracts were incubated for 30 min at 4 °C under agitation. The cell lysates were centrifuged for 15 min at 13,000 rpm and supernatants were separated. Protein G-sepharose beads (40 μl; Santa Cruz Biotech., CA, USA) were washed 3 times with 500 μl IPB buffer (150 mmol/L NaCl, 1% NP40, 10 mmol/L HEPES, 10% glycerol; pH 7.8), centrifuged for 1 min at 5000 rpm and subsequently incubated with 2 μg of anti-KCNQ1 antibody (Santa Cruz Biotech. CA, USA) under rotation for 2 h at 4 °C. After incubation, the beads were centrifuged (5000 rpm for 1 min), washed 3 times with 500 μl IPB and incubated overnight with 300 μg of protein extract under orbital shaking. After centrifugation (5000 rpm for 1 min) and washing with 500 μl IPB at 4 °C (3 times), the beads were resuspended in 30 μl SDS 1x, denaturated at 55 °C for 5 min and centrifuged at 5000 rpm for
5 min. 20 μl of the immunocomplex was then loaded and run in SDS-PAGE (7% acrylamide/bisacrylamide) with Mini-Protean Tetra-Cell (Bio-Rad, Hercules, CA, USA) and transferred to a PVDF membrane (Amersham, GE Healthcare, Fairfield, CT, USA) using Mini Trans-Blot cell (Bio-Rad).

Mouse monoclonal anti-KCNQ1 (1:1000, Neuromab, UCDavis, Ca, USA) and rabbit polyclonal anti-KCNQ1-S27-phospho (1:500, kindly provided by Prof. Robert S. Kass, University of Columbia, NY, USA) were used to test KCNQ1-channel phosphorylation at Serine 27. Anti-KCNQ1 (Neuromab) and anti-Yotiao (Santa Cruz Biotech, CA, USA) were used for co-immunoprecipitation studies. Secondary antibodies were horseradish peroxidase linked rabbit anti-mouse (1:2500; DAKO, Glostrup, Denmark) or goat anti-rabbit (1:2500; Santa Cruz Biotech., CA, USA). Proteins were detected using ECL-Plus Western blotting reagent (Amersham, GE Healthcare, Fairfield, CT, USA). The Yotiao band at 230 kD and the KCNQ1-monomer band of approximately 70 kD were quantified using Image J (http://rsb.info.nih.gov/ij; developed by Dr. Wayne Rasband, National Institutes of Health, Bethesda, MD), in both S27-phospho and total KCNQ1 blots and the fraction of Yotiao interaction or phosphorylated KCNQ1 was quantified.

1.5 Confocal Imaging
CHO cells seeded in sterilized coverslips were cultured in Ham’s F12 medium containing 10% FBS and 1% penicillin/streptomycin antibiotics until they reached 60% confluency. Cells were then transiently transfected with KCNQ1-GFP, KCNQ1-A341V-GFP or, KCNQ1-myc + A341V-GFP + KCNE1 + Yotiao cDNAs using Lipofectamine LLTX and PLUS Reagent (Invitrogen Co.). 48 Hours after transfection, cells were incubated in vivo, under 5% CO₂ atmosphere and at 37 °C during 1 hour with 1:500 mouse monoclonal anti-myc antibody (Sigma-Aldrich). Cells were washed 3 times with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Coverslips were further incubated with Texas Red conjugated goat anti-mouse antibody (1:80, ITK Southern Biotech, Uithoorn, The Netherlands) during 45 min. Stainings with a secondary antibody were performed at room temperature and cells were washed 3 times in PBS after all of the steps. No permeabilization was applied in the immunostainings. Finally, coverslips were mounted in 75% glycerol, 0.02 mol/L TRIS-HCl (pH = 8.0), 0.8% NaN₃, 2% 1,4-diazabicyclo[2.2.2]octane (DABCO; pH = 8.0).
Confocal analysis was performed at least 24 hours after the stainings, and the immunostained samples were visualized using a Leica TCS-SPE (Leica Microsystems GmbH, Wetzlar, Germany) confocal microscope and further analyzed by Image J. Transfection efficiency was determined by counting the fraction of cells that showed both GFP signal and DAPI staining. Determination of cells showing membrane GFP signal (Online Figure II) was performed independently by multiple investigators who were blinded with respect to transfection type (WT versus A341V). For cells showing clear membrane expression, fluorescence intensity at the membrane was subsequently determined at 3 separate membrane sites per cell for identical settings of the microscope using ImageJ.

1.6 Data Analysis
The voltage dependence of activation (evaluated from normalized tail-current amplitudes) was fitted with a Boltzmann equation, \( \frac{G}{G_{\text{max}}} \) (or \( \frac{I}{I_{\text{max}}} \)) = \( \frac{1}{1 + \exp((V-V_{1/2})/k)} \) to determine the membrane potential for half-maximal activation (\( V_{1/2} \)) and the slope factor (\( k \)). The time courses of activation and deactivation were fitted with a single exponential function \( \frac{I}{I_{\text{max}}} = A \times (1 - \exp(-t/\tau)) + C \) where A is amplitude and \( \tau \) is time constant.

1.7 Computational Modeling
Parameters of the 17-state \( I_{\text{Ks}} \) Markov model proposed by Silva and Rudy\(^1\) (Online Figure IVA.b, below) were fitted to the normalized I-V relationship, time constants of activation and time constants of deactivation obtained in CHO cells expressing WT or mutant \( I_{\text{Ks}} \) channels in the absence or presence of cAMP/OA (Online Figure V). Quantitative values of time constants of activation and deactivation of WT unstimulated \( I_{\text{Ks}} \) were scaled to those obtained in canine ventricular myocytes\(^2,3\) at 37 °C (at +20 mV and -80 mV, respectively) to compensate for differences due to temperature and effects of the expression system. We determined one correction factor for the activation time-constant and one for deactivation for WT \( I_{\text{Ks}} \) in our CHO cells at room temperature at these holding potentials to obtain similar half-maximal activation and deactivation times. All WT time constants were then scaled by these values, to maintain the voltage dependence of activation and deactivation measured in CHO cells. The adjusted time constants were used as input in the parameter fitting procedure. The time constants in the presence of adrenergic stimulation and/or A341V mutation were subsequently scaled to produce a
similar relative difference as observed in the heterologous expression systems (e.g., the time constant of activation at +20 mV is 2.2 fold slower in A341V compared to WT). Similarly, baseline current amplitude of WT unstimulated $I_{Ks}$ was fit to that in canine epicardial myocytes. The approximately 60% increase in current amplitude observed with cAMP/OA stimulation in CHO cells was scaled to resemble the approximately 200% increase in $I_{Ks}$ observed in ventricular myocytes during β-adrenergic stimulation and the relative difference in tail current amplitude between WT and A341V observed in CHO cells was preserved. $I_{Ks}$ model parameters were as follows:

**WT $I_{Ks}$ parameters:**

\[
\alpha_{Ks}^{NP} = \frac{8.7313 \cdot 10^{-3}}{1 + \exp\left(\frac{-V_m - 3.3032 \cdot 10^1}{1.1946} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\beta_{Ks}^{NP} = \frac{1.1068 \cdot 10^{-2}}{1 + \exp\left(\frac{V_m - 1.9519 \cdot 10^{-2}}{1.4447} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\gamma_{Ks}^{NP} = \frac{1.5226 \cdot 10^{-1}}{1 + \exp\left(\frac{-V_m + 5.3866 \cdot 10^{-2}}{5.3191 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\delta_{Ks}^{NP} = 1.0372 \cdot 10^{-2} \cdot \exp\left(4.7872 \cdot 10^{-3} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\eta_{Ks}^{NP} = 1.5447 \cdot 10^{-2} + \frac{6.9977 \cdot 10^{-2} - 1.5447 \cdot 10^{-2}}{1 + \exp\left(\frac{V_m + 1.0318 \cdot 10^2}{5.2369 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\theta_{Ks}^{NP} = 1.7695 \cdot 10^{-3}
\]

\[
\omega_{Ks}^{NP} = 5.6517 \cdot 10^{-4} \cdot \exp\left(-1.5643 \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\psi_{Ks}^{NP} = 2.8148 \cdot 10^{-5} \cdot \exp\left(1.9762 \cdot 10^{-4} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\alpha_{Ks}^{P} = \frac{6.9994 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m - 5.6717 \cdot 10^{-1}}{5.7717 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]
\[
\beta_{Ks}^P = \frac{1.3731 \cdot 10^{-2}}{1 + \exp\left(\frac{V_m - 3.8608 \cdot 10^{-3}}{1.7486} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\gamma_{Ks}^P = \frac{1.6490 \cdot 10^{-1}}{1 + \exp\left(-\frac{V_m - 2.0329 \cdot 10^{-2}}{48136 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\delta_{Ks}^P = 4.7241 \cdot 10^{-3} \cdot \exp(1.6283 \cdot 10^{-2} \cdot \frac{V_m \cdot F}{R \cdot T})
\]

\[
\eta_{Ks}^P = 9.6896 \cdot 10^{-3} + \frac{1.4242 \cdot 10^{-1} - 9.6896 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m + 1.1338 \cdot 10^{-2}}{5.5965 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\theta_{Ks}^P = 3.2528 \cdot 10^{-3}
\]

\[
\omega_{Ks}^P = 2.3813 \cdot 10^{-3} \cdot \exp(-1.9602 \cdot \frac{V_m \cdot F}{R \cdot T})
\]

\[
\psi_{Ks}^P = 4.4670 \cdot 10^{-5} \cdot \exp(3.9068 \cdot 10^{-8} \cdot \frac{V_m \cdot F}{R \cdot T})
\]

**A341V het \(K_s\) parameters:**

\[
\alpha_{Ks}^{NP} = \frac{7.8749 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m - 5.1876 \cdot 10^1}{1.0212} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\beta_{Ks}^{NP} = \frac{4.5532 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m - 1.4922 \cdot 10^{-2}}{4.2919} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\gamma_{Ks}^{NP} = \frac{1.0993 \cdot 10^{-1}}{1 + \exp\left(-\frac{V_m + 1.1599 \cdot 10^{-3}}{6.2763 \cdot 10^{-1}} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\delta_{Ks}^{NP} = 2.6854 \cdot 10^{-2} \cdot \exp(2.3346 \cdot 10^{-3} \cdot \frac{V_m \cdot F}{R \cdot T})
\]

\[
\eta_{Ks}^{NP} = 9.0527 \cdot 10^{-3} + \frac{6.5743 \cdot 10^{-2} - 9.0527 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m + 3.0409 \cdot 10^1}{0.7658} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\theta_{Ks}^{NP} = 1.2691 \cdot 10^{-3}
\]
\[ \omega_{Ks}^{NP} = 5.6517 \cdot 10^{-4} \cdot \exp(-1.5643 \cdot \frac{V_m \cdot F}{R \cdot T}) \]

\[ \psi_{Ks}^{NP} = 2.8148 \cdot 10^{-5} \cdot \exp(1.9762 \cdot 10^{-4} \cdot \frac{V_m \cdot F}{R \cdot T}) \]

\[ \alpha_{Ks}^{P} = \frac{1.1355 \cdot 10^{-2}}{1 + \exp(-\frac{V_m - 6.8892 \cdot 10^1}{1.4039} \cdot \frac{F}{R \cdot T})} \]

\[ \beta_{Ks}^{P} = \frac{3.8524 \cdot 10^{-3}}{1 + \exp(-\frac{V_m - 1.0399 \cdot 10^{-2}}{3.3478} \cdot \frac{F}{R \cdot T})} \]

\[ \gamma_{Ks}^{P} = \frac{1.2805 \cdot 10^{-1}}{1 + \exp(-\frac{V_m + 1.1741 \cdot 10^{-3}}{0.4837} \cdot \frac{F}{R \cdot T})} \]

\[ \delta_{Ks}^{P} = 2.1670 \cdot 10^{-2} \cdot \exp(2.4782 \cdot 10^{-3} \cdot \frac{V_m \cdot F}{R \cdot T}) \]

\[ \eta_{Ks}^{P} = 5.8191 \cdot 10^{-3} + \frac{4.4396 \cdot 10^{-2} - 5.8191 \cdot 10^{-3}}{1 + \exp(-\frac{V_m + 4.2618 \cdot 10^1}{1.1263} \cdot \frac{F}{R \cdot T})} \]

\[ \theta_{Ks}^{P} = 6.2849 \cdot 10^{-4} \]

\[ \omega_{Ks}^{P} = 5.6517 \cdot 10^{-4} \cdot \exp(-1.5643 \cdot \frac{V_m \cdot F}{R \cdot T}) \]

\[ \psi_{Ks}^{P} = 2.8148 \cdot 10^{-5} \cdot \exp(1.9762 \cdot 10^{-4} \cdot \frac{V_m \cdot F}{R \cdot T}) \]

**A341V**

\[ \alpha_{Ks}^{NP} = \frac{6.4929 \cdot 10^{-3}}{1 + \exp(-\frac{V_m - 8.4272 \cdot 10^1}{1.1093} \cdot \frac{F}{R \cdot T})} \]

\[ \beta_{Ks}^{NP} = \frac{2.6793 \cdot 10^{-3}}{1 + \exp(-\frac{V_m - 1.4346 \cdot 10^{-2}}{3.1158} \cdot \frac{F}{R \cdot T})} \]
\[
\gamma_{Ks}^{NP} = \frac{6.6132 \cdot 10^{-2}}{1 + \exp\left(-\frac{V_m + 1.5649 \cdot 10^{-3}}{0.2105} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\delta_{Ks}^{NP} = 3.0593 \cdot 10^{-2} \cdot \exp\left(3.8659 \cdot 10^{-3} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\eta_{Ks}^{NP} = 7.7273 \cdot 10^{-3} + \frac{8.7107 \cdot 10^{-2} - 7.7273 \cdot 10^{-3}}{1 + \exp\left(\frac{V_m + 1.3602 \cdot 10^1}{0.8158} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\Theta_{Ks}^{NP} = 3.3856 \cdot 10^{-4}
\]

\[
\omega_{Ks}^{NP} = 5.6517 \cdot 10^{-4} \cdot \exp\left(-1.5643 \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\Psi_{Ks}^{NP} = 2.8148 \cdot 10^{-5} \cdot \exp\left(1.9762 \cdot 10^{-4} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\alpha_{Ks}^P = \frac{7.2469 \cdot 10^{-3}}{1 + \exp\left(-\frac{V_m - 8.0876 \cdot 10^1}{0.9005} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\beta_{Ks}^P = \frac{3.3092 \cdot 10^{-3}}{1 + \exp\left(V_m - 1.4431 \cdot 10^{-2} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\gamma_{Ks}^P = \frac{6.6278 \cdot 10^{-2}}{1 + \exp\left(-\frac{V_m + 1.4175 \cdot 10^{-3}}{0.1937} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\delta_{Ks}^P = 3.0039 \cdot 10^{-2} \cdot \exp\left(3.6233 \cdot 10^{-3} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\eta_{Ks}^P = 7.7647 \cdot 10^{-3} + \frac{7.7303 \cdot 10^{-2} - 7.7647 \cdot 10^{-3}}{1 + \exp\left(\frac{V_m + 1.3807 \cdot 10^1}{0.9041} \cdot \frac{F}{R \cdot T}\right)}
\]

\[
\Theta_{Ks}^P = 3.4785 \cdot 10^{-4}
\]

\[
\omega_{Ks}^P = 5.6517 \cdot 10^{-4} \cdot \exp\left(-1.5643 \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]

\[
\Psi_{Ks}^P = 2.8148 \cdot 10^{-5} \cdot \exp\left(1.9762 \cdot 10^{-4} \cdot \frac{V_m \cdot F}{R \cdot T}\right)
\]
And $\tilde{g}_{K_s} = 0.6603 \cdot \left(1 + \frac{0.6}{1 + \left(3.8 \cdot 10^{-5} \frac{[Ca^{2+}]_{i}}{[Ca^{2+}]_{j}}\right)}\right)$ for all conditions.

These adjustments were necessary to apply the results obtained in the heterologous expression system in the model. The phosphorylated and non-phosphorylated $I_{K_s}$ models were incorporated into a recently developed model of the canine epicardial myocyte incorporating detailed localized effects of β-adrenergic stimulation (Online Figure IVA.a). This model uses a population-based approach to distinguish between phosphorylated and non-phosphorylated channels. The net current is the sum of the currents produced by the channels in each population. The WT $I_{K_s}$ profile during the cardiac action potential in the presence or absence of β-adrenergic stimulation was similar to that of the original model, based entirely on canine data (not shown).

The complete model consists of a coupled system of ordinary differential equations. Numerical integration was performed in custom C++ software using a forward Euler method with adaptive time step. The Matlab (The Mathworks®, Natick, MA) ODE15s steady-state solution and C++ forward Euler method produced identical action potentials and calcium transients.

The model was paced for 2000 seconds at any given setting (cycle length and isoproterenol concentration) with a conservative $K^+$ stimulus to achieve a pseudo steady-state in which action-potential duration (determined at 95% of repolarization) and maximum intracellular ion concentrations change less than 0.05% on a beat-to-beat basis. Isoproterenol levels ranged between 0 µmol/L (no β-adrenergic stimulation) and 1.0 µmol/L (maximum stimulation). Simulations were performed using WT and mutant $I_{K_s}$ formulation in the absence and presence of adrenergic stimulation.
2. Supplemental Figures

A.

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**Online Figure I.** Location and conservation of A341 in KCNQ1. **A,** Multiple sequence alignment of the KCNQ1-S6 region with other members of the Kv7.x family and other voltage-gated potassium channels. Conserved residues are bold and highlighted in blue, partially conserved residues are highlighted in green and red. The conserved K⁺ selectivity filter and the PAG (in Kv7.x) or PXP (in other Kv channels) hinge motifs are indicated below the alignment. A341 is indicated in red and is extremely conserved among all Kv channels. **B,** Location of A341 in the closed-state tetrameric KCNQ1 homology model described by Kang et al. Left panel shows top view from the extracellular side. Residues F340, A341 and A344 are shown as ‘ball-and-stick’ models. Black arrow indicates the viewing direction for the right panel. Right panel shows front view of two KCNQ1-subunits (only S5, pore and S6 region are shown). Residues F340 – A344 are shown. Distances between residue A341 of the right subunit and its two nearest residues are indicated with dashed lines.
Online Figure II. Membrane expression of KCNQ1-WT and KCNQ1-A341V. A, Confocal image of a representative CHO cell expressing KCNQ1-WT-GFP (left panel) or KCNQ1-A341V-GFP (right panel). The white scale bar at the bottom of the right panel indicates 10 μm. Bottom panels show relative fluorescence intensity along the cross sections indicated with white lines. Both signals show increased intensities at the cell boundaries, indicating membrane expression. B, Average data from confocal images of KCNQ1-WT or KCNQ1-A341V cells for transfection efficiency (left panel, determined as percentage of cells showing GFP signal compared to the number showing DAPI staining), percentage of cells with clear membrane signal (middle panel), and fluorescence intensity at the membrane for cells with clear membrane signal. Numbers in bars indicate the number of cells analyzed. There is no difference in transfection efficiency, the number of cells showing a clear membrane signal, or the fluorescence intensity at the membrane between WT and A341V.
Online Figure III. A341V phenotype is not mediated by effects on KCNH2. A, Averaged current traces during depolarization to +60 mV in CHO cells transfected with KCNH2 alone (solid black line), KCNH2 + KCNQ1-WT (dashed grey line) or KCNH2 + KCNQ1-A341V (solid grey line) before application of HMR1556 (left) or after application of HMR1556 (right; giving the HMR-resistant KCNH2 current). Inset on the right shows voltage-clamp protocol used. B, Tail I-V relationships before (left) or after (right) application of HMR1556 for the conditions in panel (A). KCNH2-only current is indicated with triangles, KCNH2+KCNQ1-WT with circles and KCNH2+KCNQ1-A341V with squares. Cotransfection of KCNQ1-WT increased the KCNH2 tail current, consistent with previous results. A similar increase in KCNH2 could be observed with KCNQ1-A341V.
Online Figure IV. Computational modeling approach. **A.a**, Schematic overview of the structure of a single cell model, including all ion channels, pumps, exchangers, Ca\(^{2+}\)-handling proteins, Ca\(^{2+}\)/Calmodulin-dependent kinase and compartmental βAR signaling pathway, as published recently.\(^5\) **A.b**, Structure of the 17-state Markov model that was fitted to WT I\(_Ks\), heterozygous or homozygous mutant channel characteristics in the absence and presence of βAR stimulation.
Online Figure V. Validation of $I_{ks}$ model properties. A, Relative $I_{ks}$-tail amplitude in WT (black/white), heterozygous A341V (blue) and homozygous A341V (red) conditions in the absence (left) and presence (right) of cAMP stimulation in experiments (symbols, identical to Figure 1 in the manuscript) and model (lines). B, Same as panel A for time constants of activation based on a mono-exponential fit. C, Same as panel A for time constants of deactivation. Time constants were scaled to match values found in canine ventricular myocytes at 37 °C (Section 1.7 of extended methods). Insets show voltage-clamp protocols used.
3. References


