Calmodulin Is Essential for Cardiac $I_{KS}$ Channel Gating and Assembly
Impaired Function in Long-QT Mutations

Liora Shamgar, Lijuan Ma, Nicole Schmitt, Yoni Haitin, Asher Peretz, Reuven Wiener, Joel Hirsch, Olaf Pongs, and Bernard Attali

Abstract—The slow $I_{KS}$ K$^+$ channel plays a major role in repolarizing the cardiac action potential and consists of the assembly of KCNQ1 and KCNE1 subunits. Mutations in either KCNQ1 or KCNE1 genes produce the long-QT syndrome, a life-threatening ventricular arrhythmia. Here, we show that long-QT mutations located in the KCNQ1 C terminus impair calmodulin (CaM) binding, which affects both channel gating and assembly. The mutations produce a voltage-dependent macroscopic inactivation and dramatically alter channel assembly. KCNE1 forms a ternary complex with wild-type KCNQ1 and Ca$^{2+}$-CaM that prevents inactivation, facilitates channel assembly, and mediates a Ca$^{2+}$-sensitive increase of $I_{KS}$ current, with a considerable Ca$^{2+}$-dependent left-shift of the voltage-dependence of activation. Coexpression of KCNQ1 or $I_{KS}$ channels with a Ca$^{2+}$-insensitive CaM mutant markedly suppresses the currents and produces a right shift in the voltage-dependence of channel activation. KCNE1 association to KCNQ1 long-QT mutants significantly improves mutant channel expression and prevents macroscopic inactivation. However, the marked right shift in channel activation and the subsequent decrease in current amplitude cannot restore normal levels of $I_{KS}$ channel activity. Our data indicate that in healthy individuals, CaM binding to KCNQ1 is essential for correct channel folding and assembly and for conferring Ca$^{2+}$-sensitive $I_{KS}$-current stimulation, which increases the cardiac repolarization reserve and hence prevents the risk of ventricular arrhythmias. (Circ Res. 2006;98:0-0.)

Key Words: KCNQ $|$ potassium channels $|$ Kv7 $|$ calmodulin $|$ KCNE $|$ long QT

$K$CNQ channels represent a family of voltage-gated K$^+$ channels (Kv7) that plays a major role in brain and cardiac excitability.1,2 Mutations of human KCNQ genes lead to severe cardiovascular and neurological disorders such as the cardiac long-QT syndrome (LQT) and neonatal epilepsy. Coassembly of KCNQ1 with KCNE1 β subunits produces the $I_{KS}$ current that is crucial for repolarization of the cardiac action potential.3–5

The cytoplasmic KCNQ C-termini were shown to feature 4 α helices.6 We previously identified the last α helix of the C terminus (helix D, aa.589–620) as a region important for the tetrameric assembly of KCNQ1 α subunits.7 This region also binds Yotiao, an A-kinase–anchoring protein that targets PKA on the $I_{KS}$ channel complex.8 The first 2 α helices of KCNQ1–5 form a calmodulin-binding domain (CBD), including an IQ motif that mediates apoCaM binding.6,9 Although KCNQ channels bind calmodulin (CaM), the role of CaM in channel function remains controversial. Recent studies found a role for CaM as a Ca$^{2+}$-sensor of KCNQ2/4/5 channels,10,11 whereas others suggested a role in channel assembly.9 So far, no information has been available about the interaction of calmodulin with cardiac $I_{KS}$ channels and its pathophysiological impact to KCNQ1-related LQT channelopathies. Here, we show that LQT mutations located near the IQ motif of KCNQ1 C terminus impair CaM binding, alter channel assembly, stabilize inactivation, and decrease current density. In healthy individuals, CaM binding to KCNQ1 is necessary for proper channel assembly and for conferring Ca$^{2+}$-sensitive $I_{KS}$-current stimulation, which is essential for maintaining the repolarization reserve that prevents excessive action potential prolongation.

Materials and Methods
The molecular interactions between CaM and the various KCNQ1 channel constructs (wild-type [WT] and LQT mutants) were investigated using complementary methods, including the yeast 2-hybrid screen, CaM-agarose pulldown assays, bacterial expression systems, in vitro cotranslations, and coimmunoprecipitations techniques. The steady-state expression levels of the channel proteins were assessed by cell surface biotinylation experiments. The functional analysis of channel activities was studied using the whole-cell patch-clamp technique in transfected Chinese hamster ovarian (CHO) cells and

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the whole-cell 2-electrode and inside-out macro-patches in microinjected Xenopus oocytes. The reagents, experimental protocols, and data analyses are described in detail in the online-only data supplement available at http://circres.ahajournals.org.

Results

The KCNQ1 C Terminus Interacts With CaM in the Presence and Absence of Ca2+

Figure 1A shows that KCNQ1 and CaM interact, as demonstrated by immunoprecipitation from lysates of CHO cells stably expressing KCNQ1. To delineate the CaM interacting region in KCNQ1 C terminus, we used the yeast 2-hybrid system (Figure 1B). Deletion of helix A (CT390–676 and CT390–620) showed a weak yeast growth, which indicates that as for KCNQ2–5,6,9,11 this domain is important for CaM interaction. Deletion of the proximal C terminus up to helix A (CT361–676 and CT370–676) led to normal yeast growth. Similarly, CaM interaction was normal when truncating both extremities of the C terminus (CT354–620 and CT370–620). Unexpectedly, deletion of the C-terminal end (CT354–589), including helix D, which corresponds to the putative assembly domain, did not yield yeast growth (Figure 1B). Myc-tagged KCNQ1 C-terminal constructs and CaM were either translated separately or cotranslated. Immunoprecipitation showed that CaM bound directly to the KCNQ1 C-terminus both in the absence and presence of Ca2+ (Figure 1C and 1D).

In line with the yeast 2-hybrid data, binding of CaM was abolished when helix D was truncated (CT-589, Figure 1C). CaM binding possibly requires an oligomerized KCNQ1 C terminus, as previously observed for CaM binding to SK-channels.12

LQT Mutations in KCNQ1 C terminus Impair CaM Binding and Stabilize Inactivation

Various LQT mutations, eg, R366W, R366P, A371T, S373P, and W392R,13–15 as well as a mild polymorphism K393N,16 are located near the KCNQ1 IQ motif (Figure 2). A yeast 2-hybrid assay indicated that A371T and S373P mutants do not interact with CaM, as no yeast cells grew on the selection medium (Figure 2A). R366W and W392R mutants exhibited a weak CaM interaction, whereas the mutant K393N mildly affected yeast growth. The weak interaction obtained with the R366P mutant was not significant in pulldown assays (see below).

We then used CaM-agarose pulldown assays and examined the profile of CaM interaction with WT KCNQ1 and LQT mutants in the presence and absence of Ca2+ in human embryonic kidney (HEK) 293 cells transfected with full length myc-tagged channels. As impaired CaM binding to LQT mutants drastically affected channel assembly (see Figure 6), we incubated CaM-agarose beads with comparable amounts of channel proteins expressed in cell lysates to
estimate CaM binding (Figure 2B, row c). Anti-myc antibodies specifically labeled KCNQ1 channels at a molecular weight of ~75 kDa. Results showed that in the presence of Ca\(^{2+}\), the LQT mutants S373P, W392R, and A371T (not shown) exhibit a much weaker CaM binding compared with WT (27% and 12% of WT, respectively; Figure 2B, row a, and 2C). The mutants K393N, R366W, and R366P bound less CaM, though at levels not significantly different from WT. In the absence of Ca\(^{2+}\), 5- to 10-fold less CaM bound to WT KCNQ1 (Figure 2B, row b, and 2C). Although K393N and R366P mutants bound apoCaM with the same strength as WT, significantly less apoCaM was bound to the mutants R366W, S373P, and W392R (74%, 19%, and 11% of WT, respectively). These results were confirmed by CaM-agarose pulldown and immunoprecipitation from in vitro translated KCNQ1 C-termini (supplemental Figure I). Immunoprecipitation of in vitro cotranslated CaM and myc-tagged KCNQ1 C-terminal constructs confirmed the lack of interaction of apoCaM with the mutant W392R (Figure 2D).

Next, WT KCNQ1 and LQT mutants were expressed in CHO cells because of their low K\(^+\) conductance background compared with HEK 293 cells. Except for K393N, the LQT mutants produced small current densities with a dramatic change in inactivation (Figure 3A through 3G). Compared with WT, the mutants exhibited a voltage-dependent macroscopic inactivation (Figure 3A through 3F and 3I) and produced a significant right shift in the voltage dependence of channel activation, with up to a +44 mV shift for mutant S373P (Figure 3H and supplemental Table II). Similar results were obtained in transfected HEK 293 cells and in Xenopus oocytes (data not shown).

**Impact of KCNE1 on CaM Binding and Inactivation Produced by LQT Mutants**

As native \(I_K\) channels result from the coassembly of KCNQ1 and KCNE1 subunits, we examined the pattern of CaM interaction with WT KCNQ1 and LQT mutants cotransfected with KCNE1 in HEK 293 cells. CaM-agarose pulldown assays showed that in the presence of Ca\(^{2+}\), the mutants W392R, S373P, and, to a lower extent, R366W exhibit a significantly weaker CaM binding than WT \(I_K\) (15%, 43%, and 58% of WT, respectively) (Figure 4A, row a, and 4C). In contrast, mutants K393N and R366P bound CaM in amounts comparable to those bound with WT. It is noteworthy that in the presence of Ca\(^{2+}\), CaM-agarose beads could also pull-down...
down KCNE1, which exists in a ternary complex with KCNQ1 and CaM (Figure 4B). Significantly less apoCaM was bound to the mutants R366W, S373P, and W392R (32%, 13%, and 3% of WT, respectively) compared with WT, K393N, and R366P (Figure 4A, row b, and 4C).

Next, we examined the biophysical properties of each mutant cotransfected with KCNE1 in CHO cells. Except mutant R366P, all mutant channels coexpressed with KCNE1 generated noninactivating currents with typical slow $I_{KS}$ activation kinetics (Figure 5). However, most of the LQT mutants produced significant lower current densities compared with WT. They also showed a significant right shift of their activation curve (eg, $V_{50}$-2.222.2 mV and $V_{50}$-21.6 mV for WT and S373P, respectively; n=6 to 16) (Figure 5G and supplemental Table II). Coexpression of R366W with KCNE1 caused a significant rescue of current density, reaching up to 55% of WT $I_{KS}$ (Figure 5H).

Impact of CaM in Channel Assembly

The marked decrease in current density observed for most mutants suggested to us that the LQT mutations affecting CaM binding might also alter KCNQ1 subunit folding and/or assembly. We tested whether we could isolate a stable complex between CaM and the C terminus of KCNQ1 by use of a bacterial expression system. We first examined whether pET-21 bacterial expression of His-tagged KCNQ1 C terminus (352–622) yielded a soluble protein. Bacterial cell lysates were prepared and centrifuged, and resulting supernatants and pellets were analyzed by SDS-PAGE followed by immunoblotting with anti-His antibodies (Figure 6A). Very little if any of the cell extract was soluble, and the protein was aggregated into inclusion bodies in the pellet fraction. By contrast, coexpression of KCNQ1 C terminus (352–622) with CaM in pET-Duet produced soluble, nonaggregated material. Conversely, coexpression of CaM with KCNQ1 C terminus deleted from CBD (helix A and B) did not express a soluble protein and the material was found in aggregates (Figure 6A). The data suggest a critical role of CaM for folding the KCNQ1 C terminus.

Next, we monitored steady-state levels of WT and mutant KCNQ1 proteins expressed at the cell surface. Cell surface proteins were biotinylated using the membrane impermeable reagent sulfo-NHS-LC-biotin. Biotinylated proteins were
As control, cell lysates were probed with either anti-Gβ and probed with anti-c-myc (a) and anti-KCNE1 antibodies (b). Proteins were resolved by 10% and 15% SDS-PAGE, blotted, surface biotinylation of intracellular Gβ (Figure 6B, lysates). Blots of total cell lysates were probed pulled down by streptavidin-agarose beads and channel proteins were detected by Western blotting with anti-myc antibodies. Equal amounts of cell lysates were also run to detect CaM binding to LQT mutants coexpressed with KCNE1 (Figure 6B). KCNE1 considerably increased both cell surface expression (3.9±0.7-fold, n=3, P<0.01) and total steady-state cellular content (3.9±2.0-pfold, n=3, P<0.01) of WT KCNQ1. This observation suggested that KCNE1 significantly improves WT IKS channel expression or assembly. Similar data were obtained for the LQT mutants. The presence of KCNE1 markedly increased cell surface expression of R366W, S373P, and W392R mutants (Figure 6B; n=3). A similar effect of KCNE1 was observed for cell surface expression of R366P and K393N mutants representing 89±21%, 132±22%, respectively, of WT IKS channels (n=3, P<0.01). Total cellular contents of K393N, R366P, R366W, S373P, and W392R were 92±27%, 64±27%, 28±21%, 22±21%, and 19±21%, respectively, of WT IKS channels (Figure 6B; n=3), reflecting the improvement of channel expression by KCNE1.

We asked whether the cellular deficit in LQT mutant expression arose from channel misfolding/misassembly and subsequent degradation along the ubiquitin-proteasome pathway. When proteins fail to fold or assemble properly, this pathway could play a major role in their ER-associated degradation. Results shown in the Data Supplement suggest that LQT mutants are targets of proteasomal degradation (supplemental Figure II).

To obtain additional evidence for a role of CaM in KCNQ1 channel assembly, we transfected HEK 293 cells with WT KCNQ1 and the defective CaM-binding mutant S373P with or without CaM overexpression and performed a biotinylation assay. Transfection with CaM increased the levels of CaM by ~2- to 3-fold over endogenous CaM, as detected with anti-CaM antibodies (Figure 6C, third row). CaM overexpression produced a striking increase in cell surface expression of WT KCNQ1 and S373P by more than 5-fold and 100-fold, respectively (Figure 6C, first row). A comparably drastic stimulation of total cellular channel content was observed in cell lysates, with a 2.8±22.0-fold increase for WT KCNQ1 and S373P, respectively (Figure 6C, second row; n=4, P<0.01).

**Figure 4.** Impact of KCNE1 on CaM interaction with WT KCNQ1 and LQT mutants. A, KCNE1 and full-length myc-tagged channels were coexpressed in HEK293 cells. Comparable amounts of WT and mutant lysate proteins were incubated with CaM-agarose in the presence of either 1 mmol/L Ca2+ (a) or 5 mmol/L EGTA (b). Proteins were resolved by 8% SDS-PAGE, blotted, and probed with anti-myc antibodies. B, Ternary complex of KCNQ1, KCNE1, and CaM. Myc-tagged WT KCNQ1 was expressed either alone or with KCNE1. Lysates were incubated with CaM-agarose in the presence of 1 mmol/L Ca2+ or 5 mmol/L EGTA. Signals (mean ±2SEM) were expressed as in Figure 2C. The normalized signal ratios were significantly lower for S373P, R366P, and K393N, respectively, compared with WT (n=3). Proteins with impaired CaM binding like R366W, S373P, and W392R were hardly detectable at the cell surface (Figure 6B, left panel, first row). Detection levels of R366W, S373P, and W392R mutants corresponded to ~1% of WT KCNQ1. In contrast, detection levels of R366P and K393N that bound CaM almost normally were 15’222% and 53’228% of WT, respectively (n=3). We also investigated levels of WT and mutant proteins in total cell lysates. Mutations with defective CaM binding (R366W, S373P, and W392R) dramatically altered channel expression, as reflected by a profound reduction in expressed mutant proteins (Figure 6B, left panel, third row). When normalized to Gβ levels, total cell expression of R366W, S373P, and W392R corresponded to 5’222%, 15’2210%, and 3’222% of WT, respectively (n=3, P<0.05). In contrast, expression levels of R366P and K393N mutants were 87’226% and 78’229% of WT, respectively (Figure 6B, left panel, third row; n=3). We performed similar biotinylation experiments on WT KCNQ1 and LQT mutants coexpressed with KCNE1 (Figure 6B). KCNE1 considerably increased both cell surface expression (3.9±220.7-fold, n=3, P<0.01) and total steady-state cellular content (3.9±220.9-fold, n=3, P<0.01) of WT KCNQ1. This observation suggested that KCNE1 significantly improves WT IKS channel expression or assembly. Similar data were obtained for the LQT mutants. The presence of KCNE1 markedly increased cell surface expression of R366W, S373P, and W392R mutants (Figure 6B; n=3). A similar effect of KCNE1 was observed for cell surface expression of R366P and K393N mutants representing 89±221% and 132±222%, respectively, of WT IKS channels (n=3, P<0.01). Total cellular contents of K393N, R366P, R366W, S373P, and W392R were 92±227%, 64±227%, 28±221%, 22±221%, and 19±221%, respectively, of WT IKS channels (Figure 6B; n=3), reflecting the improvement of channel expression by KCNE1.

**Signalizing of Ca²⁺-CaM to KCNQ1 and IKS Channels**

In addition to its role in KCNQ1 subunit assembly, CaM might also function as a Ca sensor to modulate KCNQ1 and
Iks channel gating. We tested this possibility on inside-out patches of Xenopus oocyte membranes. Bath application of the CaM antagonist W7 (50 μmol/L) potently and reversibly inhibited both WT KCNQ1/KCNE1 (Iks) and R366W/KCNE1 currents (Figure 7A). In the absence of KCNE1, R366W current was also reduced by W7 (supplemental Figure III). Increasing free Ca2+ concentrations in the bath from nominally 0 to 290 nmol/L produced a significant left-shift in the voltage dependence of channel activation, with a maximum Ca2+-induced shift ΔV50 = 22'226 mV (n=4) for WT KCNQ1 and ΔV50 = 79'228 mV (n=5) for WT KCNQ1/KCNE1 (Figure 7A and 7B; supplemental Table I).

Figure 6. Impact of CaM on KCNQ1 and Iks channel expression. A, Representative experiment that was repeated at least 10 times and shows that CaM allows KCNQ C terminus solubility. It demonstrated that 6X His-KCNQ constructs (352–622 and 540–622) were expressed alone (pET-21a) and with CaM (pET-Duet). After lysis and centrifugation, the supernatant (S) and pellet (P) were analyzed by SDS-PAGE, blotted, and probed with anti-His antibodies. B, Cell surface biotinylation (first row) of WT KCNQ1 and LQT mutants without (left panel) and with KCNE1 (middle and right panels); blots were probed with anti-myc antibodies. No cell surface biotinylation of intracellular Gβ protein was detected with anti-Gβ antibodies, showing the specificity of surface protein labeling (second row). Equal amounts of cell lysate protein were probed with anti-myc antibodies to compare surface versus total cellular channel levels (third row). Cell lysates were also probed with anti-Gβ protein antibodies to monitor inputs (fourth row). C, CaM overexpression produced a prominent increase in the expression of WT KCNQ1 and S373P (n=4, P<0.01) at the cell surface (measured by biotinylation, first row) and in full lysates (second row). Cell lysates were probed by anti-CaM antibodies to appraise CaM overexpression (third row) and by anti-Gβ protein antibodies to monitor inputs (fourth row).
The left-shift in $V_{50}$ was not observed on bath perfusion with Mg$^{2+}$ at concentrations of up to 5 mmol/L (Figure 7C). Conspicuously, $z$ (equivalent gating charge) and $V_{50}$ varied inversely for WT and mutant channels, but their product remained constant (Figure 7D). In line with inside-out patch data, elevation of internal Ca$^{2+}$ by the Ca$^{2+}$-ionophore ionomycin (5 μmol/L) increased the amplitude of WT KCNQ1 by producing a left-shift of the activation curve ($V_{50}$: 24 mV; n = 7) when measured by 2-electrode voltage-clamp in Cl$^{-}$-free solutions (supplemental Figures III and IV). In addition, ionomycin accelerated the activation kinetics of KCNQ1. Ionomycin stimulated the R366W mutant current, but the resulting amplitude was far from reaching that of WT KCNQ1 (supplemental Figure III).

Though inside-out patch records were done in the absence of ATP and in the presence of phosphatase inhibitors, we checked whether the Ca$^{2+}$-mediated stimulation of $I_{KS}$ was mediated by CaM kinase II. KN93 (5 μmol/L), an inhibitor of CaM kinase II, did not affect ionomycin-mediated stimulation of $I_{KS}$ currents (supplemental Figure IVE). Altogether, the data indicate that CaM is essential for $I_{KS}$ channel activity and conveys Ca$^{2+}$ sensitivity. In agreement with this idea, coexpression of KCNQ1 or $I_{KS}$ channels with a Ca$^{2+}$-insensitive CaM mutant (CaM1234) markedly suppressed the currents (by 81%) and produced a right-shift in the voltage-dependence of channel activation ($V_{50}$: 25 mV) (supplemental Figure V).

**Discussion**

In this study, we showed that KCNQ1 and $I_{KS}$ channels need CaM binding for not only proper channel expression and assembly but also correct gating. CaM binding confers Ca$^{2+}$-sensitive stimulation of $I_{KS}$ current. LQT mutations located near the IQ motif of KCNQ1 impair CaM binding, confer inactivation, and reduce current amplitude.

The physical interaction of CaM with KCNQ2–5 $\alpha$ subunits has been recently investigated.6,9,11 However, no infor-
mation has been available so far regarding KCNQ1 and I<sub>Ks</sub> channels. Our in vitro results clearly showed that CaM directly interacts with the C terminus of KCNQ1 in not only the presence of Ca<sup>2+</sup> but also its virtual absence. Pulldown experiments indicated that KCNQ1 channels interact more strongly with Ca<sup>2+</sup>-CaM than with apoCaM.

Mutations in KCNQ1 have been shown to be associated with either the autosomal-dominant or -recessive form of LQT, the Romano-Ward syndrome and the Jervell and Lange-Nielsen syndrome, respectively. We previously showed that mutations in the distal C terminus associated with the recessive Jervell and Lange-Nielsen syndrome phenotype impair KCNQ1 subunit assembly. Here, we investigated the impact of various LQT mutations located near the IQ motif of KCNQ1. The mutants A371T, S373P, and W392R showed severely impaired binding of CaM in both its Ca<sup>2+</sup>-loaded and unloaded form. Although less severe, the mutant R366W had a weaker CaM interaction compared with WT, whereas replacement of arginine by proline at the same location (R366P) bound normally CaM. Probably because of its proximity to the S6 C terminus, the mutant R366P displayed a prominent gating defect. Despite their ability to bind CaM, R366P channels mediated inactivating tiny currents. Except for R366P, decreases in current density of the other LQT mutants tightly correlated with both the severity of the CaM binding defect and the impairment of channel expression. Our data also showed that the LQT mutants analyzed in this study produced a voltage-dependent macroscopic inactivation. However, modulation of macroscopic inactivation is not restricted to the CaM binding domain, as other sites in KCNQ1 are known to confer macroscopic inactivation. KCNE1 association with LQT mutants prevents macroscopic inactivation and significantly improves mutant channel amplitude. However, the marked right-shift in channel activation and the resulting decrease in current amplitude cannot restore normal levels of I<sub>Ks</sub> channel activity. Hence, in these heterozygous Romano-Ward syndrome mutations, even if there is a dominant-negative effect of the mutant channel subunit, the resulting decrease in I<sub>Ks</sub> current density (Figure 5H) will significantly alter the cardiac repolarization reserve.

CaM was suggested to be important for the assembly and trafficking of ion channels like SK4/IK1 Ca<sup>2+</sup>-activated K<sup>+</sup> channels. CaM binding to KCNQ1 C terminus is essential for not only proper channel gating but also channel folding and assembly (Figure 8). First, similar to Cav1.2 and Nav1 channels, recombinant production of a soluble KCNQ1 C terminus required bacterial coexpression of CaM, suggesting that CaM is necessary for proper folding of the C terminus. Second, LQT mutations with impaired CaM binding (R366W, A371T, S373P, and W392R) profoundly disrupted channel expression, as revealed in biotinylation experiments. This disruption may involve not only channel assembly but also processes like degradation pathways (see below). Third, we found that overexpression of WT CaM greatly improved the expression WT KCNQ1 and even more dramatically that of S373P mutant channels. Fourth, deleting helix D compromised CaM interaction with KCNQ1, a feature which suggests a link between KCNQ1 channel assembly and CaM interaction. However, a recent work has shown that KCNQ1 channels lacking helix D are not functional but can coassemble as tetramers, suggesting that helix D is not the sole determinant of subunit assembly. Interestingly, we showed that KCNE1 forms a ternary complex with KCNQ1 and Ca<sup>2+</sup>-CaM. KCNE1 greatly increased not only cell surface expression but also total cellular content of WT and mutant channels, indicating that it may facilitate I<sub>Ks</sub> channel assembly. We suggest that KCNE1 assemblies with CaM-tethered KCNQ1 early in biogenesis of the channel complex, forming a ternary complex that possibly stabilizes the pre-association of CaM to KCNQ1.

Our data with the proteasomal inhibitor MG132 indicate that the deficit in LQT mutant channel expression arises from channel misfolding/assembly and subsequent degradation along the proteasome pathway. Because the LQT mutations with impaired CaM binding are likely misfolded, blocking the proteasome pathway is ineffective, which explains why the MG132 treatment did not rescue the S373P and R366W mutant expression to the cell surface. Instead, the proteasome blocker probably leads the mutant proteins to accumulate in intracellular aggregates, which was previously shown to be a cellular response to...
misfolded proteins. In contrast, CaM overexpression was effective in retrieving the S373P mutant expression to the cell surface, suggesting that by providing a larger amount of CaM, one could improve mutant channel assembly.

There are prominent differences in the role played by Ca2+-CaM in KCNQ2–5 and KCNQ1 channel signaling. Recently, overexpression of CaM in CHO cells was found to robustly reduce currents of KCNQ2, KCNQ4, and KCNQ5, but not those of KCNQ1 and KCNQ3. Here, we showed in Xenopus oocytes that KCNQ1, R366W, and IKS currents are markedly inhibited by CaM antagonists. Remarkably, intracellular Ca2+ produced a left-shift in the voltage-dependence of activation of WT IKS as determined from inside-out patch recording. Although the LQT mutations are right-shifted in their activation curve, an increase in intracellular Ca2+ still produced a marked left-shift in the activation seen for R366W/KCNE1. Similarly, ionomycin left-shifted the voltage-dependence of KCNQ1 activation and accelerated its inactivation, suggesting that by providing a larger amount of CaM, Ca2+-CaM fit within the time frame of a single action potential [see comments].

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Interestingly, previous studies showed that elevation of [Ca2+], in guinea pig ventricular myocytes enhances IKS currents. Noteworthy, IKS-currents increase ∼3-fold on free [Ca2+], rise from 10−6 M to 10−7 M, suggesting that there is substantial Ca2+-CaM bound to IKS channels under resting Ca2+ levels. This stimulation may be accounted for by the Ca2+-induced left-shift of IKS activation. Our preliminary data indicate that the kinetics of Ca2+-induced rise in IKS fit within the time frame of a single action potential (unpublished data). Our results are also consistent with a recent study showing that at high stimulation rates, there is a Ca2+-induced increase in cardiac IKS currents that plays a dominant role in shortening action potential duration. Interestingly, this Ca2+-induced IKS stimulation was sensitive to the CaM antagonist W7.

In all, CaM exerts a dual action on IKS channels, being necessary for both correct channel assembly and gating. As such, CaM enhances IKS channel activity after increases in internal Ca2+ (Figure 8). This IKS current boosting is crucial for increasing the repolarization reserve. Given the marked loss of channel function produced by the defective CaM binding LQT mutants, it is not surprising that a decrease in cardiac repolarization reserve would prolong the action potential and increase the risk of ventricular arrhythmias.

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CALMODULIN IS ESSENTIAL FOR CARDIAC $I_{KS}$ CHANNEL GATING AND ASSEMBLY: IMPAIRED FUNCTION IN LONG QT MUTATIONS

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Supplemental Materials and Methods

*Molecular biology and cloning*

For the yeast two-hybrid assay, fragments of KCNQ1 were amplified by standard PCR and cloned in frame into the Gal4 activation domain of pGAD424 (clontech). CaM was cloned in frame into Gal4 DNA binding domain of pAS2-1. For immunoprecipitation from *in vitro* translated proteins, KCNQ1 C-termini were fused to a myc-tag (EQLISEEDLN) and cloned into pcDNA3.1 (Invitrogen). For expression in mammalian cells, KCNQ1 wild-type (WT) and mutants were cloned into pcDNA3.1. For calmodulin agarose pulldown experiments of transfected cells, KCNQ1 WT and LQT mutants were introduced into pcDNA4 bearing a myc and His tag. WT CaM and Ca$^{2+}$-insensitive CaM$_{1234}$ mutant in the pBEF vector were used in this study and were kindly provided by Dr. JP Adelman (Vollum Institute, Portland).

*Yeast Two-hybrid Assay*

cDNA encoding different regions of KCNQ1 and calmodulin were co-transformed into the CG1945 yeast strain according to the manufacturer’s instructions (clontech). Co-transformants were first plated onto synthetic dropout medium lacking tryptophan and leucine to select for colonies containing both hybrid plasmids and then transferred onto medium that also lacked histidine to select for protein-protein interactions. Media were
supplemented with 6 mM 3-amino-1,2,4-triazole to suppress background growth of the CG1945 yeast strain.

**Antibodies and reagents**

Rabbit polyclonal antibodies anti-KCNQ1 was raised against a C-terminal peptide of KCNQ1 (EQLTVPRRGPDEGS; Eurogentec, Belgium) and affinity-purified. Mouse monoclonal anti-His6 (Roche), anti-Myc (9E10, Santa Cruz), anti-calmodulin (Upstate Biotechnology), rabbit anti-Gβ (Santa Cruz) and goat anti-KCNQ1 (C20, Santa Cruz) antibodies were used. In addition, rabbit anti-KCNQ1 and anti-KCNE1 antibodies were kindly provided from Alomone Labs (Jerusalem, Israel). CaM agarose beads, Ionomycin, and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) were purchased from Sigma and Calbiochem.

**Immunoprecipitation, cell surface biotinylation and pulldown experiments**

For immunoprecipitation from in vitro translated proteins, ^35^S-labelled proteins were synthesized using the TNT® Quick Translation System (Promega). The labelled proteins were diluted 20-fold into IP buffer (20 mM Tris [pH7.9]; 150 mM NaCl; 1% Triton X-100; 0.1% bovine hemoglobin; with 2 mM EGTA, respectively). The proteins were immunoprecipitated by addition of anti-myc antibody and then protein A-Sepharose (Pharmacia). Immunoprecipitates were washed twice in IP buffer and twice in washing buffer (20 mM Tris–HCl [pH7.9]; 150 mM NaCl; with 2 mM EGTA, respectively), then analyzed by 15% SDS-PAGE. Labelled proteins were visualized by autoradiography.

For CaM agarose pulldown experiments from intact cells, HEK 293 cells were transfected using the calcium phosphate method. Cells were lysed with a buffer containing 50 mM Tris-HCl [pH 7.5], 150 NaCl, 10 mM sodium pyrophosphate, protease inhibitor cocktail
(Sigma), 0.1% SDS, 1% triton X-100, 1% deoxycholate, with either 1 mM CaCl₂ or 5 mM EGTA, respectively. Equal amounts of lysate proteins were incubated overnight at 4°C with CaM-agarose beads in lysis buffer, washed 3 times in 20 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM sodium pyrophosphate, 10% glycerol, 1% triton X-100 and protease inhibitor cocktail, then resolved by 8% or 15% SDS-PAGE and Western blotted.

For immunoprecipitation from CHO cell lysates, cells were washed in phosphate-buffered saline, solubilized in ice-cold reaction buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mg/ml bovine serum albumin, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail), and rotated at 4°C for 3 hours. Unsolubilized material was removed by centrifugation (100 000 × g). Lysates were incubated with anti-KCNQ1 antibody for 3 h at 4°C, followed by incubation with protein A-Sepharose (Pharmacia) for 3 h. Immune complexes were washed once in reaction buffer and twice in washing buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl), then resolved by 15 % SDS-PAGE and Western blotted.

Biotinylation of surface proteins was carried out by incubating cells with 1mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS containing 1mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail 10µM/ml (solution A), for 30 min at room temperature. Reaction was terminated by incubating cells for 5 min in solution A containing 20 mM glycine followed by three washes in solution A. Cells were lysed in IP buffer (1 hour at 4°C under rotation). Cell lysate was cleared by centrifugation and biotinylated proteins were pulled down by incubation with streptavidin agarose beads (Pierce) overnight at 4°C. The beads were washed six times with IP buffer, proteins were eluted by incubation with sample buffer at room temperature, then resolved by SDS-PAGE and Western blotted.
**Bacterial expression**

CaM cDNA was subcloned into multiple cloning site II of an E-coli coexpression vector pETDuet (Novagen). Two different constructs of KCNQ1 C-terminus (352-622 and 540-622) were subcloned into multiple cloning site I of the vector, and into the pET21d (Novogen) expression vector as well. KCNQ1 with and without CaM expression plasmids were transformed into Tuner codon plus cells by electroporation. Cells were grown to an OD$_{600} = 0.5$ and expression was induced by addition of 150 µM IPTG for 16 hr at 16°C. Cell extracts were prepared by passage through a French press. The lysates were then centrifuged at 18,000 rpm. The resulting supernatant and pellet were analyzed by SDS-PAGE and immunoblotted with anti His antibodies (Roche).

**Cell line culture and transfection**

For electrophysiology, Chinese Hamster Ovary (CHO) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal calf serum and antibiotics. Cells were seeded on poly-D-lysine-coated glass coverslips and transfected using Fugene 6 (Roche) with pIRES-CD8 (0.5µg) as a marker for transfection and with KCNQ1 WT or its LQT mutants (0.5 µg), KCNE1 (1 µg) or with CBD-SK2 (2 µg). Transfected cells were visualized about 40 hours following transfection, using anti-CD8 antibody-coated beads 1. For biochemistry, HEK 293 cells were grown as for CHO cells and transfected using the calcium phosphate method.

**Whole-cell patch-clamp of CHO cells**

Recordings were performed using the whole-cell configuration of the patch-clamp technique. Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), sampled at 2 kHz and filtered at 800 Hz via a 4-pole Bessel low pass filter. Data were
acquired using pClamp 8.2 software in conjunction with a DigiData 1322A interface. The patch pipettes were pulled from borosilicate glass (Warner Instrument Corp, USA) with a resistance of 4-7 MΩ. The intracellular pipette solution contained (in mM): 130 KCl, 1 MgCl₂, 5 K₂ATP, 5 EGTA, 10 HEPES, adjusted with KOH to pH 7.4 (290 mOsm). The external solution contained (in mM): 140 NaCl, 4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 11 glucose, 5.5 HEPES, adjusted with NaOH to pH 7.4 (310 mOsm). Series resistances (8-15 MΩ) were compensated (75-90%) and periodically monitored.

**Two-electrode voltage clamp of Xenopus oocytes**

The cDNAs encoding human WT KCNQ1 and the R366W LQT mutant in the pcDNA3 vector were directly microinjected into Xenopus oocyte nuclei (1 ng into 10 nl). Oocytes were placed into a 100 μl recording chamber and perfused under constant rate of 0.7 ml/min. Oocytes were recorded at room temperature (20°C-22°C) using a GeneClamp 500 amplifier (Axon Instruments) in a modified ND96 solution containing (in mM: 96 Na-gluconate, 2 K-gluconate, 1 Mg-gluconate₂, 1.8 Ca-gluconate and 5 HEPES titrated to pH = 7.4 with NaOH). Current measurements were performed as previously described².

**Inside-out recording in Xenopus oocytes**

Inside-out patch-clamp recording was performed from macropatches of Xenopus oocytes with an internal bathing solution containing 60 mM KMeSO₃, 5 mM KF, 0.1 mM Na₃VO₄, 10 mM K₄P₂O₇, 10 mM HEPES, pH 7.4 and 5 mM EGTA plus CaCl₂ titrated at the indicated free Ca²⁺ concentrations. The external pipette solutions contained 2 mM KCl, 91 mM NaMeSO₃, 1 mM MgCl₂, 5 mM NaOH, and 5 mM HEPES, pH 7.4 for WT KCNQ1/KCNE1 and 104 mM KMeSO₃, 1 mM MgCl₂, 5 mM NaOH, and 5 mM HEPES,
pH 7.4 for R366W/KCNE1. The external pipette solution contained high K⁺ concentrations for R366W/KCNE1 in order to record measurable tail currents at -120 mV.

**Data analyses**

Data analysis was performed using the Clampfit program (pClamp 8.2, Axon Instruments), Microsoft Excel (Microsoft), SigmaPlot 8.0 and Prism 4.0 (GraphPad). Leak subtraction was performed off-line, using the pClamp 8.2 software. Chord conductance (G) was calculated by using the following equation:

\[
G = \frac{I}{(V - V_{\text{rev}})}
\]

where I corresponds to the tail current amplitude measured at -60 mV and V_{\text{rev}}, the calculated reversal potential (-90 mV in CHO cells). G was estimated at various test voltages V and then, normalized to a maximal conductance value, G_{\text{max}}. Activation curves were fitted by one Boltzmann distribution:

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp[(V_{50} - V)/s]}
\]

where \(V_{50}\) is the voltage at which the current is half-activated and s is the slope factor.

To analyze LQT mutant channel inactivation, a single exponential fit was applied to the inactivating currents. In inside-out patch-clamp experiments, the parameters were obtained at the [Ca^{2+}]_{i} as indicated below the plots. \(\Delta V_{50} = V_{50} \text{ at lower } [\text{Ca}^{2+}]_{i} - V_{50} \text{ at higher } [\text{Ca}^{2+}]_{i}\) (middle) and \(\Delta(zV_{50}) = zV_{50} \text{ at lower } [\text{Ca}^{2+}]_{i} - zV_{50} \text{ at higher } [\text{Ca}^{2+}]_{i}\) (right) were calculated from each patch according to Cui and Aldrich \(^3\) and then averaged. All data were expressed as mean ± SEM. Statistically significant differences were assessed by paired two-sample for means t-test (two-tail) and two-sample assuming equal variances t-test (two-tail).
Supplemental Results

**LQT mutations in KCNQ1 C-terminus impair CaM binding**

WT KCNQ1 C-terminus and mutated constructs were *in vitro* translated and incubated with CaM-agarose in the absence of Ca$^{2+}$ (Supplemental Figure 1). Our results corroborate the severely impaired binding of CaM to the mutants S373P, A371T and W392R. The data also confirmed the good interaction of apoCaM with WT and the mutants K393N and R366P obtained in the CaM-agarose pulldown (5 mM EGTA) from transfected HEK 293 cells. There is, however, a difference for R366W which has a significant weaker interaction to apoCaM in pulldown from transfected HEK 293 cells compared to the assay performed on *in vitro* translated R366W C-terminus.

**Impact of CaM in channel assembly**

WT KCNQ1 and two CaM binding-defective mutants, S373P and R366W, were transfected in HEK 293 cells. The proteasome inhibitor MG132 was applied to the cells for 4 hours (10µM) following 24 hours transfection. Then, a cell surface biotinylation assay was performed (Supplemental Figure 2). At the cell surface, a moderate (~30%) but significant increase of WT KCNQ1 was obtained in the presence of MG132, while under the same conditions both S373P and R366W mutants could not be detected (Supplemental Figure 2, 1st row). For mutant proteins that are unable to fold correctly, inhibition of proteasome function may not be effective in allowing diseased channels to be expressed at the plasma membrane. Instead, proteasome inhibitors may lead the mutant protein to accumulate either in the ER or in intracellular inclusion bodies resembling aggresomes\(^4\). To test this possibility, cells were lysed in harsher detergent conditions (1% triton X100, 0.5% Na$^+$ deoxycholate and 0.5% SDS) and lysates were analyzed by Western blotting using anti-myc antibodies.
Because of the stronger lysis conditions, anti-myc antibodies revealed the presence of nuclear c-myc immunoreactive proteins (MW ~ 60-65 kDa, two lowest bands); nevertheless, myc-tagged channels could be clearly identified as the upper band (arrow) of the blot. In the presence of MG132 (4 hours, 10µM), there was a dramatic increase in total cellular steady-state mutant protein content with 148 ± 44-fold and 24 ± 14-fold stimulation for R366W and S373P, respectively, compared to 3.5 ± 0.9-fold enhancement of WT KCNQ1 (Supplemental Figure 2, 2nd row; n = 4, p < 0.01). This result suggests that the LQT mutants are targets of proteosomal degradation.

**Signaling of Ca^{2+}-CaM to KCNQ1 and I_{KS} channels**

We investigated the signaling of Ca^{2+}-CaM to WT KCNQ1, WT I_{KS} and the impaired CaM-binding mutant R366W. First, we explored this issue in Xenopus oocytes by examining the impact of the CaM antagonist W7 on the expressed currents and by measuring the effect of the Ca^{2+} ionophore, ionomycin, on channel activities. Two-electrode voltage-clamp recordings were made in gluconate containing extracellular solutions to suppress the contribution of endogenous Ca^{2+}-activated Cl^- conductance. First, we examined the effect of the CaM antagonist W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide) that binds to calcium-loaded CaM in place of its target protein. Currents were evoked by trains of a +30 mV depolarization step from a -80 mV holding potential. Superfusion of 30 µM W7 produced 43 ± 3 % inhibition of WT KCNQ1 currents (Supplemental Figure 3A upper panel and 3E; n = 5, p < 0.003). This inhibition elicited by W7 suggests that in Xenopus oocytes there is substantial Ca^{2+}-CaM bound to WT KCNQ1 channels under resting Ca^{2+} levels. A similar degree of inhibition was obtained with 30 µM W7 on WT I_{KS}-currents (46 ± 5 % inhibition; n = 5, p < 0.003). The mutant R366W was significantly more sensitive to W7.
inhibition than WT KCNQ1 or WT $I_{KS}$, with 81 ± 5 % inhibition (Supplemental Figure 3C upper panel and 3E; n = 5, significance compared to WT KCNQ1 p < 0.00005). Similar results were obtained with another CaM antagonist trifluoperazine (data not shown).

WT KCNQ1 current was increased upon exposure to the Ca$^{2+}$ ionophore ionomycin (5 µM) with 32 ± 3 % stimulation of current amplitude at +30 mV which could be subsequently inhibited by 30 µM W7 (Supplemental Figure 3A lower panel and 3D; n = 5, p < 0.03). Similarly, WT $I_{KS}$ currents were stimulated by ionomycin with 31 ± 7 % increase in current amplitude at +30 mV which was also reduced by W7 (Supplemental Figure 3B, lower panel and 3D; n = 5, p < 0.03). Ionomycin stimulated also the inactivating R366W mutant current with 137 ± 72 % increase of channel activity but the resulting amplitude was far from reaching that of WT KCNQ1 (at +30 mV, 0.23 ± 0.06 µA versus 4.55 ± 0.25 µA for R366W and WT KCNQ1, respectively; Supplemental Figure 3C, lower panel and 3D; significance compared to WT KCNQ1 p <0.04; n = 6). While stimulation of WT KCNQ1 and WT $I_{KS}$-currents by ionomycin was subsequently inhibited by 30 µM W7 to control unstimulated current levels, the Ca$^{2+}$-stimulated mutant current R366W was inhibited by W7 to a much larger extent up to 36 ± 5 % of control unstimulated currents levels (Supplemental Figure 3C lower panel; n = 6, p < 0.01). Noteworthy, the ionomycin-mediated increase of R366W current was accompanied by a marked slowing of the macroscopic inactivation kinetics with $\tau_{\text{inact}} = 167 ± 9$ ms and $\tau_{\text{inact}} = 475 ± 12$ ms before and after ionomycin exposure, respectively (Supplemental Figure 3C lower panel and 3F; n = 6, p < 0.01). Inside-out patch-clamp recordings indicate that intracellular Ca$^{2+}$ exquisitely left-shifted the voltage-dependence of activation of WT KCNQ1/KCNE1 and of R366W/KCNE1 (supplemental Table 1). In agreement with inside-out patch data, elevation of internal Ca$^{2+}$ concentrations by the Ca$^{2+}$-
ionophore ionomycin (5 µM) increased the amplitude of WT KCNQ1 by producing a left-shift $\Delta V = -24$ mV of the activation curve (from $V_{50} = -28.3 \pm 1.0$ mV to $V_{50} = -52.0 \pm 0.3$ mV; $n = 7$) when measured by two-electrode voltage-clamp in Cl$^-$-free solutions (Supplemental Figure 4B). In addition, ionomycin accelerated the activation kinetics of KCNQ1 (Supplemental Figure 4A,C,D). At 0 mV, the KCNQ1 activation kinetics could be fitted by a two exponential function. Without ionomycin, the activation time constants were $\tau_{\text{fast}} = 230 \pm 13$ ms and $\tau_{\text{slow}} = 1541 \pm 80$ ms ($n = 7$; at 0 mV). In the presence of ionomycin, the activation time constants were significantly reduced to $\tau_{\text{fast}} = 143 \pm 8$ ms and $\tau_{\text{slow}} = 841 \pm 174$ ms (at 0 mV) (Supplemental Figure 4C; $n = 7$; $p < 0.006$) with a great decrease of the relative contribution of the slow component of activation kinetics (from 0.43 to 0.05 without and with ionomycin, respectively; Supplemental Figure 4D; $n = 7$; $p < 0.006$). Although inside-out patch experiments were performed in the absence of ATP and in the presence of protein phosphatase inhibitors, we wanted to exclude the possible involvement of CaM kinase II in the Ca$^{2+}$-mediated stimulation of $I_{KS}$ currents. Thus, we used KN93 (5 µM), a specific inhibitor of CaM kinase II as well as its inactive analog KN92 (5 µM). As shown in Supplemental Figure 4E, neither KN93 nor KN92 affected the ionomycin-mediated stimulation of $I_{KS}$ currents at $+30$ mV. Supplemental Figure 5A shows that when compared to coexpression of KCNQ1+WT CaM, the coexpression of KCNQ1 with a Ca$^{2+}$-insensitive CaM mutant (CaM$_{1234}$) markedly suppressed the currents (by 81%) and produced a right-shift of $\Delta V_{50} = +25$ mV in the voltage-dependence of channel activation (from $V_{50} = -32 \pm 2$ mV to $V_{50} = -7 \pm 2$ mV; $n = 8$; Supplemental Figure 5B). Similarly, when comparing coexpression of $I_{KS}$+WT CaM, the coexpression of $I_{KS}$ with a Ca$^{2+}$-insensitive CaM mutant (CaM$_{1234}$) markedly suppressed the currents (not shown) and led to a pronounced right-shift
of $\Delta V_{50} = +35 \text{ mV}$ in the voltage-dependence of channel activation (from $V_{50} = 37.5 \pm 3.1 \text{ mV}$ to $V_{50} = 72.7 \pm 2.6 \text{ mV}$; $n = 4$; Supplemental Figure 5F). Interestingly, the Ca$^{2+}$-insensitive CaM mutant (CaM$_{1234}$) significantly slowed the activation kinetics of KCNQ1 (Supplemental Figure 5C-E). With WT CaM, the KCNQ1 activation time constants were $\tau_{\text{fast}} = 86 \pm 1 \text{ ms}$ and $\tau_{\text{slow}} = 980 \pm 48 \text{ ms}$ ($n = 8$; at $+40 \text{ mV}$). With the Ca$^{2+}$-insensitive CaM mutant (CaM$_{1234}$), the KCNQ1 activation time constants were $\tau_{\text{fast}} = 92 \pm 2 \text{ ms}$ and $\tau_{\text{slow}} = 1196 \pm 49 \text{ ms}$ ($n = 8$; at $+40 \text{ mV}$; $p < 0.015$) with an increase of the relative contribution of the slow component of activation kinetics (from 0.36 to 0.46; $n = 8$; $p < 0.0003$, Supplemental Figure 5D,E).

**Supplemental References**


**Supplemental Figure Legends**

**Supplemental Figure 1**
KCNQ1 C-terminal constructs were *in vitro* translated and incubated with CaM-agarose in the absence of Ca$^{2+}$. The upper panel shows the results of a typical pulldown experiment; the lower panel shows the corresponding *in vitro* translation products (IVT). This experiment was replicated 3 times and gave similar results.

**Supplemental Figure 2**
Effect of the proteasome inhibitor MG132 (4 hours, 10µM) on cell surface expression of WT KCNQ1, R366W and S373P LQT mutants, measured by surface biotinylation and probed with anti-myc antibodies (1<sup>st</sup> row). The total cellular channel content was determined by lysing cells with harsh detergent conditions (1% triton X100, 0.5% Na$^+$ deoxycholate and 0.5% SDS) (2<sup>nd</sup> row). In these conditions, anti-myc antibodies also revealed the presence of nuclear c-myc immunoreactive proteins (MW ~ 60-65 kDa, two lowest bands); myc-tagged channels could be clearly identified as the upper band (arrow) of the blot. MG132 dramatically increased the cellular mutant channel content compared to a mild enhancement of WT KCNQ1 (n = 4, p < 0.01). Blots of total cell lysates were reprobed with anti-Gβ protein antibodies to monitor inputs (3<sup>rd</sup> row).
**Supplemental Figure 3**

Impact of Ca$^{2+}$-CaM on WT KCNQ1, $I_{KS}$ and R366W currents expressed in *Xenopus* oocytes. (A-C) Effects of the CaM antagonist W7 (upper panels) and the Ca$^{2+}$ ionophore ionomycin (lower panels) on WT KCNQ1, WT $I_{KS}$ and R366W currents evoked by trains of +30 mV depolarization steps from a -80 mV holding potential. (D) WT KCNQ1 and WT $I_{KS}$-currents were significantly increased upon superfusion with ionomycin (n = 5, * p < 0.03). R366W currents were drastically stimulated by ionomycin, compared to WT KCNQ1 (n = 6, # p < 0.04). (E) W7 produced a significant inhibition of WT KCNQ1 and WT $I_{KS}$-currents (n = 5, * p < 0.003). R366W mutant was more sensitive to W7 inhibition, compared to WT KCNQ1 (n = 5, # p < 0.00005). (F) When fitted by a single exponential function, inactivation kinetics of R366W were markedly slowed down by ionomycin treatment (n = 6, * p < 0.01).

**Supplemental Figure 4**

Impact of the Ca$^{2+}$ ionophore ionomycin and of a CaM kinase II inhibitor on WT KCNQ1 and $I_{KS}$ currents expressed in *Xenopus* oocytes. (A) Ionomycin accelerates the activation kinetics of WT KCNQ1 channels. Representative normalized traces of KCNQ1 at 0 mV in the absence and presence of ionomycin (5 µM). (B) Ionomycin produces a left-shift of -24 mV of the voltage dependence of KCNQ1 from $V_{50} = -28.3 \pm 1.0$ mV and slope = 12 $\pm$ 1 to $V_{50} = -52.0 \pm 0.3$ mV and slope = 10 $\pm$ 1; n = 7. (C) Fast and slow time constants of KCNQ1 activation kinetics measured at 0 mV, in the absence (empty bars) and presence of ionomycin (black bars) as described by a two exponential function (n = 7; * p < 0.006). (D) Relative contribution of the slow amplitude component of the two exponential activation kinetics of
KCNQ1 in the absence (empty bar) and presence of ionomycin (black bar) (n = 7; * p < 0.006). (E) Effects of a specific inhibitor of CaM kinase II (KCN93, 5 µM) and of its inactive analog (KCN92, 5 µM) on I_KS current stimulated at +30 mV by ionomycin (5 µM).

Supplemental Figure 5
Impact of the WT CaM and the Ca^{2+}-insensitive CaM_{1234} mutant on WT KCNQ1 and I_KS currents expressed in Xenopus oocytes. (A) Current-voltage relations of KCNQ1 channels co-expressed with either WT CaM (black squares) or the Ca^{2+}-insensitive CaM_{1234} mutant (empty squares) (n = 8). (B) Normalized conductance-voltage relation of KCNQ1 channels co-expressed with either WT CaM (black squares) or the Ca^{2+}-insensitive CaM_{1234} mutant (empty squares) which produces a right-shift of ΔV_{50} = +25 mV (from V_{50} = -32 ± 2 mV and slope = 16 ± 1 to V_{50} = -7 ± 2 mV and slope = 17 ± 2; n = 8. (C) Representative normalized traces of KCNQ1 at +40 mV coexpressed with either WT CaM or the Ca^{2+}-insensitive CaM_{1234} mutant. (D) Fast and slow time constants of KCNQ1 activation kinetics measured at +40 mV, with WT CaM (empty bars) and with the Ca^{2+}-insensitive CaM_{1234} mutant. (black bars) as described by a two exponential function (n = 8; * p < 0.015). (E) Relative contribution of the slow amplitude component of the two exponential activation kinetics of KCNQ1 coexpressed with WT CaM (empty bar) or the Ca^{2+}-insensitive CaM_{1234} mutant (black bar) (n = 8; * p < 0.0003). (F) Half activation values (V_{50}) deduced from normalized conductance-voltage relations of WT I_KS co-expressed with either WT CaM (empty bar) or the Ca^{2+}-insensitive CaM_{1234} mutant (black bar) (n = 4; * p < 0.01).
Supplemental Table 1

Parameters of activation gating of WT KCNQ1/KCNE1 and R366W/KCNE1 channels at various intracellular Ca\(^{2+}\) concentrations.

<table>
<thead>
<tr>
<th>Ca(^{2+}) Concentration</th>
<th>WTKCNQ1/KCNE1</th>
<th>R366W/KCNE1</th>
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<tr>
<td></td>
<td>n</td>
<td>V(_{50})</td>
</tr>
<tr>
<td>0 Ca(^{2+})</td>
<td>3</td>
<td>80.0±4.3</td>
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<tr>
<td>25 nM Ca(^{2+})</td>
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<td>34.2±2.1</td>
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<td>70 nM Ca(^{2+})</td>
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<tr>
<td>100 nM Ca(^{2+})</td>
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<td>27.0±3.4</td>
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Currents were recorded from inside-out macropatches bathed at indicated Ca\(^{2+}\) concentrations. V\(_{50}\) and z values were determined from the Boltzmann fits of normalized conductance-voltage relations. Conductances were determined at -120 mV tail potential after voltage steps (3 s) from -60 mV to +100 mV. Data points were fitted to one Boltzmann function.

Supplemental Table 2

Parameters of activation gating of WT KCNQ1 and the LQT mutants expressed in the absence and presence of KCNE1 in CHO cells.

<table>
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<th>V(_{50}) (mV)</th>
<th>slope factor</th>
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<tbody>
<tr>
<td>WT KCNQ1</td>
<td>-31.4 ± 5.8</td>
<td>21.7 ± 3.7</td>
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<tr>
<td>K393N</td>
<td>-32.7 ± 4.2</td>
<td>20.6 ± 2.6</td>
</tr>
<tr>
<td>A371T</td>
<td>1.3 ± 1.4</td>
<td>10.6 ± 1.3</td>
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<tr>
<td>R366P</td>
<td>-8.8 ± 1.6</td>
<td>15.3 ± 1.6</td>
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<tr>
<td>R366W</td>
<td>0.9 ± 1.6</td>
<td>19.8 ± 1.9</td>
</tr>
<tr>
<td>S373P</td>
<td>13.3 ± 0.7</td>
<td>15.1 ± 0.7</td>
</tr>
<tr>
<td>W392R</td>
<td>-5.0 ± 1.7</td>
<td>11.9 ± 1.6</td>
</tr>
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<td>WT KCNQ1+KCNE1</td>
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<td>15.7 ± 0.6</td>
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<td>K393N+KCNE1</td>
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<td>A371T+KCNE1</td>
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<td>17.8 ± 2.0</td>
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<td>R366P+KCNE1</td>
<td>32.3 ± 8.9</td>
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<td>R366W+KCNE1</td>
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<td>S373P+KCNE1</td>
<td>46.1 ± 1.6</td>
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<td>W392R+KCNE1</td>
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Currents were recorded from the whole-cell configuration of the patch-clamp technique. V\(_{50}\) and slope factor were determined from the Boltzmann fits of the normalized conductance-voltage relations. Conductances were determined at -60 mV tail potential after voltage steps (3 s) from -70 mV to +60 mV. Data points were fitted to one Boltzmann function (n = 6-16).
Supplemental Figure 1
Supplemental Figure 2