KCNQ1 Assembly and Function Is Blocked by Long-QT Syndrome Mutations That Disrupt Interaction With Calmodulin

Smita Ghosh, Deborah A. Nunziato, Geoffrey S. Pitt

Abstract—Calmodulin (CaM) has been recognized as an obligate subunit for many ion channels in which its function has not been clearly established. Because channel subunits associate early during channel biosynthesis, CaM may provide a mechanism for Ca$^{2+}$-dependent regulation of channel formation. Here we show that CaM is a constitutive component of KCNQ1 K$^+$ channels, the most commonly mutated long-QT syndrome (LQTS) locus. CaM not only acts as a regulator of channel gating, relieving inactivation in a Ca$^{2+}$-dependent manner, but it also contributes to control of channel assembly. Formation of functional tetramers requires CaM interaction with the KCNQ1 C-terminus. This CaM-regulated process is essential: LQTS mutants that disrupt CaM interaction prevent functional assembly of channels in a dominant-negative manner. These findings offer a new mechanism for LQTS defects and provide a basis for understanding novel ways that intracellular Ca$^{2+}$ and CaM regulate ion channels. (Circ Res. 2006;98:1048-1054.)

Key Words: KCNQ1 ■ K$_v$LQT1 ■ I$_{Ks}$ ■ calmodulin ■ long-QT syndrome

The long-QT syndrome (LQTS) is a collection of inherited and acquired arrhythmogenic diseases characterized on ECG by a prolonged QT interval. This ECG manifestation reflects an abnormally prolonged ventricular action potential, which can be the substrate for life-threatening arrhythmias that lead to syncope or sudden cardiac death. LQTS is a "channelopathy," all except one LQTS loci reside in genes encoding ion channels that regulate the cardiac action potential, and characterization of LQTS mutations in specific channels reveals defects that promote excessive depolarization or weakened repolarization.1–3 Nevertheless, development of arrhythmias is rare, and the specific triggers that induce them are not well understood.

Intracellular Ca$^{2+}$ is one factor with a large potential impact that has only recently been explored.3–7 As Ca$^{2+}$ is the fundamental second messenger of electrical activity in excitable cells, it is not surprising that many ion channels are subject to feedback modulation by resultant Ca$^{2+}$ fluxes, a process often mediated by the ubiquitous Ca$^{2+}$-binding protein calmodulin (CaM).8–13 The recent identification of two possible CaM interaction sites within the C-terminus of KCNQ1,14 the most commonly mutated locus (LQT1) in congenital LQTS,15 offered a window into how Ca$^{2+}$ may contribute to LQTS. As the pore-forming subunit of the cation-selective cardiac I$_{Ks}$ current that determines action potential duration on a beat-to-beat basis,16,17 KCNQ1 is well positioned to integrate changes in intracellular Ca$^{2+}$ into an alteration in action potential duration, consistent with previous reports showing that I$_{Ks}$ is a Ca$^{2+}$-responsive current.18–20 Moreover, the neuronal homologs KCNQ2 and KCNQ3 form heteromultimers that are responsible for the Ca$^{2+}$-sensitive M current21 in the brain and have also been shown to bind CaM,14,22 suggesting that Ca$^{2+}$/CaM could be a general regulator for the KCNQ K$^+$ channel family. Nevertheless, attempts to define whether and how Ca$^{2+}$/CaM regulates any of the KCNQ homologs have produced conflicting results.22–24 Moreover, KCNQ1 stood apart in these initial studies because Ca$^{2+}$/CaM did not appear to regulate KCNQ1 or I$_{Ks}$ currents in a heterologous system and because CaM was not found to interact with KCNQ1. Here, we report that CaM binds to the KCNQ1 C-terminus, where it contributes to channel assembly. On mature channels, CaM appears to relieve channel inactivation in the presence of basal levels of intracellular Ca$^{2+}$. Further relief of inactivation may augment KCNQ1 current when Ca$^{2+}$ is elevated.

Materials and Methods

Additional Materials and Methods are available in the online-only data supplement at http://circres.ahajournals.org.

Molecular Biology

Bacterial and oocyte expression constructs were generated with standard approaches. Cloning of the KCNQ1 concatemer yielded Gly-Ser dipeptide between the 2 KCNQ1 repeats.

Electrophysiology

Two electrode voltage-clamp recordings from Xenopus oocytes were performed as previously described25 with the use of ND-96 bath.
solution. Intracellular Ca\(^{2+}\) manipulations were performed by micro-injection of 50 to 100 nL of 100 mmol/L BAPTA, 100 mmol/L EGTA, 100 μmol/L CaCl\(_2\), or 500 mmol/L sucrose (in 10 mmol/L HEPES, pH 7.4).

**Protein Expression and Purification**

The KCNQ1 CT constructs, with and without CaM or CaM\(_{1234}\), were isolated from BL-21 (DE3) cells and purified with Talon metal affinity resin (Clontech). Gel filtration was performed over a Superdex 200 HR 10/30 column on an AKTA FPLC (Amersham Biosciences) in 500 mmol/L NaCl, 20 mmol/L Tris, pH 7.5, and 10 μmol/L CaCl\(_2\). The KCNQ1 CT constructs, with and without CaM or CaM\(_{1234}\), were isolated from BL-21 (DE3) cells and purified with Talon metal affinity resin (Clontech). Gel filtration was performed over a Superdex 200 HR 10/30 column on an AKTA FPLC (Amersham Biosciences) in 500 mmol/L NaCl, 20 mmol/L Tris, pH 7.5, and 10 μmol/L CaCl\(_2\). The KCNQ1 CT constructs, with and without CaM or CaM\(_{1234}\), were isolated from BL-21 (DE3) cells and purified with Talon metal affinity resin (Clontech). Gel filtration was performed over a Superdex 200 HR 10/30 column on an AKTA FPLC (Amersham Biosciences) in 500 mmol/L NaCl, 20 mmol/L Tris, pH 7.5, and 10 μmol/L CaCl\(_2\)).

**Chemical Cross-Linking**

Disuccinimidyl glutarate 2.5 mmol/L was added for 5 minutes at room temperature to the purified KCNQ1 CT/CaM complexes before the addition of SDS-PAGE sample buffer.

**Statistical Analysis**

Data are presented as mean ± SEM. Significant differences were assessed using Student’s t test at P<0.05.

**Results**

**CaM Interacts Directly With the KCNQ1 C-Terminus**

To directly assess whether CaM interacted with the KCNQ1 CT, we used paradigms successful in defining CaM interaction with other voltage-gated ion channels.6,26 We first attempted to generate recombinant protein under detergent-free conditions for quantitative binding assays with a 6xHis-tagged KCNQ1 construct of the entire CT (aa 354–676; Figure 1A), but despite abundant protein expression, none was soluble (Figure 1B). This result was similar to that observed for the CaM-binding regions in the CTs of Ca,1,2, Na,1,2, and Na,1.5 channels, so we coexpressed CaM with the KCNQ1 CT and found that the KCNQ1 CT became soluble (Figure 1B). Not only did CaM promote KCNQ1 CT solubility, but it was also a KCNQ1 CT binding partner, as indicated by CaM copurification with the 6xHis-tagged KCNQ1 CT by metal affinity chromatography (MAC) (Figure 1B). This interaction was specific, as CaM was not purified in the absence of KCNQ1 CT coexpression (not shown). Further, this interaction could be Ca\(^{2+}\)-independent, as CaM\(_{1234}\), a CaM mutant deficient in Ca\(^{2+}\) binding,9 also copurified when coexpressed with the KCNQ1 CT (Figure 1B; see below). The interaction also was Mg\(^{2+}\)-independent, as no Mg\(^{2+}\) was added to the buffers. Although consistent with previous findings that CaM interacts with the CTs of other KCNQ family members,14,22,24 this was the first direct demonstration that CaM interacted with KCNQ1.

**The Stoichiometry Between CaM and the KCNQ1 CT is 1:1**

Although KCNQ CTs contain two potential CaM binding sites (IQ\(_1\) and IQ\(_2\) in Figure 1A),14 the stoichiometry between CaM and any KCNQ channel has not been determined. As a first estimate, we evaluated the purified KCNQ1 CT/CaM complex on size exclusion chromatography. The complex ran as a single species with an apparent M\(_s\) significantly greater than the sum of the 6xHis-tagged KCNQ1 CT and CaM (Figure 2A). The elution profile for the complex was unchanged whether saturating Ca\(^{2+}\) (1 mmol/L) or EGTA (5 mmol/L) was added to the column buffer, further supporting the hypothesis that the interaction between CaM and the KCNQ1 CT did not require Ca\(^{2+}\) (but see below). These data, however, did not yield an accurate assessment of the complex’s M\(_s\) or the stoichiometry between CaM and KCNQ1 CT. We therefore performed chemical cross-linking with disuccinimidyl glutarate (DSG), a homobifunctional, non-cleavable reagent reactive toward free amino groups, and subsequent polyacrylamide gel electrophoresis. After 5 minutes’ exposure to DSG at 25°C, none of the 36 kDa KCNQ1 CT input material remained; instead, a single predominant band migrating at ≈240 kDa was visible after Coomassie staining (Figure 2B, left). CaM was not visible in the input material (lane 1), having migrated off the 7% polyacrylamide gel (arrow depicting the gel’s dye-front), but it could be readily detected in the higher M\(_s\) cross-linked material by immunoblot (Figure 2B, right). Similar results were obtained with the KCNQ1 CT/CaM\(_{1234}\) complex (Figure 2B, right). The apparent M\(_s\) of the complex, which included both CaM and the KCNQ1 C-terminus, was less than 250 kDa on SDS-PAGE and was therefore consistent with a 4:4 (KCNQ1 CT:CaM) heteromultimer (estimated M\(_s\) of 212 kDa), rather than a 4:8 heteromultimer (estimated M\(_s\) of 280 kDa). This was consistent with one CaM molecule bound for each putative CaM binding site.

To establish whether one CaM molecule per KCNQ1 CT was sufficient, we fixed the stoichiometry at 1:1 by expressing a construct in which CaM or CaM\(_{1234}\) was joined by a 12× glycine (G\(_{12}\)) linker at the KCNQ1 construct’s CT (CT-G\(_{12}\)-CaM or CT-G\(_{12}\)-CaM\(_{1234}\)). These constructs were soluble without the coexpression of unlinked CaM (Figure 2C), and DSG cross-linking of the purified material yielded a product that migrated at ≈4× the M\(_s\) of the untreated material (Figure 2D). The concatemers also allowed us to determine relative affinities of CaM and CaM\(_{1234}\) for the KCNQ1 CT.

Figure 1. The KCNQ1 CT binds CaM. A, Schematic of KCNQ1 showing the CT construct expressed in Escherichia coli, the locations of the consensus CaM-binding IQ motifs (IQ\(_1\) and IQ\(_2\)), and the congenital long QT syndrome mutations studied. B, Coomassie-stained gel showing expression and purification of the KCNQ1 CT constructs in the presence and absence of CaM or CaM\(_{1234}\). Ext indicates bacterial cell extract; Sup, 100 000g supernatant; and P, metal-affinity purified protein.
CaM, but not unlinked CaM1234, co-purified on MAC (Figure 2F, double arrowhead); and \( \approx 200 \text{ kDa} \), consistent with a tetramer of complexes (Figure 2F, asterisk). These results suggested that the KCNQ1 CT/CaM complexes were able to assemble independent of amino acids 589 to 620.

**LQTS Mutations in Either IQ1 or IQ2 Disrupt CaM Interaction**

Because several LQTS mutations fall within or near the region bordered by the 2 IQ motifs, we next evaluated the effects of a subset of these mutations on the interaction with CaM. We chose to examine S373P in IQ1 (see ref. 29); R518X, which truncates the channel before IQ1 (see ref. 30); and R539W (see ref. 31), which lies just C terminal to IQ1; and thus outside of a predicted CaM interaction domain. Using the strategy detailed in Figure 1, we did not detect any interaction between CaM and the KCNQ1 CT with the S373P mutation (Figure 3A). This mutant KCNQ1 CT was excluded from the soluble fraction of the bacterial lysate, where all of the CaM was found. The expression of the R518X mutant was poor, suggesting that both IQ motifs were necessary for recombinant expression and CaM interaction. Further, CaM1234 did not interact with either construct, suggesting that CaM and CaM1234 bound at the same site. In contrast, the KCNQ1 CT with R539W could be copurified with CaM, similar to the wild-type (WT) KCNQ1 CT. These data suggested that both putative CaM binding sites contributed to the CaM interaction domain.

CaM binding to the KCNQ1 CT correlated with the resultant functional currents of these LQTS mutants. When expressed in oocytes, KCNQ1 channels with S373P or R518X failed to generate K+ currents (current amplitude was significantly different from those recorded from uninjected control oocytes over a range of test potentials from \(-60\) to \(+60\) mV (Figure 3B and 3C); for R518X this was not unexpected, as these channels lack the trafficking domain\(^{28}\) and may also lack key elements that determine assembly (Figure 2F and 2G). R539W channels generated K+ currents (current amplitude was significantly greater than uninjected oocytes for isochronic current ampli-
tude at the end of a 2-second test pulse to \(+60\) mV; \(n=5\) for each, \(P<0.01\)), but peak current amplitude was markedly reduced compared with WT channels (19\(\pm\)4\% ; \(n=5\) for each, \(P<0.01\) at \(+60\) mV) and the \(V_{50}\) for activation was 6.9 mV more depolarized (\(-19.3\) mV for WT versus \(-12.4\) mV for R539W). The reduction in peak current, although consistent with the expected loss-of-function phenotype for LQT1 mutations,\(^{32}\) differed from findings in a previous study.\(^{31}\) This may be due, however, to use of different heterologous expression systems. Thus, the functional data suggested that the pathogenesis of at least some LQT1 mutations may be due to their effects on CaM interaction with the KCNQ1 CT.

When coexpressed with the CT-G\(_{12}\)-CaM construct, unlinked CaM, but not unlinked CaM1234, co-purified on MAC (Figure 2E, arrow). This suggested that unlinked CaM but not CaM1234, could complete with linked CaM for association with the KCNQ1 CT. Thus, although CaM could bind to the KCNQ1 CT in the absence of Cu\(^{2+}\), Cu\(^{2+}\)/CaM had a higher relative affinity.

We used the in vitro formation of the KCNQ1 CT tetramer to test definitively whether amino acids 589 to 620 were necessary for assembly, as this region had been previously identified as the KCNQ1 assembly domain,\(^{27}\) but a recent study suggested instead that it functioned as a trafficking signal.\(^{28}\) We generated a KCNQ1 CT truncated after amino acid 555 (\(\Delta S555, M_0 \approx 24\) kDa) to exclude 589 to 620. As with the full length KCNQ1 CT, the \(\Delta S555\) required CaM coexpression to generate soluble material suitable for MAC purification (not shown). Chemical cross-linking with DSG generated products of \(\approx 45\) kDa, consistent with the inclusion of one \(\Delta S555\) KCNQ1 CT and one CaM molecule (Figure 2F, single arrowhead); \(\approx 100\) kDa, consistent with a dimer of the \(\Delta S555\) KCNQ1 CT/CaM complex (Figure 2F, double arrowhead); and \(\approx 200\) kDa, consistent with a tetramer of complexes (Figure 2F, asterisk). These results suggested that the KCNQ1 CT/CaM complexes were able to assemble independent of amino acids 589 to 620.
LQTS Mutations That Disrupt CaM Interaction Are Dominant-Negative

On the basis of the CaM-dependent assembly of KCNQ1 CTs (Figure 2), we hypothesized that defects in channel assembly could explain the pathogenesis of LQTS mutations that disrupt CaM interaction. The specific expectation was that these autosomal dominant mutants would prevent assembly in a dominant-negative manner. We generated a concatemer of a WT and a mutant KCNQ1 subunit, which allowed us to fix the stoichiometry between the WT and mutant channels at 1:1. Although other stoichiometries are possible (ie, 3:1 or 1:3), this method provided a straightforward way to quantitatively assess the effects of including mutant channels within the tetramer and assured that the mutant was expressed in the context of the WT channel, thereby abolishing any concern about differential expression or translation efficiency when WT and mutant channel cRNAs are injected separately. Concatemers of WT KCNQ1 subunits generated WT-like currents (Figure 3D). Inclusion of R539W in the concatemer, however, reduced current amplitude, suggesting that these currents were CaM insensitive. As a first step, we tested whether changes in intracellular Ca\(^{2+}\) altered channel function by measuring effects on KCNQ1 currents in oocytes after chelation of [Ca\(^{2+}\)]\(_i\) to subphysiological levels by injection of the Ca\(^{2+}\) chelator BAPTA. We recorded currents from the same oocyte 2 minutes after injection of 50 to 100 nL of 100 mmol/L BAPTA (in 10 mmol/L HEPES, pH 7.5), which, in oocytes with a volume of \(\approx 1\) \(\mu\)L, achieved an estimated intracellular BAPTA concentration of 5 to 10 mmol/L. For homeric KCNQ1 channels, inactivation is readily revealed by a transient increase in the magnitude of the tail current but is not usually obvious during depolarizing pulses.\(^{35}\) Ca\(^{2+}\) chelation, however, unmasked prominent inactivation during the depolarizing pulses (Figure 4A, arrow). Moreover, as seen in Figure 4B, injection of 100 nL BAPTA reduced current amplitude by more than 50% over the entire range of test potentials. In the presence of the \(\beta\) subunit KCNE1, which eliminates voltage-dependent inactivation under standard recording conditions,\(^{35}\) chelation of Ca\(^{2+}\) did not produce inactivation during the depolarizing pulses (Figure 4C), although it was even more potent at reducing current amplitude; 100 nL BAPTA reduced current amplitude to \(\approx 85\%\) (Figure 4C). These results were not due to a non-specific effect of BAPTA, as we obtained similar results with EGTA, nor were they due to cell damage during the injection, change

**KCNQ1 Channels Are Sensitive to Ca\(^{2+}\) and CaM**

The interaction of CaM with the KCNQ1 CT and the dominant-negative effect of mutations that abolished CaM interaction prompted us to test next whether associated CaM could provide Ca\(^{2+}\) sensitivity to channel function. A Ca\(^{2+}\)-dependent increase of \(I_{Ks}\) in the heart had been observed\(^{19}\) and attributed to CaM,\(^{24}\) but other studies had suggested different Ca\(^{2+}\)-sensitive mediators\(^{20}\) or suggested that KCNQ1 currents were Ca\(^{2+}\) insensitive. As a first step, we tested whether changes in intracellular Ca\(^{2+}\) altered channel function by measuring effects on KCNQ1 currents in oocytes after chelation of [Ca\(^{2+}\)]\(_i\) to subphysiological levels by injection of the Ca\(^{2+}\) chelator BAPTA. We recorded currents from the same oocyte 2 minutes after injection of 50 to 100 nL of 100 mmol/L BAPTA (in 10 mmol/L HEPES, pH 7.5), which, in oocytes with a volume of \(\approx 1\) \(\mu\)L, achieved an estimated intracellular BAPTA concentration of 5 to 10 mmol/L. For homeric KCNQ1 channels, inactivation is readily revealed by a transient increase in the magnitude of the tail current but is not usually obvious during depolarizing pulses.\(^{35}\) Ca\(^{2+}\) chelation, however, unmasked prominent inactivation during the depolarizing pulses (Figure 4A, arrow). Moreover, as seen in Figure 4B, injection of 100 nL BAPTA reduced current amplitude by more than 50% over the entire range of test potentials. In the presence of the \(\beta\) subunit KCNE1, which eliminates voltage-dependent inactivation under standard recording conditions,\(^{35}\) chelation of Ca\(^{2+}\) did not produce inactivation during the depolarizing pulses (Figure 4C), although it was even more potent at reducing current amplitude; 100 nL BAPTA reduced current amplitude to \(\approx 85\%\) (Figure 4C). These results were not due to a non-specific effect of BAPTA, as we obtained similar results with EGTA, nor were they due to cell damage during the injection, change
in pH, or the result of an increased intracellular osmotic load. Microinjection of 10 mmol/L HEPES with 500 mmol/L sucrose caused no diminution of current (Figure 4A and 4C). Further, these effects were specific to KCNQ1, as we observed no changes in currents in oocytes expressing Cav2.1 Ca\(^{2+}\)/H11001 channels after an identical BAPTA injection (not shown). Thus, reducing [Ca\(^{2+}\)/H11001]i acutely to subphysiological levels had pronounced effects on channel inactivation and current amplitude.

We next examined the effects of raising intracellular Ca\(^{2+}\)/H11001 by injecting 100 nL of 100 mmol/L CaCl\(2\)/H11001 in 10 mmol/L HEPES. To minimize activation of the endogenous Ca\(^{2+}\)/H11001-activated Cl\(^-\)/H11002 current, we added 0.5 mmol/L niflumic acid to the bath solution. This had no effect on KCNQ1 currents during our standard test protocols (not shown), but was successful in eliminating the contamination of this endogenous current. We observed no significant change in currents after injection of Ca\(^{2+}\)/H11001 during a repetitive steps to 40 mV for oocytes uninjected with cRNA (Figure 4E). Peak current amplitudes recorded from control oocytes with this protocol ranged from 0.05 to 0.22 \(\mu\)A. In oocytes expressing KCNQ1, however, the same treatment caused a time-dependent increase in peak current that lasted for more than 2 minutes (Figure 4E). This was seen in five different batches of oocytes, and the observed fractional increase was evident when the peak amplitude ranged from 2.6 to 10.7 \(\mu\)A before intracellular injection of CaCl\(2\)/H11001. These results were consistent with previous reports showing that Ca\(^{2+}\)/H11001 enhances I\(\text{Ks}\)/H11001, and, together with the Ca\(^{2+}\)/H11001 chelator experiments (Figure 4A through 4D), were the first demonstrations of an effect of Ca\(^{2+}\)/H11001 on KCNQ1 in a heterologous expression system.

We used two approaches to ascertain whether these Ca\(^{2+}\)/H11001-regulated effects were mediated by CaM. First, we attempted to replace endogenous CaM with the Ca\(^{2+}\)/H11001-insensitive CaM\(_{1234}\) by overexpression and to test whether the Ca\(^{2+}\)/H11001 sensitivity remained. Although coinjection of CaM cRNA increased isochronic current amplitude (52 \(\pm\) 24% larger at the end of a 2-second test pulse to 60 mV compared with currents from oocytes injected with KCNQ1 only), coinjection of CaM\(_{1234}\) had no effect (Figure 5A) on current amplitude. Coexpression of neither CaM nor CaM\(_{1234}\) cRNA altered KCNQ1 current sensitivity to changes in [Ca\(^{2+}\)/H11001]. Injection of 50 nL BAPTA caused an \(\approx\)25% reduction of current amplitude whether CaM or CaM\(_{1234}\) cRNA had been coinjected (n=6 for each, \(P>0.05\) for all test potentials between \(-20\) and \(+50\) mV); similarly, coinjection of neither CaM nor CaM\(_{1234}\) cRNA affected the Ca\(^{2+}\)/H11001-mediated increase in current amplitude over 150 sec-
onds after injection of 100 nL CaCl₂ (n = 5 to 6, P > 0.05 for all time points between 0 and 150 s) or altered inactivation. The absence of an apparent effect with CaM₁₂₃₄ coinjection could not be attributed to ineffectual CaM₁₂₃₄ expression because in control experiments, this same cRNA effectively blocked Ca²⁺/CaM-mediated Ca²⁺-dependent inactivation of Ca₁.2 Ca²⁺ channels (not shown). Instead, we expected that the ineffectiveness of CaM₁₂₃₄ could be explained by a failure of CaM₁₂₃₄ to compete with endogenous CaM, as suggested by the apparent higher affinity of CaM for the KCNQ1 CT compared with CaM₁₂₃₄ (Figure 2E). We therefore used a concamer strategy, linking CaM₁₂₃₄ to the KCNQ1 N terminus to increase its local concentration in the vicinity of KCNQ1 and provide a more effective competitor for endogenous CaM. This also restricted CaM₁₂₃₄ to the channel and thus avoided any pleiotropic effects of CaM₁₂₃₄ overexpression on other CaM-signaling pathways. CaM₁₂₃₄–KCNQ1 concamers revealed prominent inactivation during the depolarizing pulse (Figure 5B, arrow), similar to that observed with Ca²⁺ chelation (Figure 4B). CaM–KCNQ1 concamers, in contrast, produced currents similar to those from WT KCNQ1 and revealed inactivation only in the transient increase in tail currents (Figure 5B). A quantitative assessment of inactivation was made by comparing the ratio of the peak current during the first 50 ms to the current at the end of the 2-second depolarizing pulse (Figure 5C). For currents from KCNQ1 channels or CaM-KCNQ1 channels, current amplitude continued to rise after the first 50 ms, so that the ratio was significantly less than unity. For currents from CaM₁₂₃₄–KCNQ1 channels or KCNQ1 channels after BAPTA injection (Figure 4B), prominent inactivation during the depolarizing pulse increased the ratio significantly.

**Discussion**

Formation of ion channels requires a complex series of coordinated events, beginning with protein synthesis and ending with insertion of functional channels into the appropriate target membrane. For channels that function as multimeric complexes, subunit assembly with the appropriate stoichiometry and types of auxiliary subunits occurs early in this process in the endoplasmic reticulum. CaM has been recognized as a dissociable subunit for several ion channels and as a constitutive subunit for others, and thereby confers on mature channels that have reached their target membrane Ca²⁺ dependence to a variety of functions, such as channel activation or inactivation. In those cases in which CaM is a constitutive subunit, its presence during channel formation affords the opportunity to confer regulation additionally to channel biosynthesis, as has been previously shown for trafficking of SK K⁺ channels.

The data presented here showing that CaM participates in assembly of KCNQ1 homotetramers suggest that CaM regulation of channel formation may be widespread and therefore add to the diversity of roles CaM, as an auxiliary subunit, plays in ion channel regulation. The implications for disease are extensive. At a general level, these findings suggest new approaches to understanding pathophysiology in the many disease states in which alterations in levels of Ca²⁺ and CaM have been found and effects on channel function have been described, such as epilepsy and heart failure. In the particular case of CaM interaction with KCNQ1, we also provide a new example of an ion channel defect that leads to LQTS: mutations that disrupt CaM interaction block proper channel assembly in a dominant-negative fashion. A role for CaM in channel assembly is consistent with studies that showed that determinants proposed to govern KCNQ2/KCNQ3 subunit interaction fall within the homologous CaM binding domain and therefore likely also explains the etiology of epileptogenic mutations in KCNQ2 that fall within this region. Indeed, two of these epileptogenic mutants and several artificial KCNQ2 mutants have been tested functionally and, similar to what we observed for the CaM binding mutants in KCNQ1 (Figure 3B and 3C), these mutant KCNQ2 channels did not yield K⁺ currents.

We also demonstrated that a stoichiometry of one CaM per KCNQ1 CT is sufficient for interaction, even though mutations in both IQ motifs result in loss of CaM binding. The possibility that both IQ motifs contribute to a complex CaM binding pocket is entirely consistent with structural data showing non-canonical interactions between CaM and multiple domains of target proteins. The ability of CaM₁₂₃₄, a surrogate for Ca²⁺-free apocalmodulin, to bind to the KCNQ1 CT is further indication that the interaction between CaM and KCNQ1 is non-canonical.

Our data also provide new evidence that Ca²⁺ regulates KCNQ1 currents and are the first data to show Ca²⁺ sensitivity of KCNQ1 in a heterologous expression system. The most striking finding was that chelation of [Ca²⁺], below physiological levels or the incorporation of a Ca²⁺-insensitive CaM induced prominent inactivation during the depolarizing pulses and reduced current amplitude. These two phenomena may not be directly coupled, as Ca²⁺ chelation reduced current amplitude from channels expressing KCNQ1 + KCNE1 but did not induce prominent inactivation. This alteration in inactivation may have important physiological effects outside the heart, where KCNQ1 is not invariably associated with KCNE1. Another finding was that increases in [Ca²⁺], above basal levels enhance KCNQ1 current. The mechanism for this is not yet clear, but it could involve partial relief of inactivation. Thus, data presented here offer new insights into mechanisms of cardiac arrhythmias and epilepsy, two major disorders that result from mutations in the KCNQ family of K⁺ channels, opening an important window onto the elusive triggers that cause the rare and episodic arrhythmias or seizures in patients carrying mutations in these channels.

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**References**


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KCNQ1 assembly and function is blocked by LQTS mutations that disrupt interaction with calmodulin

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MATERIAL AND METHODS

Molecular Biology – Bacterial expression constructs for CaM and CaM\textsubscript{1234} have been described.\textsuperscript{1} Human KCNQ1 (GenBank # AF000571), a generous gift of S.O. Marx (Columbia) was cloned by PCR into pGHE for recordings in \textit{Xenopus} oocytes. The KCNQ1 concatemer was generated by placing an additional KCNQ1 cDNA, created by PCR, into the 5′ BamHI site of the pGHE-KCNQ1 described above. This added a Gly-Ser dipeptide between the two KCNQ1 repeats. The CaM-KCNQ1 concatemers were created with a similar strategy. For expression in \textit{E. coli}, a PCR fragment containing the C terminus (encoding amino acids 354-676) was cloned into pET28a+ (Novagen). LQTS mutations were generated using QuikChange (Stratagene). Human KCNE1 (GenBank # NM_000219), obtained from an EST (Open Biosystems), was cloned into pGHE by a PCR strategy.

Electrophysiology - Two electrode voltage clamp recordings from \textit{Xenopus} oocytes followed \textit{in vitro} transcription and microinjection as described.\textsuperscript{2} Recordings were performed 2-5 days after injection of cRNA (ng / oocyte) for KCNQ1 (0.1) and, where indicated, CaM (0.1), CaM\textsubscript{1234} (0.1), and/or KCNE1 (.02). The bath solution (ND-96) contained (in mM): 96 NaCl, 2 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 HEPES (pH 7.5). For experiments to chelate or raise intracellular Ca\textsuperscript{2+}, 50-100 nl of 100 mM 1,2-bis(2-aminophenoxy)ethane-\textit{N,N,N′,N′}-tetraacetic acid (BAPTA), ethylene glycol-bis(2-aminoethylether)-\textit{N,N,N′,N′}-tetraacetic acid (EGTA), 100 \textmu M CaCl\textsubscript{2}, or 500 mM sucrose (in 10 mM HEPES, pH 7.4) was micro-injected after obtaining the baseline recording and then the recording protocol was repeated. To prevent endogenous Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents, the bath solution was supplemented with 0.5 mM niflumic acid in experiments in which CaCl\textsubscript{2} was micro-injected.
Protein expression and purification—The KCNQ1 CT constructs in pET28a+, with and without the CaM or CaM1234 expression plasmids, were transformed into BL-21 (DE3) cells by electroporation. Cells were grown to an OD_{600}=0.6 and then expression was induced with the addition of 1 mm IPTG for 72 h at 16 °C. Cells were suspended in binding buffer containing 500 mM NaCl, 20 mM Tris, 25% (v/v) glycerol, pH 7.5, supplemented with 5 mM imidazole and Complete EDTA-free protease inhibitor cocktail (Roche). Extracts were prepared by passage through a French pressure cell and then centrifuged at 100,000 x g for 90 min. The supernatants were applied to Talon metal affinity resin (Clontech) and the column was washed in binding buffer supplemented with 30 mM imidazole. Proteins were eluted in binding buffer supplemented with 250 mM imidazole, aliquoted, and stored at -20 °C in 25% glycerol for further use. Gel filtration was performed over a Superdex 200 HR 10/30 column on an AKTA FPLC (Amersham Biosciences) in 500 mM NaCl, 20 mM Tris, pH 7.5, and 10 μM CaCl₂.

Chemical cross-linking—The purified KCNQ1 CT/CaM complexes were dialyzed into 500 mM NaCl, 20 mM potassium phosphate pH 7.5, 10 μM CaCl₂, and 25% glycerol and then concentrated to 1 mg/ml. Disuccinimidyl glutarate 2.5 mM was added for 5 min at room temperature and the reaction was stopped with the addition of Tris-HCl pH 8.0 (final concentration, 0.5 M) for 15 min at room temperature before the addition of SDS-PAGE sample buffer.

Statistical analysis—Data are means ± s.e.m. Significant differences were assessed using Student’s t test at P < 0.05.
REFERENCES
