Analysis of the interactions between the C-terminal cytoplasmic domains of KCNQ1 and KCNE1 channel subunits

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Ion channel subunits encoded by KCNQ1 and KCNE1 produce the slowly activating K+ current (I\textsubscript{Ks}) that plays a central role in myocardial repolarization. The KCNQ1 \(\alpha\)-subunit and the KCNE1 \(\beta\)-subunit assemble with their membrane-spanning segments interacting, resulting in transformation of channel activation kinetics. We recently reported a functional interaction involving C-terminal portions of the two subunits with ensuing regulation of channel deactivation. In the present study, we provide evidence characterizing a physical interaction between the KCNQ1-CT (KCNQ1 C-terminus) and the KCNE1-CT (KCNQ1 C-terminus). When expressed in cultured cells, the KCNE1-CT co-localized with KCNQ1, co-immunoprecipitated with KCNQ1 and perturbed deactivation kinetics of the KCNQ1 currents. Purified KCNQ1-CT and KCNE1-CT physically interacted in pull-down experiments, indicating a direct association. Deletion analysis of KCNQ1-CT indicated that the KCNE1-CT binds to a KCNQ1 region just after the last transmembrane segment, but N-terminal to the tetramerization domain. SPR (surface plasmon resonance) corroborated the pull-down results, showing that the most proximal region (KCNQ1 amino acids 349–438) contributed most to the bimolecular interaction with a dissociation constant of \(\sim 4 \mu M\). LQT (long QT) mutations of the KCNQ1-CT, D76N and W87F, retained binding to the KCNQ1-CT with comparable affinity, indicating that these disease-causing mutations do not alter channel behaviour by disruption of the association. Several LQT mutations involving the KCNQ1-CT, however, showed various effects on KCNQ1/KCNE1 association. Our results indicate that the KCNQ1-CT and the KCNE1-CT comprise an independent interaction domain that may play a role in \(I_{Ks}\) channel regulation that is potentially affected in some LQTS (LQT syndrome) mutations.

Key words: ion channel, KCNE1, KCNQ1, long QT syndrome (LQTS), voltage-gated potassium channel.

INTRODUCTION

KCNQ1 (Kv7.1) is a voltage-gated K+ channel that performs diverse physiological functions in a variety of cell types [1]. KCNE1, a single transmembrane domain peptide, assembles with KCNQ1 to form \(I_{Ks}\) (slowly activating cardiac delayed rectifier K+ current), which is essential for normal myocardial repolarization (Figure 1A) [2,3]. Mutations in the KCNQ1 and KCNE1 genes that result in a loss of function of the channel by various means are a major cause of the inherited LQTS (LQT syndrome) characterized by ventricular arrhythmia, syncope and sudden death (often triggered by exercise) [4,5].

KCNQ1 is a member of the Kv7 channel subfamily that shares a common topology consisting of six transmembrane domains with intracellular N- and C-termini (Figure 1A). Individual channels comprise tetramers of KCNQ subunits with or without closely associated KCNE subunits for regulation. The C-termini of KCNQ channels are thought to possess multifunctional domains and play an essential role in \(K^+\) channel biological processes, including channel folding, assembly, trafficking and gating [6–10]. Many LQTS mutations in the KCNQ1-CT (KCNQ1 C-terminus) result in hereditary cardiac arrhythmia syndromes, including the autosomal-dominant Romano-Ward syndrome and the autosomal-recessive Jervell and Lange-Nielsen syndrome. Sequence analysis predicts that the C-terminus of the KCNQ family contains four \(\alpha\)-helices (Figure 1A). Helices A and B harbour binding sites for CaM (calmodulin) that is required for the proper folding of the channel C-terminus [8,11,12]. Confocal immunocytofluorescence analysis suggests that helix C may be involved in channel trafficking [10]. The tetramerization domain of the KCNQ channels is located within helix D of the C-terminus [9,10].

As a \(K^+\) ion channel ancillary subunit, KCNE1 modulates the gating of KCNQ1 and other \(K^+\) channels. Previous studies have shown that the transmembrane region of KCNE1 adopts an \(\alpha\)-helical structure [13] and interacts with the KCNQ1 pore-lining S6 domain to modulate channel activity [14]. Additional studies have identified the specific amino acids within the transmembrane region that have an impact on KCNQ1 activation gating and differentiate between KCNE1- and KCNE3-mediated effects [14–17]. A functional interaction between the KCNQ1-CT and the KCNE1-CT (KCNQ1 C-terminus) was first suggested by chimera studies we conducted between KCNQ1 and KCNQ4 in which full recapitulation of KCNQ1-dependent regulation required both the KCNQ1 pore structures and the C-terminus [14]. We also reported results supporting a functional interaction between the KCNQ1-CT and the KCNE1-CT that regulates channel deactivation [18].

Abbreviations used: CHO, Chinese-hamster ovary; FBS, fetal bovine serum; FRET, fluorescence resonance energy transfer; GST, glutathione transferase; HEK, human embryonic kidney; \(I_{Ks}\), slowly activating cardiac delayed rectifier K+ current; KCNE1-CT, KCNE1 C-terminus; KCNQ1-CT, KCNQ1 C-terminus; LQT, long QT; LQTS, LQT syndrome; MBP, maltose-binding protein; Ni-NTA, Ni\textsuperscript{2+}-nitrilotriacetate; RU, response unit(s); SPR, surface plasmon resonance; VDA, voltage-dependence of activation.

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In that study we also showed that recombinant KCNE1-CT co-precipitated with full-length KCNQ1. Recently, Haitin et al. [19] have shown that the KCNQ1-CT co-immunoprecipitated with the KCNE1-CT in vitro and provided FRET (fluorescence resonance energy transfer) evidence for a dynamic interaction during channel gating.

KCNE family sequence homology is primarily limited to a few conserved or identical residues in the transmembrane segment and the proximal intracellular segment (Figure 2A). Portions more C-terminal in the cytoplasmic tail (after KCNE Phe78) diverge within the KCNE family. LQTS mutations within the KCNE1-CT (S74L, D76N, Y81C and W87F) reduce Ik1 [5,20], suggesting that this region of the protein is involved in subunit interaction. Chimaeric and deletion analysis furthermore supports the importance of the KCNE1-CT for KCNQ1 gating [21]. Compared with the considerable knowledge concerning precise physical and functional interactions between transmembrane segments of KCNQ1 and KCNE1, less is understood with respect to the C-terminal interaction between these proteins; a lack of three-dimensional structure of the KCNQ1–KCNE1 channel complex limits a full understanding of the molecular basis of subunit interaction and the mechanism of gating modulation. In the present study, we used biophysical and biochemical methods to characterize the physical interaction between the C-terminal cytoplasmic domains of KCNQ1 and KCNE1.

MATERIALS AND METHODS
Cloning and expression of the KCNQ1-CT and the KCNE1-CT in Escherichia coli

The DNA fragments encoding the KCNQ1-CT (Q1CF, Q1C1, Q1C1A, Q1C1B, Q1C2 and Q1C3; illustrated graphically in Figure 2C) and the KCNE1-CT (E1C in Figure 2C) were obtained by PCR amplification of the human KCNQ1 and KCNE1 genes using Pfu DNA polymerase and the primers containing NdeI and HindIII restriction sites. The PCR products were cloned into an NdeI- and HindIII-digested pET23a(+)+ plasmid (Novagen) such that the His6 tag was followed by a linker (ELAA) and the KCNQ1 fragment. The amino acid boundaries for each fragment are as follows: Q1CF, 349–676; Q1C1, 349–480; Q1C1A, 349–398; Q1C1B, 349–438; Q1C2, 480–570; and Q1C3, 570–676. The MBP (maltose-binding protein) fusion proteins of KCNQ1 (MBP–KCNQ1) MBP–Q1CF, MBP–Q1C1, MBP–Q1C2 and MBP–Q1C3 were constructed by cloning the DNA fragments of Q1CF, Q1C1, Q1C2 and Q1C3 into an EcoRI- and HindIII-digested pMAL-2C vector. These recombinant plasmids were expressed in the E. coli strain BL21(DE3) pLysS grown at 37°C to an D600 of 0.5 in LB (Luria–Bertani) medium containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. Cultures were induced with 0.5 mM IPTG (isopropyl β-D-thiogalactoside).
and growth was continued for an additional 6–8 h at 25°C.

The human KCNE1 gene was obtained by PCR amplification of the human heart cDNA using Pfu DNA polymerase and the primers containing NdeI and HindIII restriction sites respectively, and a His6 affinity tag. The PCR products were cloned into an NdeI- and HindIII-digested pET23a(+) plasmid (Novagen). The recombinant plasmid was expressed using Expressway Cell-Free E. coli Expression System (Invitrogen), according to the manufacturer’s instructions. The protein synthesis reaction mixture was centrifuged at 20 000 g for 15 min at 4°C. The pellet was suspended in 20 mM Tris/HCl (pH 7.2) and 150 mM NaCl, and centrifuged three times for 15 min at 4°C. The pellet was suspended in binding buffer [20 mM Tris/HCl (pH 7.2), 150 mM NaCl, 8 M urea and 0.1% SDS] and centrifuged at 20 000 g for 15 min at 25°C to remove insoluble debris. The supernatant containing solubilized KCNE1 product was incubated with Ni2+-nitrilotriacetate resin, and shaken at 25°C for 1–2 h. The resin was then packed into a gravity-flow column and washed with 10-fold bed volumes of binding buffer, followed by washing with 5-fold bed volumes of wash buffer [20 mM Tris/HCl (pH 7.2), 150 mM NaCl, 0.5% DDM (n-dodecyl β-D-maltoside) and 5 mM 2-mercaptoethanol]. The KCNE1 product was then eluted using a wash buffer containing 200 mM imidazole (pH 6.2).

**Mutagenesis**

The LQT mutants of the KCNE1-CT (D76N and W87F) and that of KCNQ1 (Q357R, R366W, A371T, S373P, T391I and W392R) (Figure 2B) were generated using the QuikChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions, and pET23a(+):E1C as the template. The mutants were sequenced in their entirety to ensure that no undesired mutations occurred. The mutants were expressed in the E. coli strain BL21(DE3) pLysS cells as above.

**Expression of KCNQ1, KCNQ1-CT and KCNE1-CT in HEK (human embryonic kidney)-293 and CHO (Chinese-hamster ovary) cells**

The Myc-tagged human KCNQ1 expression vector and HEK-293 cells stably expressing KCNQ1 (HQ5) were generated as described previously [18]. The DNA fragments encoding the KCNE1-CT and the KCNQ1-CT were amplified by PCR with Taq DNA polymerase, and cloned into p3XFLAG-CMV-10 vector (Sigma) to generate N-terminal FLAG-tagged KCNE1-CT and N-terminal FLAG-tagged KCNQ1-CT expression plasmids. For electrophysiological experiments CHO cells were chosen for their low electrical background.

HEK-293 cells were grown in a 5% CO2 humidified atmosphere at 37°C in RPMI 1640 medium (Mediatech) containing 10% (v/v) FBS (fetal bovine serum; HyClone),
100 units/ml penicillin and 100 μg/ml streptomycin (Mediatech). CHO cells were grown in Ham’s F12 (Mediatech) supplemented with 10% (v/v) FBS (Hyclone) and penicillin/streptomycin (Mediatech). HQ5 cells, a HEK-293 clonal cell line stably expressing KCNQ1, were transfected with FLAG-tagged KCNE1-CT expression plasmids using Lipofectamine™ 2000 (Invitrogen). HEK-293 and CHO cells were co-transfected with FLAG-tagged KCNQ1-CT and FLAG-tagged KCNE1-CT using Lipofectamine™ 2000 (Invitrogen).

Electrophysiology

Transfected CHO cells were grown on sterile glass coverslips and placed in an acrylic/polystyrene perfusion chamber (Warner Instruments) mounted in an inverted microscope outfitted with fluorescence optics and patch pipette micromanipulators. The external bath consisted of 150 mM NaCl, 1.8 mM CaCl₂, 4 mM KCl, 1 mM MgCl₂, 5 mM glucose and 10 mM Hepes (pH 7.4) at room temperature (20–22°C). The internal pipette solution contained 126 mM KCl, 4 mM K-ATP, 1 mM MgSO₄, 5 mM EGTA, 0.5 mM CaCl₂ and 25 mM Hepes (pH 7.2) at room temperature. The whole-cell configuration of the patch-clamp technique was used to measure K⁺ currents using a MultiClamp 700B patch-clamp amplifier controlled via PC running pClAMP10 acquisition and analysis software (Axon Instruments). Pipette tips were heat-polished to obtain a resistance of 2–3 MΩ in the test solutions. The pipette offset potential in these solutions was set to zero just prior to seal formation. The junction potential for these solutions was set to zero just prior to seal formation. The internal pipette solution contained 126 mM KCl, 4 mM K-ATP, 1 mM MgSO₄, 5 mM EGTA, 0.5 mM CaCl₂ and 25 mM Hepes (pH 7.2) at room temperature. The whole-cell configuration of the patch-clamp technique was used to measure K⁺ currents using a MultiClamp 700B patch-clamp amplifier controlled via PC running pClAMP10 acquisition and analysis software (Axon Instruments). Pipette tips were heat-polished to obtain a resistance of 2–3 MΩ in the test solutions. The pipette offset potential in these solutions was set to zero just prior to seal formation. The junction potential for these solutions was set to zero just prior to seal formation.

Purification and analysis of the proteins

Cells (~10 g) were suspended in 40 ml of 50 mM Tris/HCl (pH 7.8), 150 mM NaCl and 1 mM 2-mercaptoethanol containing protease inhibitors (Boehringer Mannheim) and 100 μg/ml DNase I. After sonication, cells debris was removed by centrifugation at 16,060 g for 45 min at 4°C. The supernatant was dialysed against 50 mM Tris/HCl (pH 7.8) containing 150 mM NaCl and 1 mM 2-mercaptoethanol for 2 h at 4°C. After centrifugation, the supernatant was applied to a 4-ml Ni-NTA column. Briefly, cells (~10 g) were suspended in MBP buffer [20 mM Tris/HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA and 1 mM 2-mercaptoethanol containing protease inhibitors (Boehringer Mannheim) and 100 μg/ml DNase I]. Cells were lysed by sonication, and debris was removed by centrifugation. The supernatant was loaded on to a 3-ml amylose column (New England BioLabs), and the proteins were eluted with three column volumes of MBP buffer containing 10 mM maltose. After concentration to 5 ml, the proteins were applied on to a 1.6 cm × 70 cm Superdex 200 gel-filtration column. The proteins were eluted with 20 mM Hepes (pH 7.8) containing 150 mM NaCl, 5 mM potassium phosphate and 1 mM 2-mercaptoethanol at 0.5 ml/min, and the correct size fractions were pooled and concentrated.

Protein concentration was measured using a Bio-Rad Laboratories protein assay kit with BSA as a standard. The purity of proteins was determined by SDS/PAGE following the method of Laemmli. Immunoblot assay was performed to confirm the specificity of each peptide. The native molecular mass was estimated by using a Superdex 200 gel-filtration column calibrated using Bio-Rad Laboratories molecular-mass markers. ESI (electrospray ionization)–MS was performed on the purified protein to determine the subunit molecular mass of the KCNE1-CT and KCNQ1-CT fragments.

Immunoprecipitation and Western blotting

At 72 h post-transfection, cells were lysed with ice-cold NDE buffer [1% NP-40 (Nonidet P40), 0.4% deoxycholic acid, 5 mM EDTA, 25 mM Tris/HCl (pH 7.5) and 150 mM NaCl] with Complete™ protease inhibitor cocktail (Roche) for 15 min. Cell lysates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatants were incubated with a 1:100 dilution of the anti-KCNQ1 antibody (Santa Cruz Biotechnology) for 2 h at room temperature. Protein G–agarose (Pierce) was used to capture antibody–protein complexes, and bound proteins were eluted with SDS/PAGE loading buffer. Proteins were separated by SDS/PAGE (4–20% gels) and transferred on to nitrocellulose membranes.

The nitrocellulose membranes were blocked with TBS [Tris-buffered saline [25 mM Tris/HCl (pH 7.5) and 150 mM NaCl]] containing 10% (w/v) dried milk for 30 min, and then incubated with a 1:5,000 dilution of the anti-FLAG antibody (Sigma).

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for 2 h at room temperature. The membrane was washed with TBS-T (TBS containing 0.5% Tween 20) and then incubated with an HRP (horseradish peroxidase)-conjugated goat anti-(mouse IgG) secondary antibody (Pierce) for 1 h. Immunoreactivity was detected using an ECL (enhanced chemiluminescence) kit (Amersham).

**Immunofluorescence and confocal microscopy**

HEK-293 cells were transfected as above and grown on glass-bottomed 35-mm dishes. At 48 h after transfection, cells were fixed with 4% (v/v) paraformaldehyde for 20 min, permeabilized with 0.3% Triton X-100 in PBS for 5 min and blocked with PBS containing 5% (w/v) BSA for 30 min at room temperature. Fixed and blocked specimens were then incubated with a rabbit anti-FLAG antibody (dilution 1:100; Sigma), goat anti-Myc antibody (dilution 1:100; Santa Cruz Biotechnology) and mouse anti-cadherin antibody (dilution 1:100; Abcam) in PBS containing 5% (w/v) BSA and 0.1% Triton X-100 at room temperature for 2 h. After three washes with PBS, the cells were incubated with Alexa Fluor® 488 donkey anti-rabbit, Alexa Fluor® 568 donkey anti-goat and Alexa Fluor® 647 donkey anti-mouse secondary antibodies (dilution 1:800; Molecular Probes) in PBS for 1 h. Immunofluorescence was visualized using the Leica AOBS confocal microscope.

**SPR (surface plasmon resonance) analysis**

Quantitative interaction studies were carried out on a Biacore 3000 instrument using running buffer (20 mM potassium phosphate, 130 mM KCl, 3.4 mM EDTA and 0.005% Tween 20), KCNE1-CT or KCNE1-CT mutants D76N and W87F (ligand) were immobilized on the CM5 biosensor chip, and KCNQ1/KCNE1-CT fragments Q1C1, Q1C1, Q1C1A, Q1C1B, Q1C2 and Q1C3 or Q1C1 mutants (analytes) were injected over this interaction surface. An equivalent volume of each protein sample (analyte) was injected over a chip surface with no protein immobilized to serve as a blank phase for the background subtraction. MBP was injected over the surface with immobilized KCNE1-CT as the negative control. For kinetic analysis, different concentrations of analytes (0.1–12.5 μM) were tested. Data were analysed using BLAevaluation software (GE Healthcare). The binding curves of various Q1C concentrations were fitted to the two-state binding model described by eqn (1) [22]:

\[
A + B \stackrel{k_1}{\rightleftharpoons} AB^* \stackrel{k_2}{\rightleftharpoons} AB
\]

where A and B represent the analyte (Q1C domain) and the ligand (E1C domain), \(AB^*\) and AB represent an initial complex (transition state) and final docked complex (conformational change) respectively. The equilibrium constants of each binding steps are:

\[
K_{a1} = k_1/k_{-1} \quad \text{and} \quad K_{d2} = k_2/k_{-2}
\]

and the overall equilibrium binding constant \(K_a\) is calculated as

\[
K_a = K_{a1} \times (1 + K_{d2})
\]

The binding response \(R\) of the sensogram for each reaction was subsequently plotted as a function of analyte (Q1C) concentration \(A\) to determine the overall binding affinity. The data were fitted to eqn (4) [23]:

\[
R = A \times R_{max}/(A + K_a)
\]

where \(R_{max}\) is the maximum binding response value and \(K_a\) is the dissociation constant.

**RESULTS**

**Functional evidence of isolated KCNE1-CT interaction with KCNQ1 channels**

To determine whether the KCNE1-CT could interact with and affect KCNQ1 channels in situ, we co-transfected full-length KCNQ1 with the KCNE1-CT lacking the transmembrane segment into CHO cells. Subsequent voltage clamp analysis showed that the KCNE1-CT had a functional impact on the KCNQ1 K+ currents (Figure 1). The addition of free KCN1 peptide accelerated the voltage-dependent deactivation of both KCNQ1 alone and KCNQ1/KCNE1 channels (Figures 1B and 1C; \(P < 0.01\) between KCNQ1 and KCNQ1/KCNE1-CT at all voltages, and \(P < 0.05\) between KCNQ1/KCNE1 and KCNQ1/KCNE1/KCNE1-CT at \(-120\) mV). Additional changes included reduction in voltage-dependent inactivation of KCNQ1 channels, measured as the time-dependent relaxation from peak outward (illustrated by arrow marked i in Figure 1B) current during a sustained depolarizing step to 60 mV (20 ± 0.03% current inactivation for KCNQ1 compared with 5 ± 0.03%; \(P < 0.01; n = 6\)). Effects of free KCN1-CT expressed with KCNQ1 upon the voltage-dependence of activation were more complex and could not be adequately fitted to a Boltzman function (Figure 1D). Although it is an indirect measure, such an activation curve could be possible if there were various stoichiometries or saturation of possible interaction sites on the channel. We have previously observed similar results with KCNQ1 truncation mutants yielding a mixture of currents depending on the saturation of the KCNQ1 with KCN1 [18]. KCNQ1/KCNE1 channels were not appreciably perturbed by the soluble KCNE1 fragment. Activation kinetics of KCNQ1/KCNE1 channels were not significantly altered by the presence of free KCN1-CT.

**Co-immunoprecipitation of the KCNQ1-CT and the KCNE1-CT, and co-localization of KCNQ1 with the KCN1-CT in HEK-293 cells**

We have determined previously that the KCN1-CT could physically interact with full-length KCNQ1 in transfected cells [18]. To investigate the possible interaction between the KCN1-CT and the KCNQ1-CT, we conducted co-immunoprecipitation experiments. The KCN1-CT was co-expressed with the KCNQ1-CT in HEK-293 cells. At 72 h after transfection, the cells were lysed and immunoprecipitated with an antibody against KCNQ1. As shown in Figure 3(A), the KCN1-CT interacted with the KCNQ1-CT, and was immunoprecipitated with the anti-KCNQ1 antibody.

To determine whether the KCN1-CT and KCNQ1 can co-localize in HEK-293 cells, confocal microscopy was used to analyse the cellular distribution of the KCN1-CT. HQ5 cells (HEK-293 cell line stably expressing KCNQ1) transfected with the FLAG-tagged KCN1-CT were stained with antibodies to FLAG, KCNQ1 and cadherin (for surface marking) (Figure 3B). KCN1-CT immunofluorescence staining and that of KCNQ1 were largely overlapping with exclusion of the nucleus (Figures 3B and 3C, left-hand panels). When the KCN1-CT was transfected into the parent HEK-293 cell line without KCNQ1 the immunofluorescent distribution was uniform throughout the cell (Figures 3B and 3C, right-hand panels). These results were consistent and uniform between two separate transfection and immunofluorescence experiments. No visible
immunofluorescence staining was observed on untransfected HQ5 or HEK-293 cells.

Purification and properties of the recombinant KCNQ1-CT and KCNE1-CT

His$_{6}$-tagged human KCNQ1-CT domains and KCNE1-CT were purified from E. coli cells by using Ni-NTA His-Bind affinity and Superdex 200 gel-filtration column chromatography. Recombinant KCNQ1 fragments were designed to incorporate either the full-length C-terminus (Q1Cf) or smaller domains based on the predicted α-helices (Q1C1, Q1C1B, Q1C1A, Q1C2 and Q1C3) as shown schematically in Figure 2(C). Subunit molecular mass of His$_{6}$-tagged Q1Cf, Q1C1, Q1C1A, Q1C1B, Q1C2, Q1C3 and E1C recombinant proteins determined by SDS/PAGE was 37, 16, 5.8, 10, 11.5, 12 and 8.3 kDa respectively (Figures 4A–4C), consistent with the predicted molecular masses calculated from the amino acid sequences (Figure 2C). A Superose-6 gel-filtration column calibrated using Bio-Rad Laboratories molecular-mass markers was used to estimate the molecular masses of the native proteins, indicating that Q1Cf, Q1C1, Q1C2 and Q1C3 purify as higher-order oligomers. Gel-filtration chromatography on a Superdex 200 column was used to estimate the molecular mass of the native protein as 17 kDa, suggesting that the KCNE1-CT purifies as a dimer.

MBP–KCNQ1-CT domains were purified from E. coli cells by using an amylose affinity column, followed by Superdex 200 gel-filtration column chromatography. The subunit molecular mass of the MBP fusion proteins was estimated by SDS/PAGE (Figure 5).

Interaction between the cytoplasmic domains of KCNQ1 and KCNE1 in vitro

To further examine possible physical interactions between the KCNQ1-CT and the KCNE1-CT, we performed immunoprecipitation experiments using purified recombinant proteins after mixing in vitro. Physical binding of the KCNQ1-CT with the KCNE1-CT region was readily observed using His$_{6}$-tagged fusion
proteins (Figure 4D). To further characterize potential binding interactions of the KCNQ1-CT with the intracellular sequence of KCNE1, a series of MBP fusion proteins were prepared that contained the subdivided fragments of the KCNQ1-CT (Figure 5A). MBP pull-down assays using immobilized amylose showed that His6-tagged KCNE1-CT directly associated with the MBP–Q1C1 and MBP–Q1C2 regions, but not with MBP–Q1C3 (Figures 5B and 5C).

To further delineate the binding regions of KCNQ1 C1 for the KCNE1-CT, we generated Q1C1A and Q1C1B MBP fusion proteins for the MBP-binding test (Figures 1C and 4B). Pull-down assays showed that both Q1C1A and Q1C1B bind to the KCNE1 cytoplasmic domain (Figure 5D), indicating that the KCNQ1-CT region (amino acids 349–398) was sufficient for the interaction with His6-tagged KCNE1-CT.

To probe the specificity of the interaction between the C-terminal cytoplasmic domains of KCNQ1 and KCNE1 proteins in a more quantitative manner, SPR analysis was performed. KCNQ1-CT was immobilized on the biosensor chip equilibrated with running buffer, and increasing concentrations of a series of KCNQ1-CT fragments were then run over the chips and the binding of the proteins was monitored (Figures 6A and 6B). The kinetics of binding could not be globally fitted perfectly to a one-to-one or one-to-two binding model. This may reflect a higher oligomeric state or mixed stoichiometry of the purified KCNQ1-CT that could present more than one binding site with variable affinity dependent on degree of oligomer saturation. Thus the kinetics of binding determined on the sensor chip may be a summation of interactions between the KCNE1-CT on the chip surface and the oligomeric form of the KCNQ1-CT domains in solution. The sensorgrams (as shown in the example in Figures 6A and 6B) could be fitted to a two-state binding model (as outlined in Materials and methods section), indicating that full-length KCNQ1 (Q1C1) associated with KCNE1 involving a conformational change. A steady-state-binding response value...
The results of the present study characterize a physical and functional interaction between the two K⁺ channel subunits KCNQ1 and KCNE1 residing in their intracellular C-termini. The interaction is direct, not requiring other adaptor proteins or the transmembrane segments of the channel subunits. Heterologous expression of the soluble KCNE1-CT physically and functionally interacts with KCNQ1 channels in situ, and perturbs the pre-formed KCNQ1–KCNE1-CT interaction as assayed by voltage clamp analysis. Deletion analysis of KCNQ1 indicates that the KCNE1-CT binds robustly to a limited region of ~50 amino acids (349–398) encompassing helix A of the C-terminus, as measured by both MBP pull-down studies and SPR analyses. Our results suggest that more C-terminal regions (helices B–C or inter-helix linkers) play a role in the interaction with either direct binding (pull-down assays; Figure 5D) or participation in conformational changes in the binding site that allow more rapid association (SPR data; Figure 6 and Table 1). Our present results are largely in agreement with those of Haitin et al. [19] in terms of the C-terminal interaction between KCNQ1 and KCNE1. The main exception being that GST (glutathione transferase) pull-down experiments in their study showed that KCNQ1-CT truncations after the proximal helix C diminished KCNE1 binding, leading to the conclusion that helix C was the binding site. Our present results suggest that portions of helix A and its linker to the transmembrane S6 bind KCNE1 as determined using both pull-down and SPR assays, whereas the fragment encompassing isolated helices B and C binds in solution but not with immobilized KCNE1.

Several differences in the experimental approach and analyses may account for apparent discrepancies between the two studies.

## Table 1

<table>
<thead>
<tr>
<th>KCNQ1-CT</th>
<th>$K_d$ (μM)</th>
<th>$R_{max}$ (RU)</th>
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<tbody>
<tr>
<td>Q1C1</td>
<td>3.2 ± 0.7</td>
<td>4729 ± 391</td>
</tr>
<tr>
<td>Q1C1</td>
<td>4.6 ± 1.4</td>
<td>1202 ± 162</td>
</tr>
<tr>
<td>Q1C1 A371T</td>
<td>6.5 ± 0.7</td>
<td>3039 ± 161</td>
</tr>
<tr>
<td>Q1C1 D375R</td>
<td>2.4 ± 0.9</td>
<td>1011 ± 129</td>
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<tr>
<td>Q1C1 R366W</td>
<td>*ND</td>
<td>178 ± 39</td>
</tr>
<tr>
<td>Q1C1 S373P</td>
<td>7.0 ± 1.0</td>
<td>4586 ± 627</td>
</tr>
<tr>
<td>Q1C1 T391I</td>
<td>*ND</td>
<td>213 ± 10</td>
</tr>
<tr>
<td>Q1C1 W392R</td>
<td>*ND</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Q1C1A</td>
<td>*ND</td>
<td>56 ± 9.1</td>
</tr>
<tr>
<td>Q1C1B</td>
<td>2.4 ± 0.6</td>
<td>936 ± 75</td>
</tr>
<tr>
<td>Q1C2</td>
<td>*ND</td>
<td>&lt;25</td>
</tr>
<tr>
<td>Q1C3</td>
<td>*ND</td>
<td>&lt;25</td>
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</tbody>
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Values are means ± S.E.M. *ND, not determined due to low interaction signal.

## Table 2

<table>
<thead>
<tr>
<th>Immobilized KCNE1-CT</th>
<th>$K_d$ (μM)</th>
<th>$R_{max}$ (RU)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>4.5 ± 1.1</td>
<td>102 ± 11</td>
</tr>
<tr>
<td>D76N</td>
<td>3.1 ± 1.5</td>
<td>166 ± 32</td>
</tr>
<tr>
<td>W87F</td>
<td>4.1 ± 1.8</td>
<td>130 ± 25</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. WT, wild-type KCNE1-CT.

Assembly of the KCNE1 β subunit with KCNQ1 α subunits transforms the channel current into $I_{Ks}$ and also affects trafficking of the KCNQ1 to the cell surface [2,3,24]. This assembly is mediated by the interactions between specific regions of the α and β subunits. The precise region(s) that accounts for all subunit association and gating regulation is still a matter of intense investigation.

DISCUSSION

The results of the present study characterize a physical and functional interaction between the two K⁺ channel subunits KCNQ1 and KCNE1 residing in their intracellular C-termini. The interaction is direct, not requiring other adaptor proteins or the transmembrane segments of the channel subunits. Heterologous expression of the soluble KCNE1-CT physically and functionally interacts with KCNQ1 channels in situ, and perturbs the pre-formed KCNQ1–KCNE1-CT interaction as assayed by voltage clamp analysis. Deletion analysis of KCNQ1 indicates that the KCNE1-CT binds robustly to a limited region of ~50 amino acids (349–398) encompassing helix A of the C-terminus, as measured by both MBP pull-down studies and SPR analyses. Our results suggest that more C-terminal regions (helices B–C or inter-helix linkers) play a role in the interaction with either direct binding (pull-down assays; Figure 5D) or participation in conformational changes in the binding site that allow more rapid association (SPR data; Figure 6 and Table 1). Our present results are largely in agreement with those of Haitin et al. [19] in terms of the C-terminal interaction between KCNQ1 and KCNE1. The main exception being that GST (glutathione transferase) pull-down experiments in their study showed that KCNQ1-CT truncations after the proximal helix C diminished KCNE1 binding, leading to the conclusion that helix C was the binding site. Our present results suggest that portions of helix A and its linker to the transmembrane S6 bind KCNE1 as determined using both pull-down and SPR assays, whereas the fragment encompassing isolated helices B and C binds in solution but not with immobilized KCNE1.
The KCNQ1-CT fragments were not entirely the same for the two studies and multiple portions of the protein may contribute indirectly to binding efficiency. There may be differences in the solution behaviour of recombinant GST and MBP fusion proteins compared with more immobilized forms as used for SPR. We also used purification tags (His6 or MBP) on the N-termini of the KCNQ1 fragments. The use of SPR (with one ligand immobilized) may allow the recognition of qualitative and quantitative differences in protein interactions. Moreover, the in situ FRET data with full-length C-termini from Haitin’s group [19] suggest that the interaction may not be static, but rather a dynamic process that could be affected by environmental forces (voltage, immobilization, pH and ionic concentrations). At present, further experimentation using additional approaches will be needed to resolve the remaining discrepancies.

Many LQT mutations have been described within the interaction regions for KCNE1-CT and KCNQ1-CT, suggesting that this interaction may be perturbed by these disease-causing variants. The nature of the perturbation may not be the same for each mutation. This is supported by our finding that the each KCNQ1 mutant exhibited a different SPR-binding profile, whereas the KCNE1 mutants had no appreciable effect on binding. These findings may be due to differing sites within the binding interface, or alternatively allosteric effects from sites at a distance from the interaction site. Further structural studies are needed to more fully understand these results. That the KCNE1 mutations (at the conserved Asp58 and Trp57 sites) did not perturb SPR profiles suggest that they may qualitatively affect the functional result of the interaction without preventing overall binding to KCNQ1. The FRET data from the study by Haitin et al. [19] would suggest that it is the dynamic changes in the interaction that are altered by the D76N mutation rather than binding.

Previous studies have examined, in depth, the KCNQ1–KCNE1 interaction sites in the transmembrane segments and extracellular portions [14–16,21,24,25]. The physical interaction interface comprising the membrane-spanning segments appears to be extensive and the same for KCNE1 and KCNE3 in the KCNQ1 channel. Many other voltage-gated K+ channels associate with KCNE family members. The binding site for KCNE1 within the transmembrane region of various K+ channels, however, is not a conserved sequence motif, but probably a common structural conformation encompassing the pore-forming helices. The initial mapping of the functional interaction sites in the membrane-spanning segments of KCNE1 and KCNQ1 showed that activation kinetics were controlled by specific interacting residues [15]. Chimaera studies of KCNE1/KCNE3 showed that control of deactivation did not co-segregate with regions controlling activation. Functional analysis of the KCNE1-CT subsequently placed deactivation kinetics regulation of KCNQ1 in the KCNE1-CT [18]. Our present study provides additional support for this structure–function analysis. Another functional parameter controlled by the interaction of KCNE1 with KCNQ1 is the VDA (voltage-dependence of activation). VDA appears to be affected by perturbations within both the transmembrane interaction site and the C-terminus of KCNQ1/KCNE1. Whether this is due to specific interaction sites compared with more general changes in subunit anchoring and allosteric effects cannot be resolved with available data.

An anchoring effect of C-terminal interactions between KCNQ1 and KCNE1 is supported by mutant expression studies of KCNE1-CT truncation, where activation kinetics were preserved but VDA and deactivation kinetics were altered [18]. This is seemingly at odds with a previous paper [21], where investigators provided evidence that the KCNE1-CT was necessary for activation kinetics when expressed in Xenopus oocytes. Our recent finding [18] shows that KCNE1-CT truncation reduces the apparent affinity of KCNE1 for KCNQ1, but can be surmounted by relative overexpression of the KCNE1 mutant expressed in CHO cells (which lack endogenous KCNE subunits that are present in Xenopus oocytes [26]). We suggest that the anchoring effects of the C-terminus may be responsible for this apparent discrepancy and probably reflects relative amounts of subunit binding and overall structure stability of the channel complex.

Structural data from NMR studies of KCNE1 purified in liposomes suggest that the C-terminus comprises, in part, an interrupted α helix, which is consistent with a scanning mutagenesis study [27–29]. The remainder of the KCNE1-CT did not appear to exhibit structure in solution. Molecular modelling for the location of the KCNE1-CT suggested that it folded back towards the membrane, leading the authors to predict that it interacted with internal pore structures such as the S4–S5 linker [28]. Although this model is not consistent with the findings in the present study and that from Haitin et al. [19], it could however, be explained by the fact that the NMR structure did not resolve the non-helical portions of the C-terminus and its structure was determined in the absence of KCNQ1. An alternative possibility is that the KCNE1-CT has dynamic interactions with KCNQ1 that could involve an alternative interaction with differing portions of the channel during gating or a more complex interaction, such as bridging between the KCNQ1-CT and the S4–S5 linker. The crystal structure of peptides representing the distal KCNQ1-CT (helix D, ∼40 amino acids) has been solved showing a coiled-coil tetrameric structure [9,10]. In our analysis, all of the binding of the KCNE1-CT resides proximal to this region of KCNQ1, where structure is unknown.

Our results also raise several new questions. The precise functional and physical sites of C-terminal interaction are yet to be resolved. Complex dynamics of the interaction during gating is a possibility that could resolve some of the experimental differences between investigators. Solving the structure of the binding interaction may resolve some of these issues. Functional issues that must be kept in mind include not only how the interaction regulates channel deactivation rates, but how it may be involved with the newer results indicating KCNE1-CT effects on channel trafficking and recycling [30,31], response to cAMP/PKA (protein kinase A) signalling [32] and phenotypes of LQT1 and LQT5 mutations.

**AUTHOR CONTRIBUTION**

Renjian Zheng participated and helped to design all of the experiments and wrote the initial manuscript draft. Keith Thompson, Edmond Obeng-Gyimah and Dana Alessi each participated in the construction of recombinant plasmids, protein purification and in vitro binding experiments. Jerry Chen performed all of the electrophysiology experiments. Huiyong Cheng supervised and assisted in all of the surface plasmon resonance experiments. Thomas McDonald was responsible for the overall project design, data analysis and final manuscript preparation.

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**REFERENCES**


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