Intracellular domains interactions and gated motions of I_{KS} potassium channel subunits

Yoni Haitin¹, Reuven Wiener²,
Dana Shaham², Asher Peretz¹,
Enbal Ben-Tal Cohen¹, Liora Shamgar¹,
Olaf Pongs³, Joel A Hirsch² and
Bernard Attali^{1,*}

¹Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, ²Department of Biochemistry, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel and ³Zentrum für Molekulare Neurobiologie, Universität Hamburg, Hamburg, Germany

Voltage-gated K^+ channels co-assemble with auxiliary β subunits to form macromolecular complexes. In heart, assembly of Kv7.1 pore-forming subunits with KCNE1 β subunits generates the repolarizing K^+ current I_{KS} . However, the detailed nature of their interface remains unknown. Mutations in either Kv7.1 or KCNE1 produce the life-threatening long or short QT syndromes. Here, we studied the interactions and voltage-dependent motions of I_{KS} channel intracellular domains, using fluorescence resonance energy transfer combined with voltage-clamp recording and *in vitro* binding of purified proteins. The results indicate that the KCNE1 distal C-terminus interacts with the coiled-coil helix C of the Kv7.1 tetramerization domain. This association is important for I_{KS} channel assembly rules as underscored by Kv7.1 current inhibition produced by a dominant-negative C-terminal domain. On channel opening, the C-termini of Kv7.1 and KCNE1 come close together. Co-expression of Kv7.1 with the KCNE1 long QT mutant D76N abolished the K^+ currents and gated motions. Thus, during channel gating KCNE1 is not static. Instead, the C-termini of both subunits experience molecular motions, which are disrupted by the D76N causing disease mutation.

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Introduction

Most voltage-gated K^+ channel α subunits assemble with diverse accessory β subunits to form macromolecular channel complexes. These β subunits regulate channel gating or/and subcellular distribution. Kv7.1 α subunits co-assemble with KCNE proteins, a family of auxiliary β subunits, to

produce a wide variety of K^+ currents (Abbott and Goldstein, 1998; Melman *et al*, 2002; Panaghi and Abbott, 2006). Assembly of Kv7.1 with KCNE1 produces the I_{KS} potassium current that is crucial for the repolarization of the cardiac action potential (Barhanin *et al*, 1996; Sanguinetti *et al*, 1996). However, the detailed nature of their interactions remains largely unknown. Consistent with their physiological importance, mutations in either Kv7.1 or KCNE1 genes produce the long QT (LQT) or short QT syndromes, which are genetically heterogeneous cardiovascular diseases, characterized by cardiac arrhythmias (Nerbonne and Kass, 2005; Peroz *et al*, 2008; Zareba and Cygankiewicz, 2008).

Similar to all Kv channels, Kv7.1 α subunits share a common core structure of six membrane-spanning segments with a voltage-sensing domain (S1–S4) and a pore domain (S5–S6). In contrast to *Shaker*-like K^+ channels, Kv7.1 channels do not possess an N-terminal T1 tetramerization domain (Cushman *et al*, 2000; Gulbis *et al*, 2000; Minor *et al*, 2000), but exhibit a large C-terminus (Haitin and Attali, 2008). The Kv7.1 C-terminus is a multi-modular structure comprising predicted domains of α helices and coiled-coils that form potential protein–protein interfaces (Haitin and Attali, 2008). The nature and functional significance of these interactions are not fully understood yet. Proximal helices A and B form the site for calmodulin (CaM) binding, whereas distal coiled-coil helices C and D are important for channel tetrameric assembly and protein interactions (Wiener *et al*, 2008). CaM seems to be an essential auxiliary subunit of all Kv7 channels (Wen and Levitan, 2002; Yus-Najera *et al*, 2002; Gamper and Shapiro, 2003; Gamper *et al*, 2005; Ghosh *et al*, 2006; Levitan, 2006; Shamgar *et al*, 2006; Etzeberria *et al*, 2008). We and others showed that LQT mutations impairing CaM binding to Kv7.1 C-terminus affect channel gating, folding and trafficking (Ghosh *et al*, 2006; Shamgar *et al*, 2006). The Kv7.1 C-terminus also seems to convey modulation by PIP₂, which stabilizes the open state of the channel (Loussouarn *et al*, 2003; Park *et al*, 2005). The distal half of the Kv7 C-terminus directs tetramerization, using tandem coiled-coils. In Kv7.1, we showed that the proximal coiled-coil layer (helix C) is a stable dimer that undergoes concentration-dependent association to form a dimer of dimers (Wiener *et al*, 2008). In line with recent work on Kv7.4 (Howard *et al*, 2007), we found that the distal coiled-coil layer (helix D) is a tetrameric parallel coiled-coil structure, crucial for partner specificity (Wiener *et al*, 2008). In addition, the helix D module serves as the interface to the AKAP scaffold protein yotiao (Marx *et al*, 2002).

KCNE1 is known to assemble with Kv7.1 to modulate its gating functions, thereby slowing the activation kinetics, right shifting the voltage dependence of activation and increasing unitary channel conductance (Barhanin *et al*, 1996; Sanguinetti *et al*, 1996; Pusch, 1998; Sesti and Goldstein, 1998; Yang and Sigworth, 1998). It was shown that residues located at the centre of KCNE1 membrane-spanning segment have an important function for interaction with

*Corresponding author. Department of Physiology and Pharmacology, Tel Aviv University, Sackler Medical School, IL-Tel Aviv 69978, Israel. Tel.: +972 3640 5116; Fax: +972 3640 9113; E-mail: battali@post.tau.ac.il

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Kv7.1 (Melman *et al*, 2001). Likewise, residues at the Kv7.1 S6 helix were suggested to be involved in the interaction with KCNE1 (Tapper and George, 2001; Panaghie *et al*, 2006). More recently, we and others showed that KCNE1 can interact with the voltage-sensing domain of Kv7.1 (Nakajo and Kubo, 2007; Rocheleau and Kobertz, 2008; Shamgar *et al*, 2008; Xu *et al*, 2008). The dramatic effect of KCNE1 on channel gating may not only concern transmembrane segments but could also involve conformational changes of intracellular domains. Using fluorescence resonance energy transfer (FRET) and *in vitro* pull-down experiments, we found that the C-termini of Kv7.1 and KCNE1 move close to each other when I_{KS} channel was opened by depolarization. Co-expression of Kv7.1 with the KCNE1 LQT mutant D76N abolishes the K^+ currents and gated motions of the respective C-termini. Pull-down experiments indicate that the KCNE1 distal C-terminus interacts with the Kv7.1 dimeric coiled-coil helix C, demonstrating that the Kv7.1 oligomerization domain also functions as docking site for KCNE1.

Results

FRET between the intracellular domains of Kv7.1 subunits in the channel closed state

To study molecular interactions between the cytoplasmic domains of subunits forming the I_{KS} channel complex, we expressed in *Xenopus* oocytes fluorescently tagged Kv7.1 and KCNE1 subunits for FRET analysis according to the method of Zheng and Zagotta (2004) (see 'Materials and methods'). Fluorescence emission spectra from ECFP- and/or EYFP-tagged subunits were collected from the animal hemisphere of the oocyte with a confocal microscope (Zeiss LSM 510 META). With this set-up, we gathered fluorescence signals mainly from the plasma membrane (Figure 1A; Supplementary Figure 1). Electrophysiological properties of all fluorescently tagged channel constructs used in this study were similar to those of WT Kv7.1 or WT KCNE1 (Supplementary Figure 2). Only one construct in which EYFP was fused to the Kv7.1 N-terminus (EYFP-Kv7.1) exhibited a slight right shift in the voltage dependence of activation ($\approx +10$ mV). In addition, when this construct (EYFP-Kv7.1) was co-expressed with KCNE1-ECFP, there was a small left shift in the voltage dependence of activation (≈ -10 mV) (Supplementary Figure 2). To measure FRET in the channel closed state, we collected all spectral data at $V_{rest} = -70$ mV.

We fused EYFP to the Kv7.1 N-terminus (EYFP-Kv7.1) and co-expressed the construct with the ECFP-labeled Kv7.1 C-terminus (after aa. 676) (Kv7.1-ECFP) (Figure 1B). A significant FRET signal ($\text{RatioA} - \text{RatioA}_0$) was observed between the N-terminus of Kv7.1 and the C-terminus of a neighbouring subunit (0.12 ± 0.01 , $n = 15$). Co-expression of unlabeled WT KCNE1 did not significantly alter the FRET signal (0.14 ± 0.01 , $n = 15$). This result suggests that the N- and C-termini of neighbouring Kv7.1 subunits reside in close proximity at the channel closed state. Co-expression of the C-terminally tagged Kv7.1 subunits (Kv7.1-ECFP/Kv7.1-EYFP) also generated a significant FRET (0.11 ± 0.01 , $n = 44$) (Figure 1B). However, co-expression of unlabeled KCNE1 along with the C-terminally labeled Kv7.1 subunits resulted in a small but significant decrease in the FRET intensity (0.08 ± 0.01 , $n = 27$; $P < 0.05$) (Figure 1B). This

Figure 1 FRET between the intracellular domains of Kv7.1 subunits in the channel closed state. **(A)** Colour-coded emission spectra measured from *Xenopus* oocytes expressing ECFP (left), EYFP (middle) or both (FRET, right), excited with 405 nm (upper panels) or 514 nm (lower panels) laser lines. **(B)** The FRET efficiencies, expressed as the mean value of $[\text{RatioA} - \text{RatioA}_0]$ and SEM are shown for the expression of 1:1 molar ratio of Kv7.1-ECFP/Kv7.1-EYFP, EYFP-Kv7.1/Kv7.1-ECFP and for doubly labeled EYFP-Kv7.1-ECFP subunits, expressed without (grey bar) or with (red bars) unlabeled KCNE1 subunits. Asterisks indicate significance level (* $P < 0.05$; *** $P < 0.001$) by unpaired two-tailed *t* test. **(C)** Three different groups of non-interacting proteins were used as negative pair FRET controls. Numbers of oocytes are given in parentheses for each experimental condition. **(D)** No correlation ($P > 0.05$, $n = 418$) was found between the static FRET signal ($\text{RatioA} - \text{RatioA}_0$) obtained by co-expression of all FRET pairs of ECFP- and EYFP-tagged subunits and the expression level of EYFP-tagged acceptor subunits (F_{514}).

suggests that at rest, the C-termini of neighbouring Kv7.1 subunits are in proximity and that assembly with KCNE1 may increase the distance between neighbouring Kv7.1 C-termini or reorient them (see below). Subsequently, we characterized the doubly tagged Kv7.1 subunits (EYFP-Kv7.1-ECFP), in which EYFP and ECFP were fused, respectively, to the N- and C-termini (Figure 1B). A marked FRET signal was observed between the N- and C-termini of the doubly tagged channel (0.16 ± 0.01 , $n = 65$); however, we could not determine whether the FRET signal originates from inter- or intrasubunit interactions. When we co-expressed unlabeled KCNE1 along with the doubly tagged Kv7.1, a significant increase in FRET

was revealed (0.24 ± 0.02 , $n = 17$; $P < 0.001$, Figure 1B). This suggests that in the closed state the interaction of KCNE1 with the channel complex shortens the distance between the Kv7.1N- and C-termini.

No FRET signal was detected in a series of negative pair controls (Figure 1C), which include Kv7.1-ECFP co-expressed with G α i3-EYFP (-0.009 ± 0.003 , $n = 6$), CaM-ECFP with CaM-EYFP (-0.06 ± 0.002 , $n = 8$) and CaM-ECFP with KCNE1-EYFP (-0.01 ± 0.01 , $n = 34$). In addition, we found no correlation ($P > 0.05$, $n = 418$) between the FRET signal (RatioA–RatioA₀) obtained by co-expression of all FRET pairs of ECFP- and EYFP-tagged subunits and the expression level of EYFP-tagged acceptor subunits (F_{514}), indicating that our results are reliable and unbiased (Figure 1D). As members of the GFP family are known to dimerize when associated to the membrane through lipid anchors or membrane proteins (Zacharias *et al*, 2002; Snapp *et al*, 2003), we used non-dimerizing mutants (A206K) of the respective fluorescent proteins, Kv7.1-ECFP, Kv7.1-EYFP, Kv7.1 Δ 622-ECFP and KCNE1-EYFP to exclude potential artefacts produced by dimerization of the fluorophore. Results indicate that the monomeric mutants produce very similar FRET signals (RatioA–RatioA₀), compared with those obtained with the WT fluorescent fusion protein constructs (Supplementary Figure 3; see also below), suggesting that potential dimerization of the fluorophores does not bias our FRET measurements.

FRET between the C-termini of Kv7.1 and KCNE1 subunits in the channel closed state

Having established the relative spatial arrangements of the Kv7.1 intracellular domains, we asked how the C-termini of the α and β subunits are positioned. First, we fused ECFP to the distal C-terminus of Kv7.1 (after aa. 676) (Kv7.1-ECFP) and EYFP to the distal C-terminus of KCNE1 (after aa. 129) (KCNE1-EYFP). When the I_{KS} channel was in the closed state (measured at $V_{rest} = -70$ mV), a strong FRET signal was observed between Kv7.1-ECFP and KCNE1-EYFP (0.14 ± 0.01 ; $n = 53$), indicating that the C-termini of both subunits are in close proximity (less than 10 nm) (Figure 2). In contrast, co-expression of EYFP-labeled Kv7.1 N-terminus (EYFP–Kv7.1) along with KCNE1-ECFP did not result in detectable FRET signal, though both constructs are functional (-0.02 ± 0.002 , $n = 21$; $P < 0.001$, Figure 2; Supplementary Figure 2). This result suggests that within the FRET detection limit, there is no spatial proximity between the Kv7.1 N-

terminus and the KCNE1 C-terminus or/and that the fluorophore dipoles are in perpendicular orientation.

Given the large differences in C-terminal lengths of Kv7.1 and KCNE1, we asked whether truncation of the long Kv7.1 C-terminus would move the two fluorophore molecules closer to each other. Truncation of Kv7.1 C-terminus at position 622, just downstream the coiled-coil helix D, crucial for channel tetramerization (Wiener *et al*, 2008), resulted in significantly larger FRET when co-expressed with KCNE1-EYFP (0.27 ± 0.01 , $n = 50$; $P < 0.001$, Figure 2). As above, results obtained with the monomeric mutants produce very similar FRET signals (RatioA–RatioA₀), compared with those obtained with the WT fluorescent protein constructs (Supplementary Figure 3). The data suggest that at rest the distal C-terminus of KCNE1 resides close to the Kv7.1 assembly domain. Thus, the FRET experiments indicate that the Kv7.1 intracellular C-terminus may associate with its counterpart, the KCNE1 intracellular C-terminus.

KCNE1 prevents inhibition of Kv7.1 channel assembly produced by over-expression of the Kv7.1 C-terminal domain (CTD)

We identified earlier the C-terminal region encompassing helix D (aa. 589–620) to function as a minimum assembly domain for Kv7.1 (Schmitt *et al*, 2000). More recently, we showed that the distal half of Kv7.1 C-terminus (aa. 539–620) directs tetramerization, using tandem coiled-coils (Wiener *et al*, 2008). Along this line, co-expression in cells of the Kv7.1 C-terminal domain (CTD) with the full-length Kv7.1 channel may result in binding of CTD to Kv7.1 α subunits and thereby interfere with channel tetrameric assembly in a dominant-negative manner, a feature characterized earlier by Li *et al* (1992) for *Shaker* channels with the N-terminal T1 assembly domain. Thus, we examined whether CTD (aa. 510–620), which encompasses helix B and the tandem coiled-coil (helices C and D), affects Kv7.1 functional expression. When Kv7.1 was expressed alone in CHO cells, it generated large K^+ currents that were evoked by step depolarization (Figure 3A). However, co-expression of Kv7.1 with CTD in a molar ratio of 1:5, respectively, led to a marked decrease in K^+ current density with no effects on channel gating properties (Figure 3A). At +40 mV, co-expression of CTD reduced by 89% the Kv7.1 current density, from 82.2 ± 13.1 to 8.8 ± 2.3 pA/pF ($n = 16$, $P < 0.001$). For comparison, deletion of the last 86 residues of Kv7.1 (Kv7.1 Δ 590), which removes the tetrameric coiled-coil helix D and the farthest region of the C-terminus, also resulted in a strong reduction in K^+ currents (Figure 3A). The inhibitory effect of CTD was specific for Kv7.1. Under similar experimental conditions, CTD did not affect the current density of Kv2.1 (Figure 3B), a voltage-gated K^+ channel from a different subfamily, which assembles through an N-terminal T1 tetramerization domain (Misonou *et al*, 2005). The subunit trapping specificity of CTD extended to within the Kv7 channel subfamily, as it did not affect Kv7.2 K^+ current density (Figure 3C). These data suggest that co-expression of CTD specifically disrupts proper tetrameric assembly of Kv7.1. This subunit trapping likely prevents the channel from reaching the plasma membrane as could be seen in non-permeabilized HEK 293 cells, using immunocytochemical labeling of externally HA-tagged Kv7.1 (Supplementary Figure 4).

Figure 2 FRET between the C-termini of Kv7.1 and KCNE1 subunits in the channel closed state. The FRET efficiencies expressed as in Figure 1 are shown for the intracellular domains of Kv7.1 and WT KCNE1 or mutant KCNE1 (D76N). Asterisks indicate significance level (***) $P < 0.001$ by one-way ANOVA and Tukey's Multiple Comparison Test.

Figure 3 Effect of CTD on Kv7.1, Kv2.1 and Kv7.2 currents. **(A)** Representative Kv7.1 currents (left panel) and current densities–voltage relations (right panel) recorded from CHO cells in the absence or presence of CTD. From a holding potential of -85 mV, cells were stepped for 3 s from -50 to $+40$ mV in 10 mV increments and then repolarized for 1 s at -60 mV tail potential. **(B)** Representative Kv2.1 currents (left panel) and current densities–voltage relations (right panel) recorded from CHO cells in the absence or presence of CTD. The current–voltage step protocol of Kv2.1 was similar to that of Kv7.1, except that the voltage increment was 20 mV. **(C)** Representative Kv7.2 currents (left panel) and current densities–voltage relations (right panel) recorded from CHO cells in the absence or presence of CTD ($n = 8–16$).

As FRET indicated that at rest the C-termini of Kv7.1 and KCNE1 subunits reside close together, possibly associating and playing a role in I_{KS} channel assembly, we investigated whether KCNE1 co-expression altered the impact of CTD on Kv7.1 functional expression. In contrast to the strong inhibitory effect produced by CTD on homomeric Kv7.1 current density, co-expression of Kv7.1 with CTD in the presence of KCNE1 (ratio of 1:5:1) did not affect I_{KS} current density nor its channel gating properties (Figure 4A). This striking result suggests that co-expression of KCNE1 prevents CTD from interfering with the assembly of Kv7.1 α subunits in the context of I_{KS} channel formation.

KCNE1 C-terminus interacts with coiled-coil helix C of the Kv7.1 C-terminal assembly domain

An interaction between the KCNE1 C-terminus with the Kv7.1 assembly domain may account for the protective effect of KCNE1 on CTD-induced inhibition of Kv7.1 currents. To test this possibility, we first checked whether KCNE1 could interact with CTD or the whole C-terminus of Kv7.1 using immunoprecipitation experiments. FLAG-tagged CTD (aa. 510–620) or C-terminus (aa. 352–676) constructs of Kv7.1 were transfected in HEK293 cells in the absence or presence of KCNE1. The results indicated that KCNE1 potently and specifically interacts with CTD as well as with the whole C-terminus of Kv7.1 (Figure 4B). The KCNE1 immunoreactive proteins appeared as multiple bands comprising the core

Figure 4 Effect of KCNE1 on CTD-induced inhibition of Kv7.1 currents and immunoprecipitation of KCNE1 by CTD. **(A)** Representative current traces of Kv7.1, co-expressed with KCNE1 in CHO cells in the absence or presence of CTD (left panel). The current–voltage step protocol was similar to that described in Figure 5. Current densities–voltage relations of Kv7.1 + KCNE1 with or without CTD were plotted as a function of membrane potential (right panel; $n = 5–13$). **(B)** The Kv7.1 FLAG-tagged CTD (aa. 510–620) or FLAG-full-length C-terminus (C-term) (aa. 354–676) was co-expressed with KCNE1 in HEK 293 cells. Immunoprecipitation experiments were carried out using anti- α M2 (FLAG) antibodies.

Figure 5 Direct interaction of GST-fusion KCNE1 C-terminal proteins with various His-tagged Kv7.1 C-termini. **(A)** Elution profile of Superdex 200 gel filtration column of the ternary complex including the purified C-termini of Kv7.1 (352Δ386-504) and KCNE1 (aa. 67-129, CT-E1) along with CaM (solid line) and of purified CT-E1 (broken line). A standard curve (filled circles) was used to calculate molecular weights of eluted proteins on the Superdex 200 column (inset). Empty square and triangle indicate the molecular weights of the elution peaks corresponding to the ternary complex and CT-E1, respectively. Tris-Tricine-SDS-PAGE gel pattern of the eluted peak fractions are shown. A western blot probed with anti-KCNE1 antibodies is shown, confirming the identity of the KCNE1 C-terminus band. **(B)** Representative GST-KCNE1 C-terminus pull-down (upper panels) and input (lower panels) immunoblots of various His-tagged Kv7.1 deletion mutant proteins. **(C)** Quantification of the pull-down scans expressed as percentage of input (OD_{pull-down}/OD_{input}) and schematic depiction of the constructs. **(D)** Quantification of the pull-down scans expressed as percentage of input (OD_{pull-down}/OD_{input}) and schematic depiction of the constructs. Each data point represents 3-10 separate experiments. Asterisks indicate significance level (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) by one-way ANOVA and Dunnett's Multiple Comparison Test, with 352Δ(386-504) (C) or WT KCNE1 (D) serving as control.

(~16 kDa) and various glycosylated forms of the protein (~17-24 kDa).

We then asked whether we could reconstitute this complex *in vitro*, based on earlier established biochemical preparations (Shamgar *et al*, 2006; Wiener *et al*, 2008). As shown before, CaM is essential for the appropriate folding of a soluble Kv7.1 C-terminus and was therefore recombinantly co-expressed with it (Ghosh *et al*, 2006; Shamgar *et al*, 2006; Wiener *et al*, 2008). In line with the immunoprecipitation data, the purified His-tagged Kv7.1 whole C-terminus (construct 352-622) complexed with CaM interacted with the GST-tagged KCNE1 C-terminus in *in vitro* pull-down experiments (Figure 5B, first lane from left). However, we refrained using this Kv7.1 C-terminus (construct 352-622) in subsequent experiments as this construct tended to aggregate and to be degraded (see input of first left lane in Figure 5B). Instead, we used a shorter construct (352Δ386-504) which is soluble and binds CaM and in which the linker connecting helices A and B was deleted. This linker did not interact with

KCNE1 C-terminus (see below, Figure 5B and C). In addition, the linker deletion preserved functional Kv7.1 current expression in the absence or presence of KCNE1 (Supplementary Figure 5). Thus, the C-termini of Kv7.1 (352Δ386-504) and of KCNE1 were co-expressed in bacteria and co-purified (see Supplementary data; Figure 5A). The co-expressed recombinant proteins were co-purified on metal chelate and by size-exclusion chromatography. The eluted peak fractions were concentrated and analysed by SDS-PAGE (Figure 5A). The results show that both the C-termini of Kv7.1 and KCNE1 stably associate as revealed by co-elution of a main peak corresponding to a ternary complex that includes CaM. A western blot probed with anti-KCNE1 antibodies confirmed the identity of the KCNE1 C-terminus band. The chromatographic elution profile shows that the ternary complex migrates with an apparent molecular mass of about 165 kDa.

Direct interactions between the C-termini of Kv7.1 and KCNE1 were mapped by use of purified His-tagged Kv7.1 C-termini-CaM complexes and GST-tagged KCNE1 C-termini

constructs in a series of *in vitro* pull-down experiments (Figure 5B and C; Supplementary Figure 6). To narrow down the Kv7.1 C-terminal region to which the KCNE1 C-terminus interacts, we prepared a series of Kv7.1 C-terminus deletion constructs. In most of the constructs, two versions were tested; one beginning immediately after the end of the S6 transmembrane segment (aa. 352) whereas the second begins with helix A (aa. 361). Both full length (352–622) and shorter versions (352Δ386–504 and 361Δ386–504) of the Kv7.1 C-terminus all comprising helices A–D clearly bind to the whole KCNE1 C-terminus (67–129). This KCNE1 interaction was specific as GST alone was unable to bind the Kv7.1 C-terminus (Figure 5B–D). The KCNE1 C-terminus was unable to pull down Kv7.1 constructs truncated after helix B (352Δ539–676 and 361Δ539–676), suggesting that it does not interact with high affinity to the proximal half of Kv7.1 C-terminus. This region comprising helices A and B was found to bind CaM (Wen and Levitan, 2002; Yus-Najera *et al*, 2002; Gamper and Shapiro, 2003; Gamper *et al*, 2005; Ghosh *et al*, 2006; Shamgar *et al*, 2006). In contrast, the KCNE1 C-terminus strongly associated with the Kv7.1 constructs that included the helix C module (Δ563–676 and Δ572–576). However, it was unable to pull down Kv7.1 constructs that comprised helices A, B, D but lacked helix C (352Δ539–572 and 352Δ548–563) (Figure 5B and C). To check whether the proximal part of the helix C module was important for the interaction with KCNE1, we prepared the construct 352Δ547–676 that included the first nine residues of helix C. The results show that the KCNE1 C-terminus significantly bound to this Kv7.1 C-terminal construct (Figure 5B and C). Together, the data indicate that the KCNE1 C-terminus mainly interacts with the Kv7.1 coiled-coil helix C module.

To map the corresponding KCNE1 region that binds to the Kv7.1 C-terminus, we prepared three deletion constructs of KCNE1 C-terminus and proceeded with similar pull-down experiments, using the Kv7.1 C-terminal construct 352Δ386–504 (Figure 5D). The results show that deletion of the proximal KCNE1 C-terminus (CT-E1Δ69–77) significantly enhanced the binding of KCNE1 to Kv7.1 C-terminus (352Δ386–504) (more than two-fold, $P < 0.01$, $n = 3$), when compared with the WT (Figure 5D). In contrast, deletions of the more distal regions of KCNE1 C-terminus (Δ78–129 and Δ109–129) totally prevented its binding to the Kv7.1 CTD. This suggests that the most distal region of the KCNE1 C-terminus (aa. 109–129) is crucial for the interaction with the Kv7.1 C-terminus.

Gated motions of the I_{KS} channel intracellular domains

In addition to using FRET to map the topology of the cytoplasmic regions in the channel closed state, we set out to determine whether the intracellular domains of the complex undergo voltage-dependent motions on channel gating. For this purpose, FRET spectral analysis was combined with two-electrode voltage-clamp recording of K^+ currents. Briefly, time series spectral images of *Xenopus* oocytes, clamped at -80 mV and expressing various combinations of fluorophore-tagged subunits, were acquired before, during and after a $+30$ mV depolarizing pulse that opens the channel. To obtain the extent of the FRET change (dynamic FRET), we subtracted the effect of photobleaching and measured the normalized FRET ratio change ($F_{[524-534]}/F_{[481-492]}$) (and see 'Materials and methods') (Supplementary Figure 7).

Co-expression of Kv7.1Δ622-ECFP along with WT KCNE1-EYFP resulted in a significant voltage-dependent FRET increase ($\text{Ratio}_{120/50} = 1.024 \pm 0.005$, $n = 20$, $P < 0.001$), which exhibited slow kinetics (Figure 6A and E). This dynamic FRET increase indicates that the C-termini of both subunits undergo a slow spatial rearrangement when I_{KS} channels shift into the open state.

To confirm that the FRET changes we observed on depolarization, report on gated motions, we co-expressed Kv7.1 with the mutant KCNE1 (D76N)-EYFP, an LQT mutation located at the proximal C-terminus of KCNE1 (Splawski *et al*, 1997). Mutant D76N was shown earlier to alter channel gating but not trafficking nor permeation (Splawski *et al*, 1997; Bianchi *et al*, 1999), as I_{KS} channel openers, such as fenamates or stilbenes, could restore normal channel function (Abitbol *et al*, 1999). Co-expression of Kv7.1-ECFP or Kv7.1Δ622-ECFP with the LQT mutant KCNE1 (D76N)-EYFP exhibited a strong constitutive FRET signal at $V_m = -70$ mV (Figure 2), suggesting that this KCNE1 C-terminal mutation does not affect the spatial proximity of the distal C-termini of both subunits in the channel closed state. Notably, on depolarization to $+30$ mV no K^+ currents and no voltage-dependent FRET increase could be recorded ($\text{Ratio}_{120/50} = 1.003 \pm 0.003$, $n = 12$, $P > 0.05$) (Figure 6B and E), suggesting that the D76N LQT mutation abolishes the gated motions of KCNE1 and Kv7.1 intracellular C-termini.

Voltage-dependent FRET changes were not detected when 1:1 molar ratio of Kv7.1-ECFP and Kv7.1-EYFP were co-expressed ($\text{Ratio}_{120/50} = 1.006 \pm 0.005$, $n = 10$, $P > 0.05$) (Figure 6C and E). Moreover, addition of untagged WT KCNE1 to the fluorophore pair of Kv7.1 did not produce detectable voltage-dependent FRET ($\text{Ratio}_{120/50} = 1.001 \pm 0.002$, $n = 8$, $P > 0.05$) (Figure 6D and E). Thus, though the C-termini of neighbouring Kv7.1 α subunits display no relative motions during channel gating, the C-termini of Kv7.1 and KCNE1 undergo a slow voltage-dependent spatial rearrangement.

Next, we checked whether the N- and C-termini of Kv7.1 undergo gated motions that could be detected by dynamic FRET changes. Voltage step to $+30$ mV resulted in a significant FRET increase ($\text{Ratio}_{120/50} = 1.028 \pm 0.006$, $n = 14$, $P < 0.001$) between the N- and C-termini of the doubly tagged Kv7.1 (Figure 7A and D). This suggests that the intracellular N- and C-termini of Kv7.1 experience a voltage-dependent motion, which brings them closer. It should be noticed that the onset of FRET increase in the doubly tagged Kv7.1 appears faster than that recorded with Kv7.1-ECFP and KCNE1-EYFP. The FRET change recorded at $+30$ mV may reflect motions that occur between N- and C-termini within either individual or/and neighbouring Kv7.1 subunits. Thus, the doubly tagged Kv7.1 was co-expressed with unlabeled Kv7.1 in a 1:1 molar ratio. Under these conditions, intermolecular FRET would markedly decrease because of 'dilution' by the unlabeled Kv7.1. Results show that there is a slightly smaller but yet significant voltage-dependent FRET change ($\text{Ratio}_{120/50} = 1.015 \pm 0.003$, $n = 10$; $P < 0.01$, Figure 7C and D). This result suggests that the enhanced energy transfer takes place within a subunit, although we cannot exclude that FRET changes may occur between neighbouring subunits. Co-expression of unlabeled KCNE1 along with the doubly tagged EYFP-Kv7.1-ECFP did not modify the initial significant voltage-dependent FRET rise, which was observed over 15 s

Figure 6 Voltage-dependent FRET changes between the C-termini of Kv7.1 and KCNE1. (A–D) Averaged normalized $F_{[524-534]}/F_{[481-492]}$ ratio from 405 nm excited *Xenopus* oocytes under voltage-clamp conditions are shown (oocyte numbers are indicated in panel E) for Kv7.1Δ622-ECFP/KCNE1(WT)-EYFP (A), Kv7.1Δ622-ECFP/KCNE1(D76N)-EYFP (B), Kv7.1-ECFP/Kv7.1-EYFP (C) and Kv7.1-ECFP/Kv7.1-EYFP + KCNE1(WT) (D). Currents were evoked by step depolarization at $t = 60$ s from a holding potential of -80 to $+30$ mV for 60 s and back to -80 mV for another 90 s. Shown are representative simultaneously recorded current traces, using the two-electrode voltage-clamp technique (A–D, inset). Zoom-in of the onset (1.3 s) of current kinetics are shown, as inset, in panel A and C. (E) Bleach-corrected normalized voltage-dependent FRET $F_{[524-534]}/F_{[481-492]}$ change was deduced by the FRET ratio obtained at the end of the $+30$ mV depolarizing pulse (120 s) divided by the FRET ratio obtained at -80 mV before the step depolarization (50 s) (Normalized F_{EYFP}/F_{ECFP} t_{120}/t_{50}). Asterisks indicate significance level (***) $P < 0.001$ by paired two-tailed *t*-test.

after the depolarization step began (blue arrow, $\text{Ratio}_{75/50} = 1.03 \pm 0.005$, $n = 7$; $P < 0.01$, Figure 7B and D). Remarkably, this voltage-dependent FRET increase was followed by a slow decline of the FRET ratio, while the depolarization step was still maintained to $+30$ mV. At the end of the depolarizing step, no significant voltage-dependent FRET elevation was observed ($\text{Ratio}_{120/50} = 1.016 \pm 0.007$, $n = 7$; $P > 0.05$). The latter FRET value was significantly different from that observed during the initial step depolarization ($t = 75$ s; $P < 0.01$).

Discussion

In this study, we have identified crucial interactions between the cytoplasmic domains of Kv7.1 and KCNE1 subunits, which have fundamental implications for the assembly of the I_{KS} channel complex and for the gated motions of the intracellular channel modules.

We are aware of the limitations in using FRET to estimate distances. In principle, FRET efficiency could be affected by donor–acceptor distance, dipole–dipole orientation of the donor and acceptor fluorophores or alteration of the *milieu*. FRET changes because of modification of solvent environment are unlikely, as the spectral properties of both donor and acceptor subunits remained unchanged (Supplementary Figure 1). A combination of changes in distance and orientation may underlie the FRET changes. Note that the physical interaction of KCNE1 distal C-terminus with Kv7.1 helix C, as determined by pull-down experiments, is in line with the FRET data at the channel closed state, which places the farthest C-terminal region of KCNE1 close to helix D in which the fluorophores were tagged (Figure 8A).

No FRET was detected between the Kv7.1 N-terminus and the KCNE1 C-terminus. This lack of FRET signal implies that the distances between these cytoplasmic domains are larger than 10 nm or/and that the orientation of the fluorophore dipoles is not permissive (perpendicular). FRET analysis at

Figure 7 Doubly labeled Kv7.1 subunits produce voltage-dependent FRET increase. (A–C) Averaged normalized $F_{[524-534]}/F_{[481-492]}$ ratio from 405 nm excited *Xenopus* oocytes under voltage-clamp conditions are shown (oocyte numbers are indicated in panel D) for EYFP-Kv7.1-ECFP (A), EYFP-Kv7.1-ECFP/KCNE1(WT) (B) and EYFP-Kv7.1-ECFP/Kv7.1(WT) (C). (D) Bleach-corrected normalized voltage-dependent FRET $F_{[524-534]}/F_{[481-492]}$ change was deduced by the FRET ratio obtained at the end of the +30 mV depolarizing pulse (120 s) (grey bars) or after 15 s of the +30 mV depolarization step (75 s) (blue bar) and divided by the FRET ratio obtained at -80 mV before the step depolarization (50 s). Asterisks indicate significance level (** and *** $P < 0.01$; *** $P < 0.001$) by paired two-tailed *t*-test.

the channel resting state showed that the C-termini of neighbouring Kv7.1 subunits are in proximity and that assembly with KCNE1 increases the distance between neighbouring Kv7.1 C-termini, which brings the N- and C-termini of Kv7.1 closer.

Our results of voltage-dependent FRET indicate that KCNE1 is not static, but instead is engaged in slow molecular motions during I_{KS} channel gating. Although the C-termini of neighbouring Kv7.1 α subunits display no relative motion during channel gating, depolarization slowly brings the C-termini of Kv7.1 and KCNE1 close together (Figure 8A). Remarkably, the loss of K^+ currents and of voltage-dependent FRET increase with the KCNE1 LQT mutant D76N confirm that the FRET changes found with WT I_{KS} , report on gated motions.

Attempts made to quantify the kinetics of FRET changes by curve fitting were not possible because of the poor time resolution of the FRET spectral acquisition (0.2 Hz). However, a rough estimate of the time course of FRET changes of Kv7.1-ECFP and KCNE1-EYFP indicates that it is biphasic with value of the slow component being more than one order of magnitude larger than that of current kinetics. This feature is in line with a recent report (Villalba-Galea *et al*, 2009) describing at high temporal resolution the FRET changes of a fluorescently labeled voltage-sensitive phosphatase Ci-VSP. The time course of FRET changes of Ci-VSP was biphasic with a slow component that displayed a time constant of at least two orders of magnitude greater than that of the charge movement. It was suggested that the slow FRET changes reported by the fluorophores might be related to the relaxation of the voltage-sensing domain and to a global conformational change of the protein (Villalba-Galea *et al*, 2009). The slow-gated motion of Kv7.1 and KCNE1 intracellular domains is reminiscent to the slow

movement of the CLC0 chloride channel C-terminus that was determined by FRET (with $\tau > 60$ s) and was suggested to report on slow channel gating through a large conformational change of the channel protein (Bykova *et al*, 2006). Likewise, we suggest that the slow FRET changes monitor a large conformational change consequent to I_{KS} channel opening that propagates to the C-termini of both subunits. The coupling of I_{KS} channel gating to the movement of the intracellular domains may involve the CaM-binding module encompassing the proximal helices A and B of Kv7.1 C-terminus and the proximal C-terminus of KCNE1. These regions were shown to be critical for I_{KS} channel gating (Ben-Efraim *et al*, 1996; Abitbol *et al*, 1999; Tapper and George, 2000; Ghosh *et al*, 2006; Rocheleau *et al*, 2006; Shamgar *et al*, 2006).

As the distal part of KCNE1 C-terminus interacts with Kv7.1 helix C, then how can it engage in motions on channel gating? The stretch of the distal KCNE1 C-terminus (aa. 109–129) is long enough to provide both interaction with the coiled-coil helix C through few amino acids and have a distal tail with sufficient conformational freedom to allow propagation of motions-like rotation or translation. The intracellular N- and C-termini of Kv7.1 also experience a gated motion, which brings them close together (Figure 8B). However, in the presence of KCNE1 and during depolarization, the gated motion of closely associated N- and C-termini of Kv7.1 relaxes back to their resting position or moves to a different state, that only allows the constitutive FRET signal to be transferred (Figure 8B). The FRET change relaxation observed on sustained depolarization may reflect the impact of KCNE1 on the VSD, which tends to be stabilized in its resting state conformation (Nakajo and Kubo, 2007; Rocheleau and Kobertz, 2008; Shamgar *et al*, 2008; Xu *et al*, 2008). It is noteworthy that this relaxation of Kv7.1 N- and C-termini has

Figure 8 Model of the gated motions of the intracellular domains of Kv7.1 and KCNE1. (A) Cartoon of the gated motions of the C-termini of Kv7.1 (blue) and KCNE1 (grey) in frontal view. (B) Cartoon of the gated motions of Kv7.1 cytoplasmic domains in the absence or presence of KCNE1 in frontal view.

comparable slow time course to that of the oncoming of the C-termini of both subunits, which may reflect reciprocal slow motions.

We showed by immunoprecipitations that KCNE1 interacts with the Kv7.1 C-terminal assembly domain, CTD. Furthermore, when the C-termini of both subunits were co-expressed in bacteria, their interaction was revealed by the co-purification of a ternary complex that includes Kv7.1 C-terminus, CaM and KCNE1 C-terminus. *In vitro* pull-down of purified deletion mutants of both subunits indicated that the KCNE1 C-terminus directly and specifically interacts with the coiled-coil helix C of Kv7.1 assembly domain. The linker connecting S6 to helix A at the proximal C-terminus of Kv7.1 (aa. 352–361) tends to enhance this interaction, suggesting that this region provides an additional contact of low affinity. These results have important implications for I_{KS} channel assembly and stoichiometry. Recently, we showed by sedimentation equilibrium studies that the isolated helix C dimerizes, most probably by a coiled-coil interaction, and seems to undergo concentration-dependent dimer–tetramer association (Wiener *et al*, 2008). In addition, sedimentation equilibrium and velocity experiments indicated that a Kv7.1 C-terminus construct complexed with CaM and comprising helices A–C but lacking helix D is best modelled as a dimer (Wiener *et al*, 2008). We also showed that helix C plays an additional role in channel trafficking (Wiener *et al*, 2008).

Hence, during the final stages of channel complex biogenesis, helix C probably forms a dimer of dimers because of high local concentration (Wiener *et al*, 2008). In this context, the specific interaction of the KCNE1 C-terminus with the coiled-coil helix C of Kv7.1 is highly significant. First, several lines of evidence strongly suggest that the Kv7.1–KCNE1 complex assembly occurs in the endoplasmic reticulum (ER) (Krummerman *et al*, 2004; Chandrasekhar *et al*, 2006). Recent studies show that KCNE1 is retained at early stages of the secretory pathway until it co-assembles with Kv7.1 subunits, thereby mediating KCNE1 progression to the cell surface (Chandrasekhar *et al*, 2006). Second, several studies show that only two KCNE1 subunits co-assemble with the tetrameric Kv7.1 channel (Chen *et al*, 2003; Morin and Kobertz, 2008). Our results provide a plausible mechanism of I_{KS} channel biogenesis. The specific interaction of KCNE1 subunit with the dimeric coiled-coil helix C may occur in the ER and in doing so might establish the stoichiometry of Kv7.1–KCNE1 complex assembly. Though quantitative measurements of the interaction between KCNE1 C-terminus and Kv7.1 coiled-coil helix C remain to be determined, our data support the purported I_{KS} channel stoichiometry of two KCNE1 per tetrameric Kv7.1 subunits (Chen *et al*, 2003; Morin and Kobertz, 2008) and suggest a model in which one KCNE1 C-terminus binds one dimeric coiled-coil helix C. If so, how would KCNE1 then specifically bind to the relatively conserved coiled-coil helix C? Indeed, KCNE1 does not interact with Kv7.2–Kv7.5 subunits (Robbins, 2001; Panaghie and Abbott, 2006). Interestingly, seven Kv7.1 helix C residues are not conserved among other Kv7 channel members (Kv7.2–5). Most of these residues map to the solvent-exposed surface of the helix C coiled-coil and not to its hydrophobic core. It is also obvious that the interaction specificity between KCNE1 and Kv7.1 is not solely determined by the helix C interface and that other domains are likely involved, notably in the transmembrane domains. Third, the selective interaction of the KCNE1 C-terminus with helix C agrees with our electrophysiological data showing that co-expression of KCNE1 prevents the CTD-induced inhibition of Kv7.1 currents. Two non-mutually exclusive mechanisms may operate: (i) a competition for binding to helix C of full-length Kv7.1 α subunits may exist, implying that their interaction with KCNE1 is more stable than that with CTD and (ii) excess KCNE1 subunits may associate with the CTD peptides through helix C and scavenge them, thereby preventing CTD from interfering with the assembly process.

Earlier structure–function studies revealed that the KCNE1 C-terminus is an important determinant of I_{KS} channel function (Takumi *et al*, 1991; Ben-Efraim *et al*, 1996; Abitbol *et al*, 1999; Tapper and George, 2000; Rocheleau *et al*, 2006). Hence, several KCNE1 C-terminal mutants, though expressed efficiently at the plasma membrane, exhibit drastic reduction in channel activity and in some cases exerting a dominant-negative effect (Takumi *et al*, 1991). Helical periodicity perturbation analysis revealed that the proximal CTD of KCNE1 is α helical when broken into two segments separated by a proline residue (Rocheleau *et al*, 2006). The KCNE1 C-terminus was also found to be crucial for establishing the slow activation and deactivation gating kinetics (Tapper and George, 2000). Our results show that it is the KCNE1 C-terminal distal tail (aa. 109–129) that interacts with Kv7.1 coiled-coil helix C. Recent NMR studies indicate that the

KCNE1 distal C-terminus (aa. 106–129) is largely disordered and much more flexible than the rest of the protein (Tian *et al*, 2007; Kang *et al*, 2008). Along this line, our data show that if we shorten the proximal KCNE1 C-terminus (CT-E1Δ69–77), the distal KCNE1 tail binds more tightly to the Kv7.1 coiled-coil helix C as compared with WT (see Figure 5D). These data suggest that the KCNE1 flexible tail may provide the necessary conformational freedom to interact with the Kv7.1 helix C target. Shortening the KCNE1 proximal C-terminus may cause the tail to be more rigid and restrict its conformational states thereby increasing its association strength with Kv7.1. A similar mechanism seems to apply in the case of the intrinsically disordered *Shaker* channel C-terminus (Magidovich *et al*, 2007).

Taken together, this study reveals the specific interaction between the KCNE1 C-terminus and the Kv7.1 dimeric coiled-coil helix C, thereby providing a simple means to guide assembly of the I_{KS} channel complex. In addition, these subunit C-termini play a dynamic role in channel gating through voltage-dependent molecular motions.

Materials and methods

Molecular biology

For FRET microscopy, Kv7.1 and KCNE1 were amplified using standard PCR techniques and cloned in frame with primers containing *EcoRI* and *BamHI* sites in the 5'- and 3'- flanking regions, respectively, into either the pEXFP-N1 for C-terminal labeling or pEXFP-C1 for N-terminal labeling (Clontech); EXFP refers to ECFP or EYFP. Doubly labeled Kv7.1 was generated by introduction of EYFP in frame upstream of Kv7.1 in pECFP-N1. The D76N LQT5 KCNE1 mutation and Δ622 Kv7.1 truncation mutation were introduced using standard PCR techniques.

For characterization of ternary complexes, the Kv7.1 C-terminus construct 352Δ386–504 subcloning was prepared as described by Wiener *et al* (2008). The KCNE1 C-terminus gene fragment (aa. 67–129) was amplified by PCR with primers containing *BamHI* and *EcoRI* sites in the 5'- and 3'- flanking regions, respectively. Digested PCR product was ligated into the CDFDuet-1 vector (Novagen) at multiple cloning site I, having inserted earlier a sequence encoding GST and a TEV protease recognition sequence.

For constructs used in the pull-down experiments, the various Kv7.1 C-terminus constructs were cloned into the pETDuet-1 vector (Novagen) between the *BamHI* and the *NotI* restriction sites, as described (Wiener *et al*, 2008). For immunoprecipitation, the Kv7.1 C-terminus was subcloned in frame into pFLAG-CMV-2 (Sigma).

Recording of K^+ currents

Channel expression and recording of currents in *Xenopus* oocytes and in CHO cells were performed as described earlier (Gibor *et al*, 2004; Shamgar *et al*, 2006).

FRET confocal microscopy

All experiments were carried out according to the method of Zheng and Zagotta (2004). Fluorescence emission spectra from ECFP- and/or EYFP-tagged subunits were collected from the animal hemisphere of the *Xenopus* oocyte with a confocal microscope (Zeiss LSM 510 META), equipped with 405/514/622 dichroic mirror and a 20 × 0.9NA objective, using laser excitation lines of 405 and 514 nm, respectively. Fluorescence intensity spectra were quantified at 449–599 and 524–599 nm for ECFP and EYFP, respectively, with 10.2 nm binning and keeping constant PMT gain and laser intensity throughout experiments to maintain linearity. Peak ECFP

(476–497 nm) and EYFP (519–539 nm) spectral regions were used for further calculations. The level of background signal was estimated from a blank area of the same image and subtracted. The quantification of the static FRET efficiency and the voltage-dependent FRET is described in 'Supplementary data' section.

Immunoprecipitation and pull-down experiments

For M2-agarose immunoprecipitation experiments, HEK 293 cells were transfected using the calcium phosphate method. Cells were washed in PBS and lysed with a buffer containing 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail (1 h at 4°C, under rotation). Cell lysates were cleared by centrifugation (10 000 g for 15 min, 4°C). Equal amounts of lysates were incubated overnight at 4°C with M2 agarose beads (Sigma) in lysis buffer, washed three times in Tris-buffered saline (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM PMSF and a protease inhibitor cocktail), then resolved by 15% SDS-PAGE and blotted using either α KCNE1 (Alomone Labs) or α M2 (Sigma). The characterization of the Kv7.1 C-terminus/CaM/KCNE1 C-terminus ternary complex is described in 'Supplementary data' section.

The molecular reagents for the pull-down experiments were prepared as follows. Bacterial growth was performed in the presence of 100 µg/ml ampicillin, 34 µg/ml chloramphenicol and induction with 135 µM isopropyl 1-thio-β-D-galactopyranoside, as described earlier (Wiener *et al*, 2008). For Kv7.1 C-terminus/CaM, cells were lysed followed by a Ni^{2+} -NTA column purification step, fractions were pooled and applied to a pre-equilibrated Superdex 200 or Superose 6 Hi-prep gel filtration columns (GE Healthcare) with buffer F. For the GST-KCNE1 C-terminus, cells were grown as above, collected and suspended in PBS, pH 7.4 with 1 mM PMSF. Lysis and centrifugation were performed as described (Wiener *et al*, 2008). The supernatant was loaded onto a pre-equilibrated glutathione sepharose (GE Healthcare) column with PBS at a flow rate of 2.0 ml/min. Protein was eluted with buffer B (100 mM Tris-HCl, pH 8.0, 150 mM NaCl and 20 mM glutathione). Protein was applied to a pre-equilibrated Superdex 200 Hi-prep gel filtration column (GE Healthcare) with buffer F. Elution peaks were aliquoted, flash frozen in liquid nitrogen and stored at –80°C.

For GST pull-down experiments, 10 µg of GST-KCNE1 C-terminus was incubated with 50 µg of the various Kv7.1 C-terminal construct proteins in PBS containing 1% Triton X-100 for 1.5 h at 4°C. Equal amounts of glutathione sepharose (GE Healthcare) were added. After incubation under rotation for 1 h at 4°C and three washes with PBS and 1% Triton X-100, bound complexes were eluted using buffer B. Eluates were boiled together with Laemmli sample buffer and subjected to SDS-PAGE and western blotting.

Statistical analysis

All data were expressed as mean ± s.e.m. Statistically significant differences between multiple groups were performed by one-way ANOVA using *post hoc* Tukey's or Dunnett's Multiple Comparison Test, or by unpaired two-tailed *t* test. For comparing voltage-dependent FRET changes, a paired two-tailed *t* test analysis was carried out.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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