**ARTICLE ADDENDUM**

**Ca\(^{2+}\)-Calmodulin and PIP2 interactions at the proximal C-terminus of Kv7 channels**

William S. Tobelaima\(^a\), Meidan Dvir\(^a\), Guy Lebel\(^b\), Meng Cui\(^c\), Tal Buki\(^d\), Asher Peretz\(^a\), Milit Marom\(^d\), Yoni Haitin\(^d\), Diomedes E. Logothetis\(^c\), Joel A. Hirsch\(^b\), and Bernard Attali\(^a\)

\(^a\)Department of Physiology & Pharmacology, Sackler Faculty of Medicine and Sagol School of Neurosciences, Tel Aviv University, Tel Aviv, Israel; \(^b\)Department of Biochemistry & Molecular Biology, George S. Wise Faculty of Life Sciences, Institute of Structural Biology, Tel Aviv University, Tel Aviv, Israel; \(^c\)Department of Pharmaceutical Sciences, Northeastern University, Boston, MA, USA; \(^d\)Laboratory of Structural Physiology, Department of Physiology & Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

**ABSTRACT**

In the heart, co-assembly of Kv7.1 with KCNE1 produces the slow I\(_{KS}\) potassium current, which repolarizes the cardiac action potential and mutations in human Kv7.1 and KCNE1 genes cause cardiac arrhythmias. The proximal Kv7.1 C-terminus binds calmodulin (CaM) and phosphatidylinositol-4,5-bisphosphate (PIP2) and recently we revealed the competition of PIP2 with the calci

**KEYWORDS**

calmodulin; Kv7; KCNQ; Potassium channel; PIP2

**INTRODUCTION**

Kv7 (or KCNQ) channels form a subfamily of voltage-gated potassium channels (Kv) that comprise five members playing important functions in various tissues including brain, heart, kidney, stomach, pancreas or inner ear.\(^1\) The Kv7.1 pore forming \(\alpha\) subunits can interact with each of the five KCNE auxiliary \(\beta\) subunits, generating currents with distinct characteristics.\(^2\)–\(^5\) In the heart, co-assembly of Kv7.1 with KCNE1 forms the I\(_{KS}\) channel, which together with the hERG channel (I\(_{Kr}\)) form the main repolarizing currents of the cardiac action potential.\(^6\)–\(^9\) In the brain, the complexes formed by Kv7.2/3 and Kv7.3/5 subunits produce the so called “M-current”, a slowly activating, non-inactivating K\(^+\) current, modulated by muscarinic agonists and other Gq protein-coupled receptor agonists.\(^10\)–\(^15\) M-current has profound effects on neuronal excitability, as its low voltage-threshold for gating and slow activation act as a brake for repetitive firing. Kv7 channels have a prominent role in human diseases and can harbor numerous mutations that produce severe cardiovascular and neurological disorders, such as the cardiac long QT syndrome (LQT), atrial fibrillation, benign neonatal epilepsy, epileptic encephalopathy or deafness.\(^8\)–\(^10\)\(^,\)\(^13\)–\(^16\) Each Kv7 subunit features six transmembrane \(\alpha\) helices (S1–S6) comprising a voltage-sensing module (S1–S4) and a pore domain (S5–S6). Kv7 subunits possess a large C-terminus (CT), which is important for channel gating, assembly and trafficking.\(^17\)–\(^21\) The Kv7-CT comprises \(\alpha\) helices that form coiled-coil structures. A proximal antiparallel coiled-coil, adjacent to the membrane, formed by helices A and B binds calmodulin (CaM),\(^22\)–\(^24\) whereas a distal parallel tetrameric coiled-coil formed by helix D serves as an assembly domain.\(^19\)\(^,\)\(^21\) CaM appears to be an essential auxiliary subunit for all Kv7 channels.\(^17\)\(^,\)\(^20\)\(^,\)\(^24\)–\(^29\) However, the role of CaM in Kv7 channel function is not well understood yet. There are prominent differences.
in the role played by Ca\(^{2+}\)-CaM as a Ca\(^{2+}\) sensor in the signaling of Kv7.1-5 subtypes. We and others have shown that LQT mutations, which weaken CaM binding to the Kv7.1 proximal CT also affect channel gating, folding and trafficking.\(^{17,20}\) We found that Kv7.1 and I\(\text{KS}\) currents are stimulated by increases in intracellular Ca\(^{2+}\) and are markedly inhibited by CaM antagonists.\(^{20}\) In contrast, it was found that overexpression of CaM strongly reduced currents of Kv7.2, Kv7.4 and Kv7.5, but not those of Kv7.1 and Kv7.3.\(^{26}\) In a recent structural study, we revealed that CaM hugs the anti-parallel coiled-coil helices A and B with an apo C-lobe and calcified N-lobe, respectively.\(^{22}\)

Phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) is required to stabilize the Kv7 channel open state, thereby preventing current rundown.\(^{30,31}\) Previous studies mapped the PIP\(_2\) interaction site in Kv7.2-4 channels to the intervening linker connecting helices A and B.\(^{32}\) However, another study indicated that this linker is not required for PIP\(_2\) regulation of Kv7.2.\(^{33,34}\) PIP\(_2\) is also necessary for maintaining Kv7.1 channel activity.\(^{35-40}\) Various studies identified clusters of basic residues in Kv7.1 potentially forming PIP2 interaction sites, specifically at the S2-S3 and S4-S5 intracellular linkers as well as in the C-terminus, notably in pre-helix A and in helix C.\(^{37-45}\) PIP\(_2\) regulates Kv7.1 channel function by increasing the coupling between the voltage sensor domain and the pore region thereby stabilizing the channel open state and leading to increased current amplitude, slower deactivation kinetics and negative shift in the voltage dependence of activation.\(^{37-39,41,42}\) In addition, KCNE1 was found to increase PIP2 sensitivity 100-fold over that of the Kv7.1 \(\alpha\) subunit alone.\(^{36}\)

Recently, we revealed the competition of PIP\(_2\) and the calcified CaM N-lobe to a previously unidentified site in Kv7.1 helix B.\(^{46}\) We showed that residues K526 and K527 in Kv7.1 helix B form a critical site where CaM competes with PIP\(_2\) to stabilize the I\(\text{KS}\) channel open state. PIP\(_2\) and Ca\(^{2+}\)-CaM perform the same function on I\(\text{KS}\) channel gating by producing a left-shift in the voltage dependence of activation. The LQT mutant K526E revealed a severely impaired channel function with a right-shift in the voltage dependence of activation, a reduced current density and insensitivity to gating modulation by Ca\(^{2+}\)-CaM. Here we found that Kv7.1 currents expressed alone are like I\(\text{KS}\) dependent on the presence of Ca\(^{2+}\)-CaM, which prevents their rundown. Conservation of homologous residues in helix B of Kv7.2 and Kv7.3 subunits confer similar competition of Ca\(^{2+}\)-CaM with PIP2 binding to their proximal C-termini and suggest that PIP2-CaM interactions occurring in helix B could be very important for modulating channel activity in a Kv7 subtype-dependent fashion.

**Results**

Exploring the functional significance of the interaction of calcified CaM N-lobe with the Kv7.1 proximal CT, we previously showed that the I\(\text{KS}\) current density (Kv7.1+KCNE1) was dramatically decreased when the pipette solution containing 3 \(\mu\)M CaM +5 \(\mu\)M free Ca\(^{2+}\) (CaM/Ca\(^{2+}\)) was replaced by 3 \(\mu\)M CaM +zero free Ca\(^{2+}\) titrated with 5 mM Bapta (CaM/Bapta).\(^{46}\) Fig. 1 shows that a similar feature was observed when the WT Kv7.1 \(\alpha\) subunit was expressed alone in CHO cells. At +30 mV, the WT Kv7.1 current density significantly dropped from 41 ± 5 pA/pF to 19 ± 3 pA/pF with respectively, CaM/Ca\(^{2+}\) and CaM/Bapta in the pipette solution (Fig. 1B, left panel; n = 7, \(\*\)P = 0.0032, two-tailed unpaired t-test). In contrast, the voltage dependence of Kv7.1 activation was not altered by either treatment (Fig. 1B, right panel). Similar results were obtained when free calcium was titrated with 1 mM EGTA. The helix B LQT mutant K526E Kv7.1, which exhibits a lower affinity for both CaM and PIP2\(^{46}\), displayed a significant right-shift in the voltage dependence of activation when compared to WT Kv7.1 with either CaM/Ca\(^{2+}\) or CaM/Bapta in the pipette solution (Fig. 1B, left panel; n = 7, \(\*\)P = 0.05, two-tailed unpaired t-test). The V\(_{50}\) values were as follows: −26.1 ± 1.0 mV, −27.1 ± 3.0 mV and −13.1 ± 3.4 mV, −17.4 ± 2.9 mV for Kv7.1 WT CaM/Ca\(^{2+}\), Kv7.1 WT CaM/Bapta and K526E CaM/Ca\(^{2+}\), K526E CaM/Bapta, respectively. Yet, the current density of the K526E LQT mutant significantly dropped from 22 ± 4 pA/pF to 9 ± 1 pA/pF with respectively, CaM/Ca\(^{2+}\) and CaM/Bapta in the pipette solution (Fig. 1B, left panel; n = 7, \(\*\)P = 0.032, two-tailed unpaired t-test).

In the recent study by Tobelaim et al.,\(^{46}\) we showed that calcified CaM, in agreement with pulldown assays, lessened the I\(\text{KS}\) current rundown resulting from PIP2 depletion induced by Dr-VSP or by wortmannin. In Fig. 2A (right panel), we reveal that the WT Kv7.1 \(\alpha\) subunit expressed alone, exhibited a robust spontaneous rundown when the pipette
solution contained CaM/Bapta, leading to more than 56% current amplitude decrease, 9 min after membrane patch rupture. Introducing into the pipette solution CaM/Ca\(^2+\), initially induced current run up and significantly prevented current decline by maintaining 91% of the initial current amplitude, 9 min after membrane patch rupture (Fig. 2B; n = 4, P = 0.05, two-tailed unpaired t-test). Thus, like for IKS, the removal of Ca\(^2+\) from CaM was deleterious for Kv7.1 channel gating.

The proximal CT of all Kv7 subunits contain sites for modulation by PIP2 and CaM.\(^{18,41,47}\) Our recent data reveal a competition between PIP2 and calcified CaM N-lobe to a previously unidentified site in Kv7.1 helix B, which harbors a LQT mutation.\(^{46}\) This site is highly conserved in all Kv7 channels (Fig. 3A). Thus, we hypothesized that similar PIP2-CaM interactions occur in helix B of Kv7.2 and Kv7.3 subunits. We investigated the competition between WT CaM and PIP2 binding to the purified Kv7.2 CT and Kv7.3 CT. The His-tagged purified Kv7.2 and Kv7.3 CT proteins were separately pulled down using PIP2-coated agarose beads in an incubation medium containing either 0.1 mM Ca\(^2+\) or 1 mM EGTA in the presence of

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**Figure 1.** Ca\(^2+\)-CaM is required for WT Kv7.1 currents and impaired function of the helix B LQT mutant K526E Kv7.1. (A) Upper panel: representative WT Kv7.1 currents recorded from transfected CHO cells with pipette solution containing 3 μM purified recombinant WT CaM with either 5 μM free Ca\(^2+\) (left) or 5 mM Bapta-zero free Ca\(^2+\) (right). From a holding potential of −90 mV, cells were stepped for 3 s from −60 mV to +30 mV in 10 mV increments and repolarized for 1.5 s to −60 mV. Lower panel: representative currents of the LQT mutant K526E Kv7.1 recorded from transfected CHO cells with pipette solution containing 3 μM purified recombinant WT CaM with either 5 μM free Ca\(^2+\) (left) or 5 mM Bapta-zero free Ca\(^2+\) (right). The step depolarization protocol was as described above. (B) Shown are current density-voltage relations (left panel) and normalized conductance-voltage relations (right panel). The currents at +30 mV were as follows: 41 ± 5 pA/pF, 19 ± 3 pA/pF and 22 ± 4 pA/pF for WT Kv7.1 CaM/Ca\(^2+\), WT Kv7.1 CaM/Bapta and K526E CaM/Ca\(^2+\), respectively. (n = 7, *P = 0.0032 and **P = 0.032, two-tailed unpaired t-test). Activation curves were fitted by a single Boltzmann distribution. The V_{50} values were as follows: −26.1 ± 1.0 mV, −27.1 ± 3.0 mV and −13.1 ± 3.4 mV, −17.4 ± 2.9 mV for WT Kv7.1 CaM/Ca\(^2+\), WT Kv7.1 CaM/Bapta and K526E CaM/Ca\(^2+\), respectively.
increasing concentrations of WT CaM (Fig. 3B). The His-tagged purified recombinant Kv7.2 and Kv7.3 CT proteins included helices A and B with a deletion of the intervening linker connecting helices A and B (Fig. 3B). Increasing amounts of WT CaM displaced PIP2 binding to the Kv7.2 CT and Kv7.3 CT proteins, only in the presence of Ca$^{2+}$. In contrast, no competition was observed by increasing CaM concentrations in the absence of Ca$^{2+}$ (EGTA) (Fig. 3B). Thus, in line with our working hypothesis, we found that like with Kv7.1, Ca$^{2+}$-CaM competes with PIP2 binding to the Kv7.2 and Kv7.3 proximal CT in PIP2 pulldown.

**Discussion**

PIP2 is required to stabilize the open state of Kv7 channel subtypes. Clusters of basic residues are potential PIP2 interaction sites and have been identified in S2-S3 and S4-S5 intracellular linkers as well as in the C-terminus. Interestingly, the proximal CT of all Kv7 channel subtypes spanning pre-helix A and helices A and B contain sites for modulation by PIP2 and CaM, raising the question of whether the CaM and PIP2 binding modules overlap physically and functionally.\(^{18,30,41}\)

In the recent study by Tobelaim et al.,\(^{46}\) we characterized the existence of a previously unidentified site in Kv7.1 helix B, where competitive binding of PIP2 and calcified CaM N-lobe takes place. CaM and PIP2 pulldown experiments demonstrated Ca$^{2+}$-dependent competitive binding of PIP2 and CaM to the purified Kv7.1 CT. The PIP2 pulldown performed with different purified Kv7.1 CT constructs indicated that the competition of Ca$^{2+}$-CaM with PIP2 binding occurs in helix B and strictly depends on calcium. In line with our earlier structural findings,\(^{22}\) the potent reduction of IKS current density and the right-shift of the voltage-dependence of activation following introduction of Bapta-CaM or Bapta alone into the pipette solution revealed the strict requirement of calcified CaM for normal channel gating.\(^{46}\)

Here we showed...
that a similar prerequisite is necessary for Kv7.1 channel function even in the absence of KCNE1, since Kv7.1 current density considerably dropped when BaPTA was introduced together with CaM in the pipette solution. However, no right-shift was observed under these conditions, suggesting that the presence of KCNE1 was necessary to confer this sensitivity for the voltage dependence of activation.

Molecular docking, molecular dynamics simulations and functional retrospective validation using
purified protein pulldown and patch-clamp recordings indicated that K526 and K527 in Kv7.1 helix B form a critical site where CaM competes with PIP2 to stabilize the IKS channel open state. A recent cryo-EM study showed that each Kv7.1 subunit associates with one calmodulin molecule, in line with our previous structural work. Interestingly, the surface electrostatic potential of this cryo-EM structure shows two distinct positively charged pockets that could serve as potential interaction sites for negatively charged PIP2. One of the pockets faces the inner leaflet of the membrane and consists mainly of helix B and the C terminus of S6, which is in agreement with our recent data. The other pocket is located between the voltage sensor and the pore domain. It was suggested that PIP2 binds to this region to strengthen the coupling between the voltage sensor and the pore. Nevertheless, it is likely that several PIP2 molecules bind to the Kv7.1 subunit at multiple contact sites or migrate to different places, including those located at the S2-S3 and S4-S5 intracellular linkers, pre-helix A and helix B in order to achieve a specific function. The strategic location of PIP2 at the interface of CaM and Kv7.1 helix B suggests that changes in CaM conformation, such as upon calcification of its N-lobe, could disrupt the ternary complex formed by PIP2, helix B and CaM, thereby dislodging PIP2 from its binding pocket. In the present study, we showed that like for IKS, the removal of Ca2+ from CaM was deleterious for Kv7.1 channel gating as illustrated by the profound Kv7.1 current rundown obtained when CaM/Bapta was present in the pipette solution. These structural and physiological features of Kv7.1 channels are reminiscent to those described for Ca2+-activated small conductance SK2 K+ channels. In SK2 channels, the interactions of the CaM binding module at the proximal CT with the apo C-lobe and the linker of CaM are thought to be responsible for the constitutive association of CaM with the channel, while the interaction with the calcified N-lobe accounts for gating. In addition, the PIP2 binding site is located at the interface of CaM and the SK2 C terminus.

The functional importance of the helix B residue K526 interacting with both CaM and PIP2 is underscored by the existence of the LQT mutation K526E. When co-expressed with KCNE1, the LQT mutant K526E showed a severely impaired channel function with a right-shift in the voltage dependence of activation, reduced current density and insensitivity to gating modulation by Ca2+-CaM. Here we showed that the mutant Kv7.1 K526E expressed alone exhibits a significant right-shift in the voltage dependence of activation when compared to WT Kv7.1 with either CaM/Ca2+ or CaM/Bapta in the pipette solution.

Our data show that both PIP2 and Ca2+-CaM perform the same function on Kv7.1 channel gating by stabilizing the open state. What is the physiological significance of this CaM-PIP2 competitive interaction for IKS channel function? We propose that at resting cytosolic Ca2+, a ternary complex exists, where the Kv7.1 helix B can interact and accommodate with PIP2 and the calcified CaM N-lobe, while the Kv7.1 helix A binds the CaM apo C-lobe. However, following receptor-mediated PIP2 depletion and increased cytosolic Ca2+, we suggest that the calcified CaM C-lobe unbinds helix A, while the calcified CaM N-lobe interacts with helix B in place of PIP2 to limit the decrease in IKS channel activity that arises from PIP2 hydrolysis. Impairment in achieving this crucial task, as with the LQT mutation K526E leads to compromised IKS channel gating. In the present study, we show that this helix B site is highly conserved in the other Kv7 channel subtypes and we found that like with Kv7.1, Ca2+-CaM competes with PIP2 binding to the Kv7.2 and Kv7.3 proximal CT in PIP2 pull-down. Interestingly, the same helix B region in Kv7.2 exhibits epileptic encephalopathy mutations (R553Q/W/L; R554N). Thus, we hypothesize that PIP2-CaM interactions converge to helix B in all Kv7 subtypes. What is the functional outcome in Kv7.2 and Kv7.3 subunits? Does calcified CaM reduces the affinity of PIP2 and vice-versa in all Kv7 subunits? Are there physical and functional differences in PIP2 and CaM interactions between the different Kv7 channel subtypes? Future work should provide clues for a basic understanding of the integration in time and space of Kv7 channel modulation by these key signaling molecules under normal and disease conditions, notably those of neuronal hyper-excitability and cardiac arrhythmia.

Materials and methods

Human Kv7.1 was cloned into pCDNA3 vector to allow eukaryotic expression. The mutation K526E in
Kv7.1 was introduced using the PCR-based Quik-change site-directed mutagenesis (Stratagene).

Expression and Purification of Kv7.2 and Kv7.3 CT/CaM Complexes

The Kv7.2 CT (317-539, Δ369-511) and Kv7.3 CT (356-537, Δ410-508) bearing deletion of the intervening loop between helix A and B were cloned into the multiple cloning site (MCS) I of the pET-Duet vector (Novagen), located downstream of a His-tag and a Tobacco etch virus (TEV) protease site. CaM was in multiple cloning site II. Kv7 CT constructs were co-purified with WT CaM as previously described.21,59 WT CaM was purified as previously described.43

Pulldown experiments

Pulldown experiments were performed as previously published.43 In brief, for PIP2 pulldown, 5 μg of purified WT or mutants His-tagged Kv7 C-terminus proteins were incubated with equal amounts of PIP2-coated agarose beads (Echelon Biosciences) for 2 h at room temperature in binding buffer containing (in mM): 10 HEPES pH 7.4, 150 NaCl, 0.25% Igepal with either 0.1 Ca2+ or 1 EGTA. After sample centrifugation at 600 g, three washes in binding buffer were performed and followed by boiling with 4 x Laemmli sample buffer at 95°C. Samples were subjected to SDS-PAGE and followed by Western blotting. Input samples were taken for quantification purposes. Detection was performed using HRP-conjugated anti-His antibody (Roche) or with anti-calmodulin antibodies (Millipore) and ECL (Millipore).

Cell Culture and Transfection

Chinese hamster ovary CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% fetal calf serum, and antibiotics. In brief, 40,000 cells seeded on poly-D-lysine-coated glass coverslips (13 mm in diameter) in a 24-multiwell plate were transfected with pIRES-CD8 (0.3 μg) as a marker for transfection with 0.5 μg WT or mutant Kv7.1. Transfection was performed using 3.6 μl of X-tremeGENE 9 (Roche) according to the manufacturer’s protocol. For electrophysiology, transfected cells were visualized approximately 40h after transfection, using the anti-CD8 antibody coated beads method as described previously.43

Electrophysiology

Recordings were performed using the whole cell configuration of the patch clamp technique. Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), sampled at 5 kHz and filtered at 2.4 kHz via a four pole Bessel low pass filter. Data were acquired using pClamp 10.5 software in conjunction with a DigiData 1440A interface. The patch pipettes were pulled from borosilicate glass (Harvard Apparatus) with a resistance of 3–7 megohms. The intracellular pipette solution contained 130 mM KCl, 5 mM K2-ATP, 1mM MgCl2, 5 mM BAPTA, 10 mM HEPES, pH 7.3 (adjusted with KOH), CaCl2 as needed for different values of free Ca2+ concentration according to MAXCHELATOR software, with sucrose added to adjust osmolarity to 290 mosmol. The external solution contained 140 mM NaCl, 4 mM KCl, 1.8 mM CaCl2, 1.2 mM MgCl2, 11mM glucose, 5.5 mM HEPES, adjusted with NaOH to pH 7.3 (310 mOsM). Series resistances were compensated (75–90%) and periodically monitored.

Data Analyses

Data analysis was performed using the Clampfit program (pClamp 10.5; Axon Instruments), Microsoft Excel (Microsoft, Redmond, WA), and Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Leak subtraction was performed off-line, using the Clampfit program of the pClamp 10.5 software. Chord conductance (G) was calculated by using the following equation: G = I/(V-Vrev), where I corresponds to the current amplitude measured at the end of the pulse, and Vrev is the calculated reversal potential assumed to be −90 mV in CHO cells. G was estimated at various test voltages (V) and then normalized to a maximal conductance value, Gmax. Activation curves were fitted by one Boltzmann distribution: G/Gmax = 1/ [1+exp((V50-V)/s)], where V50 is the voltage at which the current is half-activated and s is the slope factor. All data were expressed as mean ± S.E.M. For electrophysiology, if not indicated otherwise, statistically significant differences were assessed by unpaired two-tailed Student’s t test.
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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