An Epithelial Ca\textsuperscript{2+}-Sensor Protein is an Alternative to Calmodulin to Compose Functional KCNQ1 Channels

Atsushi Inanobe\textsuperscript{a,b} Chizuru Tsuzuki\textsuperscript{a} Yoshihisa Kurachi\textsuperscript{a,b}

\textsuperscript{a}Department of Pharmacology, Graduate School of Medicine, Osaka University, Suita, Osaka, and \textsuperscript{b}Center for Advanced Medical Engineering and Informatics, Osaka University, Suita, Osaka, Japan

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Abstract

Background/Aims: KCNQ channels transport K\textsuperscript{+} ions and participate in various cellular functions. The channels directly assemble with auxiliary proteins such as a ubiquitous Ca\textsuperscript{2+}-sensor protein, calmodulin (CaM), to configure the physiological properties in a tissue-specific manner. Although many CaM-like Ca\textsuperscript{2+}-sensor proteins have been identified in eukaryotes, how KCNQ channels selectively interact with CaM and how the homologues modulate the functionality of the channels remain unclear. Methods: We developed protocols to evaluate the interaction between the green fluorescent protein-tagged C-terminus of KCNQ1 (KCNQ1cL) and Ca\textsuperscript{2+}-sensors by detecting its fluorescence in size exclusion chromatography and electrophoresed gels. The effects of Ca\textsuperscript{2+}-sensor proteins on KCNQ1 activity was measured by two electrode voltage clamp technique of \textit{Xenopus} oocytes. Results: When co-expressed CaM and KCNQ1cL, they assemble in a 4:4 stoichiometry, forming a hetero-octamer. Among nine CaM homologues tested, Calm13 was found to form a hetero-octamer with KCNQ1cL and to associate with the full-length KCNQ1 in a competitive manner with CaM. When co-expressed in oocytes, Calm13 rendered KCNQ1 channels resistant to the voltage-dependent depletion of phosphatidylinositol 4,5-bisphosphate by voltage-sensitive phosphatase. Conclusion: Since Calm13 is closely related to CaM and is prominently expressed in epithelial cells, Calm13 may be a constituent of epithelial KCNQ1 channels and underscores the molecular diversity of endogenous KCNQ1 channels.

Introduction

KCNQ1 is a pore-forming subunit of voltage-gated K\textsuperscript{+} channels. Inherited mutations in KCNQ1 alter the duration of cardiac action potentials, leading to life-threatening arrhythmias in the worst cases \cite{1,2}. KCNQ1 channels are also expressed in multiple types of epithelia and

\textsuperscript{a}Department of Pharmacology, Graduate School of Medicine, Osaka University, Suita, Osaka, and \textsuperscript{b}Center for Advanced Medical Engineering and Informatics, Osaka University, Suita, Osaka, Japan

E-Mail inanobe@pharma2.med.osaka-u.ac.jp or E-Mail ykurachi@pharma2.med.osaka-u.ac.jp
function in K+ recycling and maintaining resting membrane potentials [3-9]. These functions of the channels contribute to the intimate epithelial functions of excreting ions and underpin the activity of Na+-driven electrogenic transporters [10]. Like other membrane-embedded ion channels, KCNQ1 channels assemble with various auxiliary subunits to become functional [11-14]. Non-pore-forming, single membrane-spanning KCNE subunits modulate the electrophysiological properties and the subcellular localizations of KCNQ1 channels [15-17]. At the cytoplasmic domain of the channels, A-kinase anchoring proteins bind and tether the phosphorylation-related enzymes, such as protein kinase A and phosphatase, that refine autonomic nerve regulation in the heart and brain [18-22]. Therefore, formation of a macromolecular complex is essential for the physiological function of KCNQ1.

KCNQ1 binds to a prototypical Ca2+-sensor CaM at its cytoplasmic domain [23-28]. The binding of CaM seems to facilitate the proper folding of the cytoplasmic domain and confers Ca2+ sensitivity to KCNQ1 [24, 25]. Both Ca2+-bound and Ca2+-unbound CaM can interact with KCNQ1 [29-31]. Thus, CaM is a constituent of functional KCNQ1 channels. In eukaryotes, multiple genes encode CaM-like Ca2+-sensors [32-34], and these CaM-like proteins differently modulate the activity of enzymes and ion channels by direct binding [35, 36]. However, the selectivity of the interactions of KCNQ1 with Ca2+-sensors and the differences in the channel properties as a result of the distinct assemblies required clarification.

To address these questions, we developed a biochemical approach to evaluate the interactions between KCNQ1 and Ca2+-sensors by monitoring a GFP-tagged, cytoplasmic C-terminus of KCNQ1. The detection systems based on GFP fluorescence allowed us to determine that Calml3 can substitute for CaM to facilitate folding of the KCNQ1 cytoplasmic domain [37, 38]. Since Calml3 is abundant in multiple types of epithelial cells, including those from the mammary gland [39, 40] and the gastrointestinal tract [41], it may act as a binding partner for KCNQ1 to support epithelial K+ transport.

Materials and Methods

Molecular Biology

To generate GFP fusion proteins, we used GFPuv (Clontech, Mountain View, CA) as a parent. The Ncol, BamHI, and Xhol sites were eliminated, and alanine at position 206 was replaced by lysine to prevent dimer formation. The GFP mutant (amino acids 2-237) was tagged with an octa-histidine at the N-terminus, connected to the cytoplasmic domain (amino acids: 354-676) of human KCNQ1 (KCNQ1cL) [42] via a tobacco etch virus protease cleavage site, and subcloned into the pET28a vector (Novagen/Merck Millipore, Garmstadt, Germany). KCNQ1 and a canonical inward rectifier K+ channel, Kir2.3 [43], were subcloned into the pGem vector (Promega, Madison, WI). PCR fragments of the Ca2+-sensor proteins CaM (GenBank accession number: M19380.1), Calml3 (NM_027416.3), Calml4a (NM_138304.2), Calml4b (NM_001102468.1), NCS-1 (AF020184.1), NCALD (NM_134094.4), Hpc (NM_001130419.2), VILIP-1 (NM_012038.4), KChIP2a (AB044570.1) and Calp (AF305071.1), were amplified from mouse forebrain cDNA and cloned into pCDFDuet-1 using standard cloning procedures. For expression in human embryonic kidney 293T (HEK) cells, KCNQ1, CaM, and Calml3 were subcloned into the pcDNA3 (Life Technologies, CA), pEGFP-N1 (Clontech), and pCMV-Myc-N vectors (Clontech), respectively. The constructs were verified by DNA sequencing. The sequence alignment was carried out using the 'T-Coffee' program [44]. The wild type and enzyme-defective mutant (C363S) of Ciona intestinalis voltage sensitive phosphatase (Ci-VSP) were kindly provided by Dr. Yasushi Okamura [45].

Protein expression

E. coli Rosetta 2 (DE3) cells (Novagen) were used as a host strain for the protein expression. The culture medium (Terrific broth) was supplemented with three antibiotics (chloramphenicol, streptomycin, and kanamycin) for the selective growth of the transformed cells. For induction, isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.1 mM and the cells were incubated at 18°C overnight.
Fluorescence detection size exclusion chromatography (FSEC) column

The cell pellet harvested from 0.5 ml of overnight E. coli cultures was disrupted with 0.4 ml lysis buffer using an ultrasonicator UD-100 (TOMY, Tokyo, Japan). The lysis buffer contained 40 mM HEPES-NaOH (pH 8.0), 500 mM NaCl, 2 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride (PMSF) supplemented with complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was clarified by centrifugation and the aliquot (10 µL) was subjected to gel filtration on Superdex 200 5/150 GL columns (GE Healthcare, Pittsburgh, USA) equilibrated with buffer containing 10 mM HEPES-NaOH (pH 7.0) and 500 mM NaCl. The chromatography was controlled by ÄKTA UNICORN system (GE Healthcare) and the flow path coming from the SEC column was directly connected to a fluorometer RF-20A (Shimazu, Kyoto, Japan). The data acquisition was controlled and stored in the UNICORN control software.

In-gel fluorescence detection

The E. coli cell lysate (100 µl) prepared as described above was incubated at different temperatures for 10 min, and then centrifuged at 50,000 x g for 20 min. A small aliquot of the supernatant (1.25 µL) was diluted with lysis buffer, mixed with standard Laemmli sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fluorescence imaging was performed using a Typhoon 9410 scanner (GE Healthcare), where the GFP were excited by a 488-nm laser, and the images were subjected to densitometry analysis using ImageJ 1.40g software [46].

Immunoprecipitation analysis

HEK cells were transfected with plasmids carrying KCNQ1, CaM, or Calml3 using X-tremeGENE 9 DNA transfection reagent (Roche, Mannheim, Germany). Two days after transfection, the cells were rinsed and collected into ice-cold phosphate buffered saline. The cells were sonicated in 500 µL of membrane preparation buffer (20 mM HEPES, 150 mM NaCl, 12.5 mM KCl, 0.5 mM EDTA, 1 mM PMSF supplemented with complete protease inhibitor cocktail). Membrane fractions were collected by ultracentrifugation and stored at -80°C. The membrane proteins were solubilized with 2% (v/v) Triton X-100 in the membrane preparation buffer. After pre-absorbed non-specific binding with non-immune IgG and protein G Sepharose (GE Healthcare), either rabbit anti-KCNQ1 (APC-022; Alomone) or mouse anti-c-Myc antibody (Clontech) was mixed with the extract to isolate the protein complexes. The immune-complexes were separated by SDS-PAGE. Before transferring the electrophoresed proteins to polyvinylidene difluoride membranes, the gels were cut off below 60 kDa for KCNQ1 detection and above 25 kDa for Calml3 detection.

Electrophysiological recording and data analysis

The treatment of frogs (Xenopus laevis) was carried out in accordance with the Guidelines for the Use of Laboratory Animals of Osaka University Medical School. The oocytes were surgically removed from frogs anaesthetized with 0.35% tricaine methanesulfonate (Sigma-Aldrich, MO), and were defolliculated in 1 mg/mL type I collagenase solution (Life Technologies). All clones were transcribed in vitro with mMESSAGE mMACHINE Transcription kits (Life Technologies). The cRNA of KCNQ1 (0.5 ng) was injected into oocytes in various combinations with the cRNA of either CaM or Calml3 cRNA (10 ng), and the cRNA of Ci-VSP (1-5 ng). Membrane currents were recorded using a conventional two-microelectrode voltage-clamp technique with a GeneClamp 500 amplifier (Molecular Devices, CA) 3-5 days after injection of cRNA into the oocytes. All experiments were conducted at ambient temperature (20-24°C). The glass electrodes had a resistance of 0.4-1.2 MΩ when filled with 3 M KCl. The bath solution for recording KCNQ1 contained 2 mM KCl, 96 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 150 µM niflumic acid (pH 7.35 with KOH). For recording Kir2.3, the concentrations of KCl and NaCl were changed to 40 mM and 58 mM, respectively. Voltage clamp protocols are described in each figure legend. The leak subtraction was carried out by subtracting the linear ohmic function calculated from the difference in current amplitudes at -100 mV and -90 mV. Tested oocytes were prepared from at least five different frogs. Data acquisition and fitting was carried out using the Clampfit software (Molecular Devices) and SigmaPlot (Systat Software, Chicago, IL). Data are presented as the mean ± S.E.M. (n = number of observations).
Results

Since eukaryotic cells, but not prokaryotic cells, express CaM-like Ca\(^{2+}\)-sensor proteins [32-34], we selected *E. coli* as a host strain to study the interaction between KCNQ1 and CaM. The bacteria, however, are not an ideal expression system for mammalian membrane proteins to retain normal function. Thus, we expressed the cytoplasmic, C-terminus of KCNQ1 (KCNQ1cL) with CaM to reconstitute the protein complex in the cells (Fig. 1A). We were able to purify a large quantity of the hetero-octamer with a stoichiometry of 4:4, as reported previously (Fig. 1B) [24, 25]; however, the culture scale and purification procedure hampered our ability to effectively and quantitatively evaluate the biochemical properties of the complex.

Development of fluorescence-based detection techniques for KCNQ1-CaM complex

We next expressed a KCNQ1cL construct tagged with GFP on its N-terminus (Fig. 1A). The cell lysate was separated on a size exclusion chromatography (SEC) column, which is connected in series to a fluorescence monitor to detect GFP fluorescence in the eluate (FSEC) (Fig. 1C) [47]. In the absence of CaM, there are two fluorescence peaks: the first was collected at the void volume, which presumably reflects the contaminated aggregates, and the second was collected at an elution volume close to the molecular weight of GFP, suggesting degradation products. However, in the presence of CaM, a fluorescence peak was prominently observed in fractions corresponding to a molecular weight of approximately

![Fig. 1.](image-url) Fluorescence-based detection of KCNQ1-CaM complex separated by size exclusion chromatography (SEC). A. Schematic representation of KCNQ1. The KCNQ1 channel is a pore-forming subunit consisting of 6 transmembrane helices and 4 cytoplasmic helices (helix A to helix D). The cytoplasmic C-terminus of KCNQ1 (KCNQ1cL) can be expressed in a soluble fraction in the presence of CaM. KCNQ1cL accommodates a hexa His-tag and/or a GFP on the N-terminus without disrupting the CaM interaction. B. In-gel staining of purified KCNQ1cL –CaM complex. Homogenously purified complex of His-tagged KCNQ1cL and CaM was subjected to SDSPAGE and stained by CBB R-250. C. Fluorescence-detection SEC (FSEC). The lysates from the cells expressing GFP-tagged KCNQ1cL in the presence (top) or absence (bottom) of CaM were subjected to SEC column. The fluorometer was connected to the outlet of the column and used to monitor the GFP fluorescence in the eluate. Inset in top panel shows elution position of standard proteins: bovine serum albumin dimer (132 kDa; open circle), monomer (66 kDa; open triangle) and GFP (27 kDa; open square).
360 kDa, suggesting that the KCNQ1cL-CaM complex exists as a hetero-octamer with a 4:4 stoichiometry (330 kDa including 4 GFP tags). The fluorescent probe provides highly sensitive detection of the protein. Through the combination of the pre-packed SEC column and a small bed volume, the FSEC protocol allowed us to reduce the induction scale to less than 1 mL and the duration of analysis to approximately 15 min per sample.

Thermal stability is one of the biochemical characteristics of a protein complex. To assess the thermal stability of the KCNQ1cL-CaM complex, aliquots from the same E. coli cell lysate described above were incubated at different temperatures for 10 min, clarified by centrifugation, and then subjected to FSEC (Fig. 2A). The height of the fluorescence peak corresponding to the hetero-octamer was lowered by increasing incubation temperatures. On the other hand, the fluorescence peak recovered at the void volume disappeared after low heat treatment (45°C), but the fluorescence peak corresponding to the GFP was stable at temperatures up to 65°C.

GFP is resistant to low concentrations of SDS, and the fluorescence can be detected in SDS-PAGE gels, even though it is fused to other proteins [48]. Given the thermal stability of the GFP-KCNQ1cL-CaM complex in FSEC and the recovery of the majority of the full-length product in the hetero-octamer fractions, we were interested in studying its thermal stability using this in-gel fluorescence-detection technique. The lysates of cells expressing both KCNQ1cL and CaM were incubated at different temperatures for 10 min, clarified...
by centrifugation, resolved on polyacrylamide gels, and then scanned to detect the GFP-fluorescence within the gels. A single major band matching the size of the full length GFP-KCNQ1cL and bands corresponding to minor degraded products were detected (Fig. 2B). By increasing the temperature, the band corresponding to the full length of the product disappeared, while the lower bands were retained. The fluorescent intensity of the full

Fig. 3. Analysis of KCNQ1 interaction with Ca²⁺-sensor proteins. A. Phylogenic tree of Ca²⁺-sensor proteins. Comparison of the amino acid sequences of six different Ca²⁺-sensor proteins generated with the T-Coffee program [44]. B. CBB staining of the cell lysate. GFP-tagged KCNQ1cL was expressed in bacteria with or without Ca²⁺-sensor proteins as indicated below the panel. Asterisks show the migrated positions of the Ca²⁺-sensor proteins. Numbers on the left represent the molecular weights of the markers. C. Monitoring the fluorescence in the SDS-PAGE gel. The fluorescence in the crude lysates was detected using the fluorometric scanner. An arrowhead corresponds to the full length GFP-tagged KCNQ1cL. D. FSEC analysis of the elution profile of GFP-tagged KCNQ1cL. The cell lysates were subjected to the SEC column at the points indicated by the arrowheads. The elution profile of fluorescence was monitored. The fluorescence peak of the hetero-octamer was only observed in cell lysates expressing either CaM or Calml3. E. The thermal stability of KCNQ1cL-Calml3 complex. The thermal stability of KCNQ1cL-Calml3 complex was analyzed in gels and compared with that of the KCNQ1cL-CaM complex (n = 4). F. Amino acid sequence alignment of CaM and Calml3. Amino acids are described in single letter code. Two Ca²⁺-sensor proteins show 87% sequence identity. Identical residues are represented in white on red squares, conserved substitutions are colored in black and non-conserved residues are in blue.
length bands in the digitized gels was densitometrically quantified and compared with the height of fluorescence peak corresponding to the hetero-octamer in FSEC (Fig. 2C). The thermal denaturation profile suggests that denaturation occurs at temperatures between 45°C and 65°C, with a midpoint around 55.9 ± 0.1°C by FSEC (n = 4) and 57.1 ± 0.2°C by the in-gel analysis (n = 4). The full length product was eluted in the void volume fractions and in fractions containing the hetero-octamer (Fig. 2A). Since, within these fractions about 90% of the fluorescence corresponded to the hetero-octamer, the denaturation profiles measured by these two different protocols were comparable.

These quantifications are based on the measurement of the fluorescence intensity of GFP. Therefore, if GFP is unstable at lower temperatures than the midpoint of KCNQ1-CaM complex denaturation, these quantifications are inadequate for assessing the thermal stability of the complex. To address this question, we quantified the thermal denaturation of GFP alone by in-gel analysis (Fig. 2C). The fluorescent intensity was stable up to 70°C and dropped at temperatures between 75°C and 90°C [49]. The midpoint of the curve was 81.5 ± 0.2°C (n = 4). Therefore, the denaturation curves produced by measuring the fluorescence of GFP-fused KCNQ1cL can be attributed mainly to the thermal stability of the KCNQ1cL-CaM complex. Since the in-gel analysis enables us to increase the number of samples analyzed at a time, this technique provides an effective and quantitative means for analyzing the biochemical and biophysical properties of a complex.
Using biochemical techniques, we tried to assess the ability of Ca^{2+}-sensor proteins to facilitate the folding of KCNQ1 cytoplasmic domain. Marine Ca^{2+}-sensor proteins (Calml3, Calml4a, Calml4b, NCS-1, NCALD, Hpc1, VIIIP-1, KChIP2a, and Calp) were expressed in cells with GFP-KCNQ1cL. Among CaM homologues, Calml3 is highly similar and Calml4a is mildly similar to CaM (93% and 53% amino acid similarity, respectively), while other neuronal Ca^{2+}-sensor (NCS) family members share relatively low amino acid similarity (8-12%) (Fig. 3A). The protein expression of two CaM homologues (Calml3 and Calml4a) and three NCS family members (NCS-1, NCALD and NCS-1) was confirmed by Coomassie staining of SDS-PAGE gels loaded cell lysates (Fig. 3B). Expression of the homologues did not appear to perturb the overall protein expression level of GFP-KCNQ1cL (Fig. 3C). After clarifying each lysate by centrifugation, the supernatant was analyzed by FSEC (Fig. 3D). The remarkable fluorescence peak at the position of the hetero-octamer was only observed in cell lysates co-expressing GFP-KCNQ1cL and either Calml3 or CaM.
The number of amino acids in Calml3 is equal to the number in CaM (Fig. 3F). Calml3 shares a dumbbell-like crystal structure with CaM, but the structures of the two proteins show displacement of the central helix connecting the two globular domains [50, 51]. Furthermore, Calml3 has an 8-fold lower affinity for Ca\textsuperscript{2+}, but undergoes large structural changes upon metal binding [52]. These observations suggest that the two Ca\textsuperscript{2+}-sensors...
may distinctly impact KCNQ1cL. The thermal denaturation profile of the KCNQ1cL-Calml3 complex measured by the in-gel analysis indicates that the midpoint of denaturation was 49.4 ± 0.2°C (n = 4) (Fig. 3E). The slight difference in the thermal stability between the two assemblies of KCNQ1cL and Ca²⁺-sensor implies that, although Calml3 forms a complex with KCNQ1cL in a comparable manner to CaM, the two Ca²⁺-sensors confer unique property on KCNQ1 channels.

**Interaction of KCNQ1 and Calml3**

We next tested whether Calml3 interacts with the full length KCNQ1 (Fig. 4). KCNQ1 was expressed in HEK cells with or without either GFP-tagged CaM or c-Myc-tagged Calml3. After solubilization with Triton X-100, KCNQ1 was isolated with a specific antibody and then analyzed by immunoblotting or in-gel GFP fluorescence analysis. As CaM formed the complex with KCNQ1 (Fig. 4A), Calml3 was found in the immunoprecipitates with KCNQ1 (Fig. 4B). Furthermore, KCNQ1 was also immunoprecipitated with Calm13 when using an anti-c-Myc antibody. Therefore, Calml3 appears to bind to KCNQ1. To address the mode of Calml3 binding, we examined the effect of CaM over-expression on the interaction of KCNQ1 and Calml3 (Fig. 4C). CaM over-expression reduced the amount of Calml3 by 45.2 ± 8.5% (n=8) recovered in the immunoprecipitate with KCNQ1, while Calml3 over-expression lowered the amount by 29.2 ± 9.3% (n=5) (Fig. 4D). These results indicate that Calml3 binds to KCNQ1 competitively with CaM, thereby suggesting that Calml3 is an alternative to CaM.

**Effects of Calml3 on KCNQ1 channel activity**

Since Calm3 and CaM are known to differently regulate the activities of target enzymes [37, 53], we next tested their effects on KCNQ1 activity. KCNQ1 cRNA was injected into *Xenopus* oocytes with or without either CaM or Calm3 cRNA, and K⁺ currents were measured using the two electrode voltage clamp technique. Fig. 5A shows typical current traces of KCNQ1 alone and of KCNQ1 co-expressed with CaM or Calm3 obtained with depolarizing voltage steps from -100 mV at 10-mV increments in a solution containing 2 mM K⁺. KCNQ1 was rapidly activated by depolarizing step pulses. The current amplitude at the end of the test pulses was augmented in a voltage-dependent manner (Fig. 5B). Co-expression of both Ca²⁺-sensors did not modulate the current expression level or the voltage dependence of KCNQ1. Therefore, it is likely that the KCNQ1-Calml3 complex is functionally comparable to the KCNQ1-CaM complex.

PIP₂ plays pivotal roles in KCNQ1 activation [54, 55]. Amino acid residues responsible for PIP₂-dependent activation of KCNQ1 channels have been mapped to the proximal half of the C-terminus, sandwiched between putative CaM-binding sites [56-59]. Based on this result, we hypothesized that bound Ca²⁺-sensor proteins affect the affinity of KCNQ1 for PIP₂. A voltage-sensitive phosphatase (Ci-VSP) hydrolyzes PIP₂ in the inner leaflet of cell membranes in a voltage-dependent manner [45]. It is highly active at depolarization, but less active at hyperpolarization. Thus, Ci-VSP could deplete PIP₂ by increasing the membrane potential or the duration of membrane potential depolarization. Over-expression of Ci-VSP/WT did not modulate the current amplitude at low membrane potentials (Fig. 5C). However, the current amplitude of KCNQ1 was reduced at membrane potentials above +20 mV, peaked at +40 mV and decreased by 47 ± 4% at +100 mV (n = 16), when compared to the current amplitude recorded at +10 mV (Figs. 5C and D). Therefore, in the presence of Ci-VSP/WT, the current-voltage characteristics of KCNQ1 was an apparent bell-shaped curve. The Ca²⁺-sensor proteins did not modify the trend of the voltage-dependent effect of Ci-VSP. However, in the presence of Calm3, but not CaM, the bell-shaped voltage-dependent curve of KCNQ1 became tall and its peak was shifted to right by approximately 10 mV. Furthermore, the current amplitude only decreased by 21 ± 5% at +100 mV (n = 27). On the other hand, voltage-dependent modulation of the KCNQ1 current did not occur in the presence of phosphatase-dead Ci-VSP mutant (C363S) even though the gating currents were detected when membrane potentials changed. These data indicate that the expression of Calm3 lessens the KCNQ1 response to the voltage-dependent depletion of PIP₂ by Ci-VSP.
There remained the possibility that the effect of Ca\textsuperscript{2+}-sensors on the affinity of the KCNQ1 channel for PIP\textsubscript{2} was based on the modulation of phosphatase activity of Ci-VSP. To address this possibility, we expressed the sensor proteins to measure the voltage-dependent modification of a canonical inward rectifier K\textsuperscript{+} channel, Kir2.3, which requires PIP\textsubscript{2} for activation, by Ci-VSP (Fig. 6A). Expression of Ci-VSP reduced the current amplitude of Kir2.3 by one third without influencing the current voltage relationship (Fig. 6A). The membrane potential was stepped to +20 mV from the holding potential of -20 mV and then hyperpolarized to -120 mV to measure inward current amplitude passing through Kir2.3. The length of the depolarizing membrane potential was varied by increasing the duration in steps of 100 msec. Without Ci-VSP, the depolarizing membrane potential did not affect the current profile of Kir2.3. However, in the presence of Ci-VSP, the current amplitude was significantly reduced by increasing the length of the depolarizing membrane potential (Fig. 6B). The time constant was 0.56 ± 0.05 sec (n = 9) (Fig. 6C). Co-expression of CaM and Calml3 did not perturb the reduction of the overall current amplitude of Kir2.3 by Ci-VSP. Furthermore, the voltage-dependency of Ci-VSP in the presence of CaM and Calml3 (0.55 ± 0.04 sec, n = 11) and 0.56 ± 0.03 sec, n = 7, respectively) was consistent with that in the absence of Ca\textsuperscript{2+}-sensors. Therefore, neither CaM nor Calml3 appears to modify the phosphatase activity of Ci-VSP, thereby suggesting that direct association of Ca\textsuperscript{2+}-sensor proteins with KCNQ1 influences PIP\textsubscript{2}-sensitivity of the channels.

**Discussion**

In this study, we developed an evaluation system to analyze the association between KCNQ1 and Ca\textsuperscript{2+}-sensor proteins. The setup comprises two steps. The first step is the co-expression of the GFP-fused KCNQ1 cytoplasmic domain with CaM in *E. coli*, which lacks endogenous CaM-like Ca\textsuperscript{2+}-sensor proteins (Fig. 1). The second step is the measurement of the GFP fluorescence in the tagged KCNQ1-CaM complex using FSEC and in-gel detection systems (Figs. 1 and 2). Since these approaches allowed us to identify Calml3 as an alternative to CaM for the formation of functional KCNQ1 channels (Fig. 3), these biochemical methods appear to accurately detect the interaction between KCNQ1 and Ca\textsuperscript{2+}-sensor proteins.

Calml3 and CaM competitively bind to the KCNQ1 channel (Fig. 4), and the electrophysiological property of KCNQ1 in complex with Calml3 was equivalent to that in complex with CaM (Fig. 5). These results suggest that the roles of Calml3 in the folding of KCNQ1 and the channel formation are comparable to those of CaM. However, the two complexes showed slight differences in thermal stability (Fig. 3) and in channel affinity for PIP\textsubscript{2} (Fig. 5). These results are in agreement with the observations that Calml3 and CaM have distinct potencies for controlling target enzymes [37, 53]. Structural features of the two Ca\textsuperscript{2+}-sensors, such as the domain orientation and the electrostatic surface potential, may underlie the difference in their ability to control the targets [51].

To detect the effects of Calml3 on the KCNQ1-PIP\textsubscript{2} interaction, we needed to inject its cRNA at a maximal dose (Fig. 5). This interfered the evaluation of functional competition between Calml3 and CaM by injecting different ratios of the two Ca\textsuperscript{2+}-sensor cRNAs into oocytes. CaM is reported to be abundantly present in most of cells (approximately 25 µM) [32-35]. Therefore, the requirement of high expression of Calml3 could be mainly accounted for by the presence of endogenous competitor CaM. In the case of the L-type voltage-gated Ca\textsuperscript{2+} channel, competitive interaction between CaBP1 and CaM was found to modulate Ca\textsuperscript{2+}-dependent inactivation [60]. While a functional unit of Ca\textsuperscript{2+} channel harbors a single Ca\textsuperscript{2+}-sensor protein at its IQ domain, the tetrameric KCNQ1 channel associates with four Ca\textsuperscript{2+}-sensors. This implies that the number of molecules binding to KCNQ1 is also involved in the control of sensitivity to PIP\textsubscript{2}. On the other hand, it becomes apparent that the binding of cytoplasmic auxiliary proteins influences the gates at the selectivity filter of voltage-gated K\textsuperscript{+} channels [12, 61]. Therefore, although the absence of a quantitative functional analysis hampers any rigorous assessment of how Ca\textsuperscript{2+}-sensors differently modulate the affinity of KCNQ1 for PIP\textsubscript{2}, it might link to the general roles of cytoplasmic proteins in direct and
indirect modulations of the protein folding, subcellular localization and gating of the ion channels.

Many CaM-like Ca\(^{2+}\)-sensor proteins bind directly to different types of ion channels, including IP\(_3\) receptors [62-64], voltage-gated Ca\(^{2+}\) channels [65-67], voltage-gated Na\(^+\) channels [68, 69], and transient receptor potential canonical (TRPC) channels [70, 71]. These Ca\(^{2+}\)-sensor proteins share less than 15% amino acid similarity with CaM, suggesting a broad specificity in binding to these ion channels. In contrast, KCNQ1 bound only to the Ca\(^{2+}\)-sensors CaM and Calml3, which share strong amino acid similarity [38]. Thus, it seems likely that Ca\(^{2+}\)-sensor binding to KCNQ1 is considerably selective.

The C-terminus of KCNQ1 contains two putative CaM binding sites. There is a cysteine residue between these sites, at position 445, that is targeted for S-nitrosylation [72]. CaM appears to switch its mode of binding in a Ca\(^{2+}\)-dependent manner [30, 31, 73], which influences the redox-dependent modulation of the channel. Furthermore, the binding of phosphatidylinositol and CaM regulate the TRPC6 in an integrative manner [74]. Therefore, Ca\(^{2+}\)-sensors may dynamically regulate KCNQ1 channel activity and contribute to the molecular mechanisms underlying the divergence in the modulation of channel activity.

Calml3 is abundant in multiple types of epithelial cells, including those found in tissues of the breast [39], kidney, intestine, and skin [41]. On the other hand, KCNQ1 channels prominently function in the repolarization of cardiac action potential and contribute to the functions of epithelial cells; for example, gastric acid secretion from parietal cells [3-6], thyroid hormone biosynthesis [7, 9], and cerebrospinal fluid secretion [8]. Their overlapping distribution in epithelial tissues strongly suggests that KCNQ1 and Calml3 assemble to form functional channels. However, in epithelial cells, CaM is also expressed abundantly. This led us to speculate that the functional units of epithelial KCNQ1 channels harbor both Ca\(^{2+}\)-sensor proteins. Although we tried to isolate these complexes from native tissues by the immunoprecipitation, the commercially available antibodies that were specific to Calml3 and CaM did not work in this study. This is a clear limitation of this study designed to determine how Ca\(^{2+}\)-sensor proteins participate in the formation of functional KCNQ1 channels in the epithelium. Nevertheless, since a variety of cell signals modulate PIP\(_2\) metabolism, the Calml3-induced increase in PIP\(_2\) affinity may maintain KCNQ1 activity to support the intimate functions of the epithelium.

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Disclosure Statement

The authors have nothing to disclose. There are no competing interests.

References

1. Nerbonne JM, Kass RS: Molecular physiology of cardiac repolarization. Physiol Rev 2005;85:1205-1253.
2. Sanguinetti MC, Tristani-Firouzi M: hERG potassium channels and cardiac arrhythmia. Nature 2006;440:463-469.
3. Dedek K, Waldegger S: Colocalization of KCNQ1/KCNE channel subunits in the mouse gastrointestinal tract. Pflugers Arch 2001;442:896-902.
4. Demolombe S, Franco D, de Boer P, Kuperschmidt S, Roden D, Pereon Y, Jarry A, Moorman AF, Escande D: Differential expression of KvLQT1 and its regulator Isk in mouse epithelia. Am J Physiol Cell Physiol 2001;280:C359-372.
5. Grahammer F, Herling AW, Lang HJ, Schmitt-Graf A, Wittekindt OH, Nitschke R, Bleich M, Barhanin J, Warth R: The cardiac K+ channel KCNQ1 is essential for gastric acid secretion. Gastroenterology 2001;120:1363-1371.

6. Lee MP, Ravenel JD, Hu RJ, Lustig LR, Tomasselli G, Berger RD, Brandenburg SA, Litzl TJ, Bunton TE, Limb C, Francis H, Golellikow M, Gu H, Washington K, Argani P, Goldenring JR, Coffey RJ, Feinberg AP: Targeted disruption of the Kvlqt1 gene causes deafness and gastric hyperplasia in mice. J Clin Invest 2000;106:1447-1455.

7. Puret R, Paroder-Belenitsky M, Reyna-Neyra A, Nicola JP, Koba W, Fine E, Carrasco N, Abbott GW: The KCNQ1-KCNE2 K+ channel is required for adequate thyroid I- uptake. J Biol Chem 2002;277:1529-1539.

8. Roepke TK, Kanda VA, Puret K, King EC, Lerner DJ, Abbott GW: KCNE2 forms potassium channels with KCNA3 and KCNQ1 in the choroid plexus epithelium. FASEB J 2011;25:4264-4273.

9. Roepke TK, King EC, Reyna-Neyra A, Paroder M, Puret K, Koba W, Fine E, Lerner DJ, Carrasco N, Abbott GW: Kcne2 deletion uncovers its crucial role in thyroid hormone biosynthesis. Nat Med 2009;15:1186-1194.

10. Jespersen T, Groen S, Olesen SP: The KCNQ1 potassium channel: from gene to physiological function. Physiology (Bethesda) 2005;20:408-416.

11. Dai S, Hall DD, Bell JW: Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev 2009;89:411-452.

12. Haïtin Y, Attali B: The C-terminus of Kv7 channels: a multifunctional module. J Physiol 2008;586:1803-1810.

13. Pongs O, Schwarz JR: Ancillary subunits associated with voltage-dependent K+ channels. Physiol Rev 2010;90:755-796.

14. Puret K, Roepke TK, Abbott GW: Cardiac arrhythmia and thyroid dysfunction: a novel genetic link. Int J Biochem Cell Biol 2010;42:1767-1770.

15. Barhanin J, Lesage F, Guillemaire E, Fink M, Lazzunski M, Rosney G: KVLQT1 and IsK (minK) proteins associate to form the Ik5 cardiac potassium current. Nature 1996;384:78-80.

16. Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT: Coassembly of KVLQT1 and minK (IsK) proteins to form cardiac Ik5 potassium channel. Nature 1996;384:80-83.

17. Schroeder BC, Waldeger S, Fehr S, Bleich M, Warth R, Gregor R, Jentsch TJ: A constitutively open potassium channel formed by KCNQ1 and KCNE3. Nature 2000;403:196-199.

18. Chen L, Kass RS: Dual roles of the A kinase-anchoring protein Yotiao in the modulation of a cardiac potassium channel: a passive adaptor versus an active regulator. Eur J Cell Biol 2006;85:623-626.

19. Hoschi N, Zhang JS, Omaki M, Takeuchi T, Yokoyama S, Wanaverbecq N, Langeberg LK, Yonedo Y, Scott JD, Brown DA, Higashida H: AKAP150 signaling complex promotes suppression of the M-current by muscarinic agonists. Nat Neurosci 2003;6:564-571.

20. Li Y, Chen L, Kass RS, Deissauer CW: The A-kinase anchoring protein Yotiao facilitates complex formation between adenylyl cyclase type 9 and the Ik5 potassium channel in heart. J Biol Chem 2012;287:29815-29824.

21. Marx S, Kurokawa J, Reiken S, Mutoi H, D’Armiento J, Marks AR, Kass RS: Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. Science 2002;295:495-499.

22. Terrenoire C, Houslay MD, Baillie GS, Kass RS: The cardiac Ik5 potassium channel macromolecular complex includes the phosphodiesterase PDE4D3. J Biol Chem 2009;284:9140-9146.

23. Gamper N, Shapiro MS: Calmodulin mediates Ca2+-dependent modulation of M-type K+ channels. J Gen Physiol 2003;122:17-31.

24. Ghosh S, Nunziato DA, Pitt GS: KCNQ1 assembly and function is blocked by long-QT syndrome mutations that disrupt interaction with calmodulin. Circ Res 2006;98:1048-1054.

25. Shamgar L, Ma L, Schmitt N, Haitin Y, Peretz A, Wiener R, Hirsch J, Pongs O, Attali B: Calmodulin is essential for cardiac Ik5 channel gating and assembly: impaired function in long-QT mutations. Circ Res 2006;98:1055-1063.

26. Wen H, Levitan IB: Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels. J Neurosci 2002;22:7991-8001.

27. Wiener L, Haitin Y, Shamgar L, Fernandez-Alonso MC, Martos A, Chomsky-Hecht O, Rivas G, Attali B, Hirsch JA: The KCNQ1 (Kv7.1) COOH terminus, a multitiered scaffold for subunit assembly and protein interaction. J Biol Chem 2008;283:5815-5830.
Inanobe/Tsuzuki/Kurachi: Calml3 as a Constituent of Epithelial KCNQ1

Cellular Physiology and Biochemistry

Yus-Najera E, Santana-Castro I, Villarroel A: The identification and characterization of a noncontinuous calmodulin-binding site in nonactivating voltage-dependent KCNQ potassium channels. J Biol Chem 2002;277:28545-28553.

Gamper N, Li Y, Shapiro MS: Structural requirements for differential sensitivity of KCNQ K+ channels to modulation by Ca2+/calmodulin. Mol Biol Cell 2005;16:3538-3551.

Bal M, ZaiKa O, Martin P, Shapiro MS: Calmodulin binding to M-type K+ channels assayed by TIRF/FRET in living cells. J Physiol 2008;586:2307-2320.

Xu Q, Chang A, Tolia A, Minor D L Jr: Structure of a Ca2+/CaM:Kv7.4 (KCNQ4) B-helix complex provides insight into M current modulation. J Mol Biol 2013;425:378-394.

Hoeftlich KP, Ikura M: Calmodulin in action: diversity in target recognition and activation mechanisms. Cell 2002;108:739-742.

Mikhaylova M, Hradsky J, Kreutz MR: Between promiscuity and specificity: novel roles of EF-hand calcium sensors in neuronal Ca2+ signalling. J Neurochem 2011;118:695-713.

Yap KL, Kim J, Truong K, Sherman M, Yuan T, Ikura M: Calmodulin target database. J Struct Funct Genomics 2000;1:8-14.

Saimi Y, Kung C: Calmodulin as an ion channel subunit. Annu Rev Physiol 2002;64:289-311.

Kovalevskaya NV, van de Waterbeemd M, Bokhovchuk FM, Bate N, Bindels RJ, Hoenderop JG, Vuister GW: Structural analysis of calmodulin binding to ion channels demonstrates the role of its plasticity in regulation. Pflugers Arch 2013;465:1507-1519.

Edman CF, Schulman H: Identification and characterization of delta B-CaM kinase and delta C-CaM kinase from rat heart, two new multifunctional Ca2+/calmodulin-dependent protein kinase isoforms. Biochim Biophys Acta 1994;1221:89-101.

Koller M, Strehler EE: Characterization of an intronless human calmodulin-like pseudogene. FEBS Lett 1988;239:121-128.

Rogers MS, Foley MA, Crotty TB, Hartmann LC, Ingle JN, Roche PC, Strehler EE: Loss of immunoreactivity for human calmodulin-like protein is an early event in breast cancer development. Neoplasia 1999;1:220-225.

Yaswen P, Smoll A, Hosoda J, Parry G, Stamper MR: Protein product of a human intronless calmodulin-like gene shows tissue-specific expression and reduced abundance in transformed cells. Cell Growth Differ 1992;3:335-345.

Sanguinetti MC, Jiang C, Curran ME, Keating MT: A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. Cell 1995;81:299-307.

Notredame C, Higgins DG, Heringa J: T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol 2000;302:205-217.

Murata Y, Iwasaki H, Sasaki M, Inaba K, Okamura Y: Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. Nature 2005;435:1239-1243.

Schneider CA, Rasband WS, Eliceiri KW: NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-675.

Kawate T, Gouaux E: Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. Structure 2006;14:673-681.

Geertsma ER, Groeneveld M, Slotboom DJ, Poolman B: Quality control of overexpressed membrane proteins. Proc Natl Acad Sci U S A 2008;105:5722-5727.

Penna TC, Ishii M, Junior AP, Cholwa O: Thermal stability of recombinant green fluorescent protein (GFPuv) at various pH values. Appl Biochem Biotechnol 2004;113-116:49-63.

Chattopadhyaya R, Meador WE, Means AR, Quiocio FA: Calmodulin structure refined at 1.7 A resolution. J Mol Biol 1992;228:1177-1192.

Han BG, Han M, Sui H, Yaswen P, Walian PJ, Jap BK: Crystal structure of human calmodulin-like protein: insights into its functional role. FEBS Lett 2002;521:24-30.

Durussel I, Ryhner JA, Streblor EE, Cox JA: Cation binding and conformation of human calmodulin-like protein. Biochemistry 1993;32:6089-6094.
53 Edman CF, George SE, Means AR, Schulman H, Yaswen P: Selective activation and inhibition of calmodulin-dependent enzymes by a calmodulin-like protein found in human epithelial cells. Eur J Biochem 1994;226:725-730.

54 Falkenburger BH, Jensen JB, Dickson EJ, Suh BC, Hille B: Phosphoinositides: lipid regulators of membrane proteins. J Physiol 2010;588:3179-3185.

55 Park KH, Piron J, Dahimene S, Merot J, Baro I, Escande D, Loussouarn G: Impaired KCNQ1-KCNE1 and phosphatidylinositol-4,5-bisphosphate interaction underlies the long QT syndrome. Circ Res 2005;96:730-739.

56 Hernandez CC, Zaike O, Shapiro MS: A carboxy-terminal inter-helix linker as the site of phosphatidylinositol 4,5-bisphosphate action on Kv7 (M-type) K+ channels. J Gen Physiol 2008;132:361-381.

57 Loussouarn G, Park KH, Belloq C, Baro I, Charpentier F, Escande D: Phosphatidylinositol-4,5-bisphosphate, PIP2, controls KCNQ1/KCNE1 voltage-gated potassium channels: a functional homology between voltage-gated and inward rectifier K+ channels. EMBO J 2003;22:5412-5421.

58 Rosenhouse-Dantsker A, Logothetis DE: Molecular characteristics of phosphoinositide binding. Pflugers Arch 2007;455:45-53.

59 Zhang H, Craciun LC, Mirshahi T, Rohacs T, Lopes CM, Jin T, Logothetis DE: PIP2 activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. Neuron 2003;37:963-975.

60 Findeisen F, Rumpf CH, Minor DL, Jr.: Apo states of calmodulin and CaBP1 control Cav1 voltage-gated calcium channel function through direct competition for the IQ domain. J Mol Biol 2013;425:3217-3234.

61 Strutz-Seebohm N, Henrion U, Schmitt N, Schulze-Bahr E, Seebohm G: A common structural component for beta-subunit mediated modulation of slow inactivation in different Kv channels. Cell Physiol Biochem 2013;31:968-980.

62 Michikawa T, Hirota J, Kawan S, Hiraoka M, Yamada M, Furuichi T, Mikoshiba K: Calmodulin mediates calcium-dependent inactivation of the cerebellar type 1 inositol 1,4,5-trisphosphate receptor. Neuron 1999;23:799-808.

63 Schlecker C, Boehmerle W, Jeromin A, DeGray B, Varshney A, Sharma Y, Szegi-Buck K, Ehrlich BE: Neuronal calcium sensor-1 enhancement of InsP3 receptor activity is inhibited by therapeutic levels of lithium. J Clin Invest 2006;116:1667-1674.

64 Yang J, McBride S, Vardi N, Palczewski K, Haeseleer F, Foskett JK: Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca2+ release channels. Proc Natl Acad Sci U S A 2002;99:7711-7716.

65 Leal K, Mochida S, Scheuer T, Catterall WA: Fine-tuning synaptic plasticity by modulation of Cav2.1 channels with Ca2+ sensor proteins. Proc Natl Acad Sci U S A 2012;109:17069-17074.

66 Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA: Cav2.1/calmodulin binds to and modulates P/Q-type calcium channels. Nature 1999;399:155-159.

67 Zühlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H: Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature 1999;399:159-162.

68 Biswas S, DiSilvestre D, Tian Y, Halperin VL, Tomaselli GF: Calcium-mediated dual-mode regulation of cardiac sodium channel gating. Circ Res 2009;104:870-878.

69 Tan HL, Kupershmidt S, Zhang R, Stepanovic S, Roden DM, Wilder AA, Anderson ME, Balser JR: A calcium sensor in the sodium channel modulates cardiac excitability. Nature 2002;415:442-447.

70 Kinoshita-Kawada M, Tag J, Xiao R, Kaneko S, Foskett JK, Zhu MX: Inhibition of TRPC5 channels by Ca2+-binding protein 1 in Xenopus oocytes. Pflugers Arch 2005;450:345-354.

71 Zhang Z, Tang J, Tikunova S, Johnson JD, Chen Z, Qin N, Dietrich A, Stefan I, Bärnbaumer L, Zhu MX: Activation of TRp3 by inositol 1,4,5-trisphosphate receptors through displacement of inhibitory calmodulin from a common binding domain. Proc Natl Acad Sci U S A 2001;98:3168-3173.

72 Asada K, Kurokawa J, Furukawa T: Redox- and calmodulin-dependent S-nitrosylation of the KCNQ1 channel. J Biol Chem 2009;284:6014-6020.

73 Alaimo A, Alteroni A, Gomis-Perez C, Fernandez-Orth J, Bernardo-Seisdedos G, Malo C, Millet O, Areso P, Villarroel A: Pivoting between calmodulin lobes triggered by calcium in the Kv7.2/calmodulin complex. PLoS One 2014;9:e86711.

74 Kwon Y, Hofmann T, Montell C: Integration of phosphoinositide- and calmodulin-mediated regulation of TRPC6. Mol Cell 2007;25:491-503.