Intramolecular Disulfide Bonds for Biogenesis of CALHM1 Ion Channel Are Dispensable for Voltage-Dependent Activation

Jae Won Kwon, Young Keul Jeon, Jinsung Kim, Sang Jeong Kim, and Sung Joon Kim

1Department of Physiology, Seoul National University College of Medicine, Seoul 03080, Korea, 2Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul 03080, Korea, 3Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul 03080, Korea, 4These authors contributed equally to this work.

*Correspondence: physiolksj@gmail.com
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Calcium homeostasis modulator 1 (CALHM1) is a membrane protein with four transmembrane helices that form an octameric ion channel with voltage-dependent activation. There are four conserved cysteine (Cys) residues in the extracellular domain that form two intramolecular disulfide bonds. We investigated the roles of C42-C127 and C44-C161 in human CALHM1 channel biogenesis and the ionic current ($I_{\text{CALHM1}}$). Replacing Cys with Ser or Ala abolished the membrane trafficking as well as $I_{\text{CALHM1}}$. Immunoblotting analysis revealed dithiothreitol-sensitive multimeric CALHM1, which was markedly reduced in C44S and C161S, but preserved in C42S and C127S. The mixed expression of C42S and wild-type did not show a dominant-negative effect. While the heteromeric assembly of CALHM1 and CALHM3 formed active ion channels, the co-expression of C42S and CALHM3 did not produce functional channels. Despite the critical structural role of the extracellular cysteine residues, a treatment with the membrane-impermeable reducing agent tris(2-carboxyethyl) phosphine (TCEP, 2 mM) did not affect $I_{\text{CALHM1}}$, for up to 30 min. Interestingly, incubation with TCEP (2 mM) for 2-6 h reduced both $I_{\text{CALHM1}}$ and the surface expression of CALHM1 in a time-dependent manner. We propose that the intramolecular disulfide bonds are essential for folding, oligomerization, trafficking and maintenance of CALHM1 in the plasma membrane, but dispensable for the voltage-dependent activation once expressed on the plasma membrane.

Keywords: CALHM1, disulfide bond, membrane trafficking, oligomerization, reducing agent

INTRODUCTION

Calcium homeostasis modulator 1 (CALHM1) is a newly identified voltage-gated nonselective ion channel, and its functional expression has been reported in cerebral cortical neuronal cells and taste bud cells (Dreses-Werringloer et al., 2008; Taruno et al., 2013). In cerebral neurons, CALHM1 is reportedly involved in the action potential frequency-dependent regulation of excitability (Vingtdeux et al., 2016). In type II taste bud cells, CALHM1 interacts with CALHM3 as a pore-forming subunit, and the CALHM1/3 hetero-multimeric channel functions as an endogenous ATP-release channel signaling to the purinoceptors in the sensory nerve ending (Kashio et al., 2019; Ma et al., 2018).

In whole-cell patch clamp studies, CALHM1-expressing cells showed slowly activating outward currents under strong depolarized conditions, and the voltage-dependence was shifted to the left by lowering the extracellular Ca$^{2+}$ concen-
Disulfide Bonds in CALHM1 Channel Structure and Function
Jae Won Kwon et al.

MATERIALS AND METHODS

Cell culture preparation
Chinese hamster ovary (CHO) and human embryonic kidney cell line (HEK293) cells were purchased from ATCC (USA) and incubated in Dulbecco’s modified Eagle’s medium (DMEM: Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). The cells were incubated at 37°C and 5% CO₂. The cells were sub-cultured by centrifuging at 160 × g for 2 min and then trypsinized. CHO cells at less than 30 passages were used for subsequent experiments because of the very low expression of endogenous ion channels (Gamper et al., 2005).

Molecular cloning and heterologous expression of CALHM1
To insert the coding sequence (CDS), the cDNA of human CALHM1 and pEGFP-N1 vector (Takara Bio, USA) were truncated with the restriction enzymes EcoRi and BamHI. Truncated inserts were ligated, and the sequence of the CALHM1-EGFP vector was confirmed. All point mutations in CALHM1 were generated and PCR-amplified using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, USA), and the sequences of eight mutants were confirmed by DNA sequencing. All constructs were transfected into CHO cells using the TurboFect transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. The day before transfection, 3 × 10⁴ cells were seeded on a 12-well culture dish. The following day, 300 ng of the CALHM1-EGFP vector was transfected into the cells in each well. Downstream experiments were conducted 18-40 h after transfection. For co-immunoprecipitation, the FLAG (DYKDDDDK) tag and stop codon were inserted between the CALHM1 and EGFP using Gibson assembly method (Gibson et al., 2009). HEK293 cells (3 × 10⁴ cells) were seeded on 6-well dishes and co-transfected CALHM1-FLAG with WT CALHM1-EGFP or C42S CALHM1-EGFP (total amount of cDNA was 1 µg) using the Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Electrophysiology
The transfected CHO cells were transferred to a bath mounted on the stage of an inverted microscope (Ti; Nikon, Japan). Borosilicate glass pipettes were connected to the CV 203BU head stage of a patch-clamp amplifier (Axopatch 200B: Axon Instruments, USA). The current was recorded at 10 kHz and low-pass Bessel-filtered at 5 kHz. pCLAMP software version 10.6.2, and Digidata-1440A (Axon Instruments) were used to acquire data and apply command pulses. Data were processed using Clampfit version 11.2 (Axon Instruments). The temperature of the bath and cells was controlled using an in-line solution heating system (Warner Instruments, USA), which was placed in front of the bath. The temperature was monitored using a thermistor in the bath near the cells (<300 µm) and recorded using a digitizer. Pipettes with a free-tip resistance of 2.0–2.8 MΩ were used for whole-cell patch clamp. Series resistance was estimated by dividing the time constants of the capacitive current with the membrane capacitance, which was kept below 10 Ω in the whole-cell configuration. Because the electrical capacitance of the cell reflects the surface area of the plasma membrane, the current density of 〈I CALHM1〉 was defined as the amplitude of the time-dependent outward current component divided by the cell capacitance (pA·pF⁻¹). The bath was continuously perfused at a constant flow rate (5 ml·min⁻¹) with Normal Tyrode’s bath solution (NT) comprised of (in mM) 140 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 20 mannitol, and 10 HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], pH 7.4 (titrated with NaOH) for whole-cell patch clamp experiments. The Ca²⁺ concentration of NT was modified based on the experimental conditions (0 and 2 mM). The pipette solution...
used for whole-cell patch clamp contained (in mM) 140 CsCl, 0.5 MgCl₂, 10 EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid], and 10 HEPES, pH 7.2 (titrated with CSOH). A salt-agar bridge with 3 M KCl was used to compensate for the junction potential change following treatment with reducing agents (Berman and Awayda, 2013). The reagents used in this study were purchased from Sigma-Aldrich (USA).

Confocal microscopy imaging
CALHM1-overexpressed cells were imaged on an 18 mm coverslip with an A1 confocal microscope (Nikon) one day after transfection. Cell-attached coverslips were fixed with 4% paraformaldehyde and washed three times with ice-cold phosphate-buffered saline (PBS). The membranes and nucleus of cells were stained in the dark using 5 μg/ml wheat germ agglutinin (WGA) and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI), respectively. Images were scanned with a 100x immersion objective lens at 512 × 512 pixels using a digital zoom. All confocal images were processed and transferred using NIS software (Nikon) and ImageJ software (NIH, USA).

Immunoblotting and surface biotinylation
Proteins were extracted from overexpressed CHO cells using lysis buffer containing 0.5 M EDTA, 25 mM Tris-HCl, 150 mM NaCl, and 1% Triton X-100 with a phosphatase and protease inhibitor cocktail (Roche, Germany), pH 7.4. After scraping the cells and treating them with the lysis buffer, the lysates were homogenized 10 times with a sterile 26-gauge needle. The protein was quantified using the Bradford assay (Bio-Rad, USA). After incubation at 95°C with an appropriate concentration of DTT (indicated in the results), lysates were fractionated using SDS-PAGE on 3%-8% gradient gels (Thermo Fisher Scientific) and transferred onto a PVDF membrane in 25 mM Tris, 192 mM glycine, and 20% methanol.

RESULTS

The schematic figure of barrels and connecting lines demonstrates the topology and domains of CALHM1 (Fig. 1A). Amino acid sequences of the three human CALHM paralogues (CALHM1-3) and the calhm1 sequences of mice and killifish were compared (Fig. 1B). Four cysteine residues (C42, C44, C127, and C161 in human CALHM1) were conserved across all five proteins. The densities of the two disulfide bonds between the conserved cysteines (C42-C127 and C44-C161) in the killifish calhm1 cryo-EM structure were determined (Figs. 1C-1E). All these residues were located in the extracellular space, and the two disulfide bonds were located relatively close to each other (Figs. 1C and 1D, red color). Since the cryo-EM structure of human CALHM1 has not been reported before, we used the homologous model to confirm whether the less-conserved cysteine molecules also formed disulfide bonds. There were eight additional cysteine residues in cryo-EM structure, but they were too far from each other to form disulfide bonds (Fig. 1D, orange color). Taken together, we confirmed that only the four conserved cysteine residues...
formed the two disulfide bonds identified (Fig. 1E).

We compared the electrophysiological properties of cysteine mutants and wild-type (WT) CALHM1 in CHO cells. In the whole-cell patch clamp configuration, step-wise depolarization was applied from −40 mV of holding voltage to 80 mV (5 s) with various levels, followed by repolarization to −40 mV. At room temperature, slowly activating outward currents (I_CALHM1) were observed only at strong depolarizations above 80 mV (Fig. 2A, left panel) but not in the empty CHO cells (data not shown). Consistent with the known CALHM1 property (Ma et al., 2012; Tanis et al., 2017), removal of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]e) facilitated voltage-dependent activation of CALHM1 current (Fig. 2A, middle panel). We recently reported on the thermosensitivity of CALHM1 (Jeon et al., 2021) and confirmed the significant facilitation of CALHM1 activation at physiological temperature (37°C, Fig. 2A, right panel). The summarized results are presented as current-to-voltage relations (I-V curves, Fig. 2B). The voltage dependence of CALHM1 current was investigated with conductance-to-voltage relations (G-V curves, Fig. 2C) under three conditions as described above. The voltage was stepped from −40 mV to 120 mV (2 s) with 10 mV increments, followed by repolarization to −80 mV (1 s) and then back to −40 mV of holding potential. By removing the [Ca\(^{2+}\)]e, or increasing the temperature to 37°C, the half-maximal activation voltage (V_{1/2}) was shifted to the left (42.90 ± 2.515 mV, 58.25 ± 0.725 mV, re-
Disulfide Bonds in CALHM1 Channel Structure and Function
Jae Won Kwon et al.

spectively) compared with condition of 2 mM $[\text{Ca}^{2+}]_e$ at room temperature (67.76 ± 0.965 mV).

Replacement of any of the four cysteines residues with serine or alanine residues by site-directed mutagenesis completely abolished $I_{\text{CALHM1}}$. Representative patch clamp recordings of CHO cells transfected with C42S at room temperature, free calcium (0 mM $[\text{Ca}^{2+}]_e$), and at 37°C are shown in Figs. 2D and 2E. In addition, the summary of outward current densities at 60 mV in CHO cells expressing the tested mutants is shown as bar graphs (Fig. 2F), clearly indicating the loss of channel function across all the mutations.

To investigate CALHM1 trafficking to the plasma membrane, we conducted confocal microscopic analysis of CHO cells expressing CALHM1 with an EGFP tag (Figs. 3A and 3B). The cells were co-stained with WGA, which binds to the glycoproteins in the cell membrane (Fig. 3A, red color). Wild-type CALHM1 was localized to the plasma membrane, along with the puncta form of intracellular expression (Fig. 3B).
the presence of monomers, dimers, CALHM1 proteins of various sizes were detected, pointing to acrylamide gels was performed with or without a reducing technique. While WT CALHM1 was found in the plasma membrane localization, while the perinuclear expression of cysteine mutants using a surface biotinylation technique. We then evaluated whether intramolecular disulfide bonds on CALHM1, as reported by Okui et al. (2021). Notably, a considerable amount of the CALHM1 protein was located in the perinuclear region. A pattern of relatively larger cytosolic expression of WT CALHM1 has also been reported (Dreses-Werringloer et al., 2008). In contrast to WT, none of the Cys mutants showed plasma membrane localization, while the perinuclear expression pattern was evident (Figs. 3A and 3B). We also compared the plasma membrane expressions of WT with the expression of cysteine mutants using a surface biotinylation technique. While WT CALHM1 was found in the plasma membrane fraction, none of the four cysteine mutants were detected (Fig. 3C). The multi-bands of WT and Cys mutants in the whole-cell lysates indicate variations of N-glycochains on CALHM1, as reported by Okui et al. (2021).

We then evaluated whether intramolecular disulfide bonds are necessary for multimerization in the biogenesis of the CALHM1 channel. Immunoblotting using 3%-8% polyacrylamide gels was performed with or without a reducing agent (DTT, 0, 0.1, 1, and 10 mM). In the control condition, CALHM1 proteins of various sizes were detected, pointing to the presence of monomers, dimers, and tetramers (Fig. 4A). The calculated molecular weight of CALHM1-GFP is ~65.9 kDa, and the predicted molecular masses for dimeric and tetrameric forms are ~131.8 kDa and ~263.6 kDa, respectively. The multimeric forms of CALHM1 decreased with increasing concentrations of DTT, while the monomeric form increased. When either C42 or C127 was replaced with serine, multimeric bands were detected in the non-reducing condition (0 mM DTT), similar to WT (Fig. 4B). In contrast, the multimeric sizes of C44S and C161S decreased significantly, even under non-reducing conditions. We compared the intensity of monomer and tetramer bands in four immunoblots with increasing concentrations of DTT. Whereas the relative expression ratios of monomer gradually increased to 3.24 at 10 mM DTT in WT, those of tetramer decreased to 0.22, when normalized to signal intensity of 0 mM DTT (Fig. 4C). The ratios of monomer and tetramer bands in C42S and C127S were similar to WT (monomers, 3.12 and 2.97; tetramers, 0.28 and 0.14, respectively, Fig. 4D), while not significantly altered in C44S and C161S (monomers, 1.53 and 1.44; tetramers, 0.55 and 0.67, respectively).

Co-expression of WT with mutated channel units often showed dominant-negative effects, and hetero-multimerized channels showed impaired electrophysiological function (Bannister et al., 1999; Cho et al., 2000). Since the functional CALHM1 channel has an octameric structure, a putative hetero-multimeric assembly might disrupt the function of the channel. When WT and C42S cDNAs were co-transfected using constant quantities of WT and increasing quantities of C42S (10:1, 10:2, 10:5, and 10:10), the ICALHM1 amplitude did
Disulfide Bonds in CALHM1 Channel Structure and Function
Jae Won Kwon et al.

not change significantly (Fig. 5A). Furthermore, when the total amount of transfected cDNA was kept constant, the increased ratio of C42S (9:1, 1:1, and 1:9) induced only a gradual decrease in $I_{\text{CALHM1}}$. Notably, the current density was still significant even when the ratio of WT and C42S cDNA was 1:9 (30 ng:270 ng).

To determine whether WT and C42S CALHM1 form hetero-multimerized channels, we performed co-expression and immunoprecipitation of FLAG tagged WT CALHM1 and GFP tagged C42S CALHM1 cDNAs with a positive control expressed with different (FLAG or GFP) tagged WT CALHM1 cDNAs. While CALHM1 co-immunoprecipitates with itself as a homo-multimer, GFP tagged C42S CALHM1 was not pulled down with FLAG tagged WT CALHM1, indicating C42S CALHM1 could not interact with WT CALHM1 (Fig. 5B).

The intrinsically expressed ATP-releasing channel in taste buds is composed of CALHM1 and CALHM3 (Kashio et al., 2019; Ma et al., 2018). When expressed with CALHM3 alone, CHO cells showed negligible voltage-dependent currents (Fig. 5C). However, when co-expressed with CALHM1,
the voltage-dependent outward $I_{\text{CALHM1/3}}$ showed a larger amplitude (54.5 ± 3.57 pA/pF), with faster kinetics of activation compared with $I_{\text{CALHM1}}$ (Figs. 5C and 5D). These results are consistent with previous reports (Ma et al., 2018). The co-expression of CALHM3 with C42S CALHM1 could not rescue the electrophysiological activity; no outward voltage-dependent current was observed (Figs. 5C and 5D). Although the data obtained from the co-expression of WT CALHM1 and C42S excluded any interaction between the two, the significant $I_{\text{CALHM1/3}}$ compared with $I_{\text{CALHM1}}$ might suggest a rescue effect from WT to C42S through hetero-multimerization. To test this hypothesis, the current density of triple co-expression of WT CALHM1 and C42S was compared with that of WT CALHM1 and CALHM3 and with GFP, replacing the C42S cDNA. Including C42S did not affect $I_{\text{CALHM1/3}}$, amplitude, similar to GFP (empty vector), suggesting that the CALHM1 cysteine mutant did not assemble into the CALHM1/3 hetero-multimeric channel (Fig. 5D).

Finally, we investigated whether reducing agents used to break the cysteine bridges could affect CALHM1 activity. Step depolarization from −40 to 60 mV (5 s) was repetitively applied to activate CHO cells expressing WT CALHM1 every 30 s. After confirming the constant amplitudes of $I_{\text{CALHM1}}$, 2 mM DTT or TCEP was added to the bath perfusing solution, and this did not significantly change $I_{\text{CALHM1}}$ amplitude for up to 30 min (Figs. 6A and 6B). Treatment with 10 μM ruthenium red or 10 μM Gd3+, known blockers of CALHM1, almost completely suppressed $I_{\text{CALHM1}}$ (data not shown). In contrast to the acute effect of the reducing agents, prolonged pretreatment (2–6 h) of the transfected cells with 2 mM TCEP significantly reduced $I_{\text{CALHM1}}$ amplitude compared with time-matched control cells (Fig. 6C). $I_{\text{CALHM1}}$ suppression by TCEP pretreatment suggests reduced membrane expression (internalization) or degradation of the CALHM1 protein. In fact, based on
**DISCUSSION**

The structures of various transmembrane proteins, including ion channels, are stabilized by intra- or intermolecular disulfide bonds. In the CALHM family, four cysteine residues were highly conserved across and within species (Fig. 1B). Recent cryo-EM studies have confirmed the intramolecular disulfide bridges investigated here (Choi et al., 2019; Demura et al., 2020; Ren et al., 2020; Yang et al., 2020). Although the cryo-EM structure of human CALHM1 has not been reported, we indirectly confirmed the existence of two disulfide bonds between four conserved cysteine residues using homology modeling of CALHM1 based on human CALHM2 structure. These disulfide bonds are located in the extracellular space and occur approximately 7 Å apart, when measured using the homology models and killifish CALHM1 cryo-EM structure. Interestingly, eight non-conserved cysteine residues were also located in CALHM1 cryo-EM structure, but the distances between them were too large to form intermolecular or intramolecular disulfide bridges (Fig. 1D). This implies that only the four conserved cysteine residues could form two intramolecular disulfide bonds (Figs. 1C-1E).

The nascent channel proteins are targeted to the endoplasmic reticulum (ER) and are folded and multimerized within the ER prior to membrane expression (Deutsch, 2003; Isacoff et al., 2013). In the ER, oxidative protein folding, a process of disulfide bond formation, and a conformational folding reaction are generated (Narayan, 2012). It is very important that only properly folded and assembled channels are trafficked to the plasma membrane as dysfunctional channels can have serious effects. Starting with ER quality control, maturation in Golgi shuttled channel complexes is also important to proceed in forward traffic to reach the cell membrane.

Our study showed the critical roles of the intramolecular disulfide bonds (C42-C127 and C44-C161) in the biogenesis and membrane trafficking of CALHM1 ion channels. Specifically, we found that the cysteine bridge between C44 and C161 (C44-C161) is essential, while that between C42 and C127 (C42-C127) is dispensable for multimerization in the surface biotinylation and immunoblotting analysis, CALHM1 expression decreased in the plasma membrane after 2 h of TCEP treatment (Figs. 6D and 6E). The expression had reduced even further 6 h after treatment. Interestingly, the total amount of CALHM1 protein also significantly decreased after 6 h of TCEP treatment.

**Fig. 6. Different effects of the acute and sustained treatment with reducing agent on $I_{\text{CALHM1}}$ and protein expression.**

(A and B) Acute treatment with the reducing agents, DTT (2 mM, upper panel) and TCEP (2 mM, lower panel), did not change $I_{\text{CALHM1}}$ under standard condition (2 mM [Ca$^{2+}$], and 27°C). Representative trace (A) and summary of the current density (B) are shown (n = 8 and n = 13 for DTT and TCEP, respectively). n.s., not significant. (C) $I_{\text{CALHM1}}$ gradually decreased following sustained treatment with cell-impermeable reducing agent, TCEP (n = 13 and n = 15 for control and TCEP, respectively). (D and E) CALHM1 membrane expression decreased markedly from 2 h after TCEP treatment (n = 5, closed bars). Total amount of CALHM1 expression levels also decreased when TCEP was treated, but the expression decreased gradually from 2 to 6 h (n = 5, open bars, one-way ANOVA; **$P$ < 0.01, ***$P$ < 0.001).
Disulfide Bonds in CALHM1 Channel Structure and Function
Jae Won Kwon et al.

ER (Fig. 4). Nevertheless, the patch clamp and confocal microscopy demonstrated the necessity of both disulfide bonds as all mutants of the four individual cysteine residues showed impaired trafficking to the membrane, with no electrophysiological function (Figs. 2 and 3). We cautiously interpret that each disulfide bridge is critical to pass the quality check during CALHM1 localization to the plasma membrane.

While WT CALHM1 forms a homo-multimer, the single C42 mutation (C42S) lacking the C42-C127 bridge did not assemble into WT CALHM1 when it was co-expressed with WT CALHM1 (Figs. 5A and 5B). In addition, although CALHM1 and CALHM3 form a heteromeric ion channel (CALHM1/3) with higher activity than CALHM1 alone (Ma et al., 2018), the triple co-expression of WT CALHM1, CALHM3 and C42S CALHM1 did not affect the electrophysiological function (Figs. 5C and 5D). These results suggest that C42S might be unable to assemble with CALHM1/3 hetero-multimeric proteins.

Finally, we investigated the sensitivity of CALHM1 to DTT and TCEP. Surprisingly, despite the critical role of extracellular disulfide bridges in biogenesis and trafficking, %CALHM1 was not affected by acute treatment with both reducing agents (Figs. 6A and 6B). The absence of an acute response to the reducing agents indicated that, once assembled and trafficked to the membrane, the octameric CALHM1 channels can maintain their gating mechanisms even though the reducing agents may have reduced the extracellular disulfide bonds. Although there was possibility that the concentration of TCEP was not enough to break the extracellular disulfide bridges, we could not increase TCEP more than 2 mM due to toxic effects of reducing agent for maintaining tight seal.

Because TCEP is impermeable to the plasma membrane, we investigated the effect of sustained treatment on ion channel activity and membrane expression of CALHM1. We observed a decrease in %CALHM1 and the membrane expression of CALHM1 from 2-6 h, while GAPDH expression was unaltered (Figs. 6C-6E). We cautiously interpret that the conversion of disulfide bonds to sulfhydryl residues induced endocytotic removal and impaired recycling to the plasma membrane. Furthermore, the disappearance of CALHM proteins in the total preparation suggested degradation of CALHM1 with reduced cysteines that would have lost structural integrity (Figs. 6D and 6E). Considering the effects of sustained treatment of TCEP on membrane expression, we carefully interpreted that 2 mM of TCEP could break disulfide bridge of CALHM1 channel, while absence of acute effects.

The time gap between the nonsignificant acute effect on %CALHM1 and the delayed decrease in expression stood out. Here, we interpret that the disconnected disulfide bridges affected the gating function while shortening the functional lifetime of the CALHM1 channels. Based on the limited information obtained from the present results, we could not exclude the possibility that the quaternary structural change in the CALHM1 channel due to TCEP treatment might be a slow phenomenon that could not be detected within several minutes by the patch clamp technique.

A number of studies have reported that intermolecular or inter subunit disulfide bridges are important for the assembly of ion channels: shaker-type voltage-gated K+ channels (Schulteis et al., 1995: 1996), voltage-gated Na+ channels (Chen et al., 2012; Yereddi et al., 2013), voltage-gated Ca2+ channels (Calderon-Rivera et al., 2012), voltage-gated H+ channels (Fujiwara et al., 2013), and acid-sensing ion channels (Zha et al., 2009). Among the two-pore domain (K2P) K+ channel family, TWIK1 and TREK1 form a heterodimeric channel via a disulfide bridge in the extracellular cap region (Hwang et al., 2014). However, the role of disulfide bridges in the cap structure formed by the extracellular M1-P1 linkers of each K2P monomer remains controversial (Zuniga and Zuniga, 2016).

The critical role of intramolecular disulfide bridges in functional channel expression has been reported in other types of ion channels: inward rectifier K+ channels (Kir2.1 and 2.3) (Bannister et al., 1999; Cho et al., 2000), transient receptor potential channels (TRPC4 and 5) (Duan et al., 2018: 2019), transient receptor potential ankyrin channel (TRPA1) (Wang et al., 2012), TIM23 complex of mitochondria (Ramesh et al., 2016), chloride intracellular ion channel (CLC1) (Al Kharni et al., 2016), and CALHM1, identified in this study. In contrast to the role of CALHM1 in membrane trafficking, the extracellular cysteine residues of Kir2.1 and 2.3 are required for their electrophysiological function but not for expression in the plasma membrane. Furthermore, the disulfide bond on the extracellular side of the pore and the preceding small loop of TRPC5 seem to be critical for the function and pharmacological activation of the agonist, Englerin A. The functional modulation of TRPC5 that results from changing the state of disulfide bonds using reducing agents has been reported (Xu et al., 2008). In contrast to TRPC5, acute treatment with a reducing agent did not affect %CALHM1. However, the previously observed inhibitory effect of sustained TCEP treatment on TRPC5 activity (Hong et al., 2015) is similar to the results of the our present study, that is, reduction in %CALHM1 and membrane expression (Fig. 6).

As discussed above, the roles of disulfide bridges in channel complexes are widely variable and include biogenesis, gating process, and pharmacological modulation. Our study confirmed the essential role of highly conserved intramolecular disulfide bridges in the proper assembly and trafficking of CALHM1. Although TCEP treatment did not acutely change the channel activity, prolonged exposure to the reducing environment could significantly lower CALHM1 expression, and this might have pathophysiological implications in the cells and tissues where the functions of CALHM1 are being revealed.

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Disulfide Bonds in CALHM1 Channel Structure and Function
Jae Won Kwon et al.

AUTHOR CONTRIBUTIONS
J.W.K. and Y.K.J. conceived and performed the experiments. J.K. gave technical support. S.J.K. (Sang Jeong Kim) provided expertise and feedback. S.J.K. (Sung Joon Kim) wrote the manuscript and supervised the study.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

ORCID
Jae Won Kwon https://orcid.org/0000-0002-4635-319X
Young Keul Jeon https://orcid.org/0000-0001-5200-380X
Jinsung Kim https://orcid.org/0000-0001-5135-3699
Song Jeong Kim https://orcid.org/0000-0002-0289-121X

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