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Young-Woo Nam
Chapman University

Meng Cui
Northeastern University

Razan Orfali
Chapman University, orfal101@mail.chapman.edu

Adam Viegas
Chapman University

Misa Nguyen
Chapman University

See next page for additional authors

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Authors

Young-Woo Nam, Meng Cui, Razan Orfali, Adam Viegas, Misa Nguyen, Eman H. M. Mohammed, Khalid Zoghebi, Simin Rahighi, Keykavous Parang, and Miao Zhang

DR MIAO ZHANG (Orcid ID : 0000-0002-3099-155X)

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Hydrophobic interactions between the HA helix and S4-S5 linker modulate apparent Ca^{2+} sensitivity of SK2 channels

Young-Woo Nam ^{1*}, Meng Cui ^{2*}, Razan Orfali ¹, Adam Viegas ¹, Misa Nguyen ¹, Eman H.M. Mohammed ¹, Khalid A. Zoghebi ¹, Simin Rahighi ¹, Keykavous Parang ¹, Miao Zhang ¹

¹ Department of Biomedical and Pharmaceutical Sciences, Chapman University School of Pharmacy, Irvine, California 92618, USA

² Department of Pharmaceutical Sciences, Northeastern University School of Pharmacy, Boston, Massachusetts, 02115, USA

* These authors contributed equally to this work.

Correspondence should be addressed to M.Z. (zhang@chapman.edu)

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Email: zhang@chapman.edu

Phone: 1-714-516-5478

Department of Biomedical and Pharmaceutical Sciences

Chapman University School of Pharmacy

9501 Jeronimo Road

Irvine, CA 92618

Abstract

Aim: Small-conductance Ca^{2+} -activated potassium (SK) channels are activated exclusively by increases in intracellular Ca^{2+} , that binds to calmodulin constitutively associated with the channel. Wild-type SK2 channels are activated by Ca^{2+} with an EC_{50} value of $\sim 0.3 \mu\text{M}$. Here, we investigate hydrophobic interactions between the HA helix and the S4-S5 linker as a major determinant of channel apparent Ca^{2+} sensitivity.

Methods: site-directed mutagenesis, electrophysiological recordings and molecular dynamic (MD) simulations were utilized.

Results: Mutations that decrease hydrophobicity at the HA-S4-S5 interface lead to Ca^{2+} hyposensitivity of SK2 channels. Mutations that increase hydrophobicity result in hypersensitivity to Ca^{2+} . The Ca^{2+} hypersensitivity of the V407F mutant relies on the interaction of the cognate phenylalanine with the S4-S5 linker in the SK2 channel. Replacing the S4-S5 linker of the SK2 channel with the S4-S5 linker of the SK4 channel results in loss of the hypersensitivity caused by V407F. This difference between the S4-S5 linkers of SK2 and SK4 channels can be partially attributed to I295 equivalent to a valine in the SK4 channel. A N293A mutation in the S4-S5 linker also increases hydrophobicity at the HA-S4-S5 interface and elevates the channel apparent Ca^{2+} sensitivity. The double N293A/V407F mutations generate a highly Ca^{2+} sensitive channel, with an EC_{50} of $0.02 \mu\text{M}$. The MD simulations of this double mutant channel revealed a larger channel cytoplasmic gate.

Conclusion: The electrophysiological data and MD simulations collectively suggest a crucial role of the interactions between the HA helix and S4-S5 linker in the apparent Ca^{2+} sensitivity of SK2 channels.

Key words: HA helix, S4-S5 linker, SK2 channels,

Introduction

Small-conductance Ca^{2+} -activated potassium (SK) channels are a unique group of ion channels that are voltage independent. Four mammalian SK channel subtypes are encoded by the *KCNN* genes, including *KCNN1* for SK1 ($\text{K}_{\text{Ca}2.1}$), *KCNN2* for SK2 ($\text{K}_{\text{Ca}2.2}$), *KCNN3* for SK3 ($\text{K}_{\text{Ca}2.3}$) and *KCNN4* for SK4 (IK or $\text{K}_{\text{Ca}3.1}$) channels, respectively. The SK channels are constitutively associated with the Ca^{2+} -binding protein calmodulin (CaM). CaM serves as the Ca^{2+} sensor for SK channels and elevated intracellular Ca^{2+} levels activate SK channels.¹ CaM complexed with the SK2 channel is subject to modulation by casein kinase 2 (CK2) and protein phosphatase 2A (PP2A), which leads to changes in apparent Ca^{2+} sensitivity.²⁻⁴

SK channels play a key role in both the cardiovascular system and the central nervous system. In the central nervous system, SK channels contribute to the medium afterhyperpolarization (mAHP) and reduce neuronal excitability.^{5,6} SK channels expressed in the vascular endothelial cells are associated with endothelium-dependent hyperpolarization.⁷⁻⁹ SK channels have been proposed as novel drug targets for movement disorders¹⁰⁻¹⁵ and hypertension.¹⁶ As such, a tremendous deal of effort has been devoted into the development of small molecules targeting SK channels.¹⁷ The first positive allosteric modulator, 1-EBIO was identified more than two decades ago.¹⁸ 1-EBIO positively modulates the SK4 channel activity more than the activity of other SK channel subtypes.⁵ On the other hand, CyPPA positively modulates SK2 and SK3 channel activity, but not SK1 and SK4 channel activity.¹⁹ In mouse models of ataxia, positive SK channel modulators like Chlorzoxazone (CHZ), 1-EBIO and CyPPA exert beneficial effects through positively modulating the activity of the SK2 channel subtype expressed in cerebellar Purkinje cells.^{13,14,20-22} Riluzole a clinically used drug that also positively modulates the activity of SK channels showed promising results in a recent phase II studies in a mixed population of ataxia patients²³ and in inherited ataxia patients.²⁴ SKA-31, a more potent and selective riluzole derivative, has a higher potency on the SK4 channel than the other SK channel subtypes. SKA-31 positively modulates the activity of SK channel subtypes expressed in the vascular endothelial cells²⁵ and induces robust vasodilation and lowers blood pressure.²⁶⁻²⁸

There are also compounds that can negatively modulate SK channels, which include NS8593,²⁹ AP14145³⁰ and AP30663 which is in clinical trials for atrial fibrillation.³¹ NS8593 shifts the Ca²⁺ dependent activation of SK3 channels to the right and reduces its apparent Ca²⁺ sensitivity.²⁹

Both the positive and negative SK channel modulators exert their effects on the channel through influencing the apparent Ca²⁺ sensitivity of these channels.^{5,29} However, exactly how SK channel apparent Ca²⁺ sensitivity is regulated remains to be elucidated. SK channels are tetramers formed by four channel subunits. There are six transmembrane α -helical domains that are denoted S1–S6 in each channel subunit. Previous studies on the apparent Ca²⁺ sensitivity of SK channels were focused on a proximal C-terminal region named as CaM-binding domain (CaMBD) that interacts with the Ca²⁺-sensor CaM.³²⁻³⁴ We recently identified that a valine to phenylalanine mutation in this region (V407F) results in a ~6-fold increase in apparent Ca²⁺ sensitivity of SK2 channels.³⁵

A recent cryo-EM study of the SK4 channel subtype demonstrated that the CaM interacts with the linker between S4 and S5 transmembrane domains (S4-S5 linker), in addition to the CaMBD.³⁶ Utilizing this cryo-EM structure of human SK4 channels (PDB code: 6CNN) as a template, we generated a homology model of the rat SK2 channels as previously described (**Fig. 1a**).³⁵ The CaMBD forms two α -helices, HA and HB, while the S4-S5 linker consists of two α -helices, S₄₅A and S₄₅B (**Fig. 1b**). The HA helix from one channel subunit (green), the S₄₅A and S₄₅B helices from a neighboring channel subunit (yellow) and CaM (blue) closely interact with each other. The V407 residue that causes hypersensitivity to Ca²⁺ is right on the HA helix, within 5 angstroms from multiple hydrophobic residues on the S₄₅A and S₄₅B helices including I289, I295, F297 and F301. Interestingly, an isoleucine to asparagine (I289N) mutation in the S4-S5 linker results in diminished SK2 channel current and causes tremor phenotypes in rats.³⁷ All these observations point towards a crucial role of the hydrophobic interactions between the HA helix and S4-S5 linker in the regulation of SK2 channel apparent Ca²⁺ sensitivity. Here, we report

that decreasing hydrophobicity between the HA helix and the S4-S5 linker with mutations I289A, I295A, F297A or F301A results in reduced apparent Ca^{2+} sensitivity of SK2 channels. In contrast, increasing hydrophobicity either by introducing an aromatic mutation (V407F) or removing a polar amino acid residue (N293A) results in hypersensitivity to Ca^{2+} with EC_{50} values of 0.05 μM and 0.09 μM , respectively. The combination of both mutations generated a highly sensitive channel with an EC_{50} value to Ca^{2+} of 0.02 μM , suggesting the hydrophobic nature of the interactions.

Results

Mutagenesis of the hydrophobic residues in the S4-S5 linker of SK2 channel

In our previous study, we identified a stretch of amino acid residues (E404-M412) in the SK2 channel that is essential for modulation of apparent Ca^{2+} sensitivity. Using site-directed mutagenesis and electrophysiological recordings, we found that decreasing hydrophobicity by the F410A mutation led to the hyposensitivity of SK2 channels to Ca^{2+} , whereas increasing hydrophobicity by V407F mutation caused hypersensitivity of SK2 channels to Ca^{2+} .³⁵ Limited by the structural information available back then, we were only able to identify M411 as a residue that could interact with the ectopic phenylalanine in the hyperactive V407F mutant channels.

With a cryo-EM structure of the human SK4 channel published in 2018³⁶ as the template, we generated a homology model of the rat SK2 channel (**Fig. 1a and Fig. S1**) as described previously,³⁵ which allowed us to take a fresh look at the cognate V407 residue. Instead of forming a disordered fragment like in a previously determined crystal structure of the CaM/CaMBD complex,^{38,39} the stretch of amino acid residues (E404-M412) in the full-length SK2 channel model appears to form a helix named as HA (**Fig. 1b**). The HA helix makes close contacts with the two α -helices, S₄₅A and S₄₅B from the S4-S5 linker of a neighboring channel subunit. Both V407 and M411 are located at the interface between the HA helix and S₄₅A-S₄₅B helices (**Fig. 1b**). With the previous knowledge on the relationship between the hydrophobicity of HA helix and channel apparent Ca^{2+} sensitivity,³⁵ we first investigated the large hydrophobic/aromatic residues in the S4-S5 linker, using an alanine-scanning mutagenesis strategy. The effect of substituting for alanine at the isoleucine and phenylalanine residues on the Ca^{2+} -dependent channel activation was examined using inside-out patch-clamp recordings on the mutant channels heterologously expressed in HEK293 cells (**Fig. S2**). Compared with wild type (WT) SK2 channel ($\text{EC}_{50} = 0.32 \pm 0.017 \mu\text{M}$, $n = 8$), the alanine substitution at I289, I295, F297 and F301 effectively reduced the SK2 channel apparent Ca^{2+} sensitivity (**Fig. 1c**).

EC₅₀ values for Ca²⁺ were $1.06 \pm 0.032 \mu\text{M}$ ($n = 10$, $P < 0.0001$), $1.61 \pm 0.096 \mu\text{M}$ ($n = 5$, $P < 0.0001$), $0.93 \pm 0.073 \mu\text{M}$ ($n = 8$, $P < 0.0001$) and $1.24 \pm 0.098 \mu\text{M}$ ($n = 6$, $P < 0.0001$), respectively (**Fig. 1d and Table S1**).

Mutagenesis of the polar amino acid residue N293 in the S4-S5 linker of SK2 channel

As mutation of the isoleucine (I289 and I295) and phenylalanine (F297 and F301) residues in the S4-S5 linker resulted in Ca²⁺ hyposensitivity, we hypothesized that removal of a polar or charged residue at the binding interface of HA and S4-S5 linker might lead to Ca²⁺ hypersensitivity of the SK2 channel. We searched for polar or charged residues within 5 angstroms from V407 and identified N293 as the only polar or charged residue in the vicinity of V407, with its side chain facing the binding interface of HA and S4-S5 linker (**Fig. 1b and Fig. 2a**). The distance between the gamma-carbon of V407 and the delta-nitrogen of N293 is 3.7 angstroms. We examined the relationship between the size and charge of the side chain at the residue 293 and SK2 channel apparent Ca²⁺ sensitivity. Amino acids of different sizes and different charges were introduced at position 293 by site-directed mutagenesis and their impact on SK2 channel apparent Ca²⁺ sensitivity was tested with inside-out patch recordings (**Fig. 2b**). Both negative charge (N293D) and positive charge (N293K) substitutions decreased SK2 channel apparent Ca²⁺ sensitivity drastically, with EC₅₀ values of $1.32 \pm 0.10 \mu\text{M}$ ($n = 6$, $P < 0.0001$) and $1.04 \pm 0.12 \mu\text{M}$ ($n = 8$, $P < 0.0001$), respectively (**Fig. 2c**). On the other hand, neither phenylalanine N293F nor Leucine N293L substitution changed SK2 channel apparent Ca²⁺ sensitivity, with EC₅₀ values of $0.49 \pm 0.11 \mu\text{M}$ ($n = 8$, $P = 0.27$) and $0.19 \pm 0.020 \mu\text{M}$ ($n = 6$, $P = 0.64$), respectively (**Fig. 2c**). The alanine (N293A) substitution increased SK2 channel apparent Ca²⁺ sensitivity, with an EC₅₀ value of $0.086 \pm 0.0074 \mu\text{M}$ ($n = 10$, $P = 0.025$) (**Fig. 2c and Table S1**).

The effectiveness of the equivalent valine to phenylalanine (V-to-F) and asparagine to alanine (N-to-A) mutations in SK4 channels

The amino acid sequences of four mammalian SK channel subtypes at the proximal end of the HA helix, equivalent to K402-M412 in SK2, are completely identical, including the valine corresponding to V407 (**Fig. 2a**). The amino acid sequences of SK1, SK2 and SK3 subtypes at the S₄₅A and S₄₅B helices are almost identical. SK4 subtype share 59% identity with the other three subtypes, although the asparagine (N186) equivalent to N293 in SK2 is conserved among all four channel subtypes (**Fig. 2a**). We first determined if mutating N186 would change the apparent Ca²⁺ sensitivity of SK4 channels. Substitution of alanine for N186 in the S₄₅A helix of SK4 channel shifted the Ca²⁺ concentration-response curve to lower concentrations (**Fig. 3a**) and changed the EC₅₀ value from 0.25 ± 0.023 μM (n = 11) to 0.11 ± 0.0058 μM (n = 6; P < 0.0001) (**Fig. 3b**). However, the substitution of alanine for V298 in the SK4 channel does not have an obvious effect (**Fig. 3c**). The EC₅₀ values for Ca²⁺ activation of the WT SK4 and mutant SK4-V298F channels are 0.25 ± 0.023 μM (n = 11) and 0.33 ± 0.030 μM (n = 6; P = 0.14), respectively (**Fig. 3d, Table S1**). Thus, the substitution of alanine at the cognate asparagine in S₄₅A helix increases the apparent Ca²⁺ sensitivity of both SK2 and SK4 channels, whereas substitution of phenylalanine at the cognate valine in HA helix increases the apparent Ca²⁺ sensitivity of SK2 channels but not SK4 channels.

The interaction between the S4-S5 linker and the HA helix

Next, we addressed why the V407F mutation increases SK2 channel apparent Ca²⁺ sensitivity, while the equivalent V298F mutant SK4 channel did not show enhanced apparent Ca²⁺ sensitivity. The SK2 and SK4 channels share 100% identity at the proximal HA helix, while only 59% identity at the S4-S5 linker (**Fig. 2a**). We hypothesized that the interactions of the V-to-F mutation in the HA helix with the S4-S5 linker is crucial for its effects on apparent Ca²⁺ sensitivity. To test this hypothesis, we constructed two chimeric channel cDNAs by swapping the S4-S5 linkers between the two subtypes. More specifically, the SK2_{S4-S5-SK4} chimera consists of the transmembrane domains, N- and C-termini of SK2 channels, together with the S4-S5 linker of SK4 channel. On the other hand, replacing the S4-S5 linker of the SK4 channel with that of the SK2 channels gives birth to the SK4_{S4-S5-SK2} chimera (**Fig. 4a**). Both these chimeras form

functional channels in patch-clamp experiments, and Ca^{2+} -dependent potassium currents were recorded from HEK293 cells heterologously expressing these chimeras (**Fig. 4b and 5a**).

The SK4_{S4-S5-SK2} chimeric channel was activated by Ca^{2+} with an EC_{50} of $0.32 \pm 0.027 \mu\text{M}$ ($n = 11$) (**Fig. 4c**). Unlike the V298F mutation introduced into the WT SK4 channels, the introduction of the V298F mutation into this SK4_{S4-S5-SK2} chimera yielded a ~ 3.3 fold increase in apparent Ca^{2+} sensitivity (**Fig. 4c and Table S1**), with an EC_{50} of $0.098 \pm 0.0079 \mu\text{M}$ ($n = 9$, $P < 0.0001$). Certainly, the apparent Ca^{2+} sensitivity of this SK4_{S4-S5-SK2}V298F mutant chimeric channel is also significantly higher than that of the SK4_V298F channel, supporting a crucial role of the interactions between the HA helix and the S4-S5 linker in the mediating apparent Ca^{2+} sensitivity.

The SK2_{S4-S5-SK4} chimeric channel exhibited an EC_{50} value for Ca^{2+} of $0.55 \pm 0.038 \mu\text{M}$ ($n = 12$) (**Fig. 5b and Table S1**). Instead of increasing the apparent Ca^{2+} sensitivity as it did in the WT SK2 background, V407F mutation introduced into this SK2_{S4-S5-SK4} chimera yielded a ~ 2.7 fold decrease in apparent Ca^{2+} sensitivity (**Fig. 5b and Table S1**), with an EC_{50} of $1.50 \pm 0.17 \mu\text{M}$ ($n = 7$, $P < 0.0001$). The apparent Ca^{2+} sensitivity of this SK2_{S4-S5-SK4}V407F mutant chimeric channel is also significantly reduced compared to that of the SK2_V407F channel (**Fig. 5b**). Collectively, these results with chimeric SK4_{S4-S5-SK2} and SK2_{S4-S5-SK4} channels demonstrated that the interaction between the HA helix and the S4-S5 linker is crucial for the regulation of apparent Ca^{2+} sensitivity of the SK channels.

A close examination of the amino acid sequences at the S4-S5 linker revealed several differences between the two channel subtypes (**Fig. 4a**). But only two residues have their side chains facing its binding interface with the HA helix. The SK2 channel has an isoleucine at position 295 (I295) and a serine at residue 286 (S286) (**Fig. 6a**), while the SK4 channel has a

valine (V188) and tyrosine (Y179) at the equivalent locations (**Fig. S3**). In the SK2 channel, the closest distance between V407 and I295 is 3.7 angstroms, suggesting strong hydrophobic interactions. The distance between V407 and S286 is 6.6 angstroms, which might be too far to form significant interaction (**Fig. 6a**). We replaced I295 and S286 in SK2 channels with the equivalent amino acid residues in SK4 channels, to test their effects on the channel apparent Ca^{2+} sensitivity (**Fig. 6b**). The I295V and S286Y double mutations did not influence the apparent Ca^{2+} sensitivity with an EC_{50} value of $0.34 \pm 0.032 \mu\text{M}$ ($n = 6$, $P = 0.20$), compared to the WT SK2 channels' EC_{50} value of $0.32 \pm 0.017 \mu\text{M}$ ($n = 8$) (**Fig. 6c and Table S1**). In the V407F mutant SK2 channel background, however, the single mutation of I295V and the I295V/S286Y double mutations significantly decreased the apparent Ca^{2+} sensitivity of the SK2-V407F mutant channel, with EC_{50} values of $0.13 \pm 0.013 \mu\text{M}$ ($n = 11$, $P < 0.0001$) and $0.16 \pm 0.0069 \mu\text{M}$ ($n = 9$, $P < 0.0001$), respectively. The single mutation of S286Y did not affect the apparent Ca^{2+} sensitivity of the SK2-V407F mutant channel, with EC_{50} values of $0.078 \pm 0.0045 \mu\text{M}$ ($n = 11$, $P = 0.12$), echoing its distance from cognate V407 residue.

The combination of the valine to phenylalanine (V407F) and asparagine to alanine (N293A) mutations in SK2 channels

Since the asparagine to alanine (N293A, **Fig. 3a**) and the valine to phenylalanine (V407F, **Fig. 3b**) mutations can each cause hypersensitivity to Ca^{2+} in SK2 channels, we tested if these two mutations have additive effects on the apparent Ca^{2+} sensitivity (**Fig. 7a**). The combination of these two mutations (N293A/V407F) yielded a further increase in apparent Ca^{2+} sensitivity (**Fig. 7b and Table S1**), with an EC_{50} of $0.020 \pm 0.0015 \mu\text{M}$ ($n = 8$), compared to $0.051 \pm 0.0047 \mu\text{M}$ ($n = 9$, $P = 0.0019$) for the V407F single mutant and to $0.086 \pm 0.0074 \mu\text{M}$ ($n = 10$, $P < 0.0001$) for the N293A single mutant. To the best of our knowledge, this is the mutant SK2 channel with the highest sensitivity towards Ca^{2+} . To elucidate any potential conformational changes induced by these two mutations, we undertook molecular dynamic (MD) simulations to compare the conformational dynamics of WT SK2 channels and the N293A/V407F mutant channels. During the 520 ns simulation time, both structures have stabilized and the RMSD

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curves have reached a plateau (**Fig. S4**). We measured the minimum distance at the cytoplasmic gate of the WT and N293A/V407F mutant SK2 channels during the 520 ns simulation time (**Fig. 7c**). The minimum distance at the cytoplasmic gate was defined by a valine residue in the transmembrane S6 domain conserved among the four SK channel subtypes. In the SK2 channel this conserved valine is V391, which is equivalent to the V282 residue of SK4 channels described in the cryo-EM study (**Fig. 2a**).³⁶ In the SK4 channel, the radius of the cytoplasmic gate of the conductive activated state II is ~1.9 angstroms larger than the nonconductive activated state I.³⁶ In the SK4 channel cryo-EM structures, we measured the distance between the hydrogen atoms of the V282 residues from two opposite channel subunits. This distance in the conductive activated state II (PDB: 6CNO) is ~3.3 angstroms larger than the nonconductive activated state I (PDB: 6CNN). In the SK2 channel, the distance between the hydrogen atoms of the V391 residues from two opposite channel subunits was measured (**Fig. S5**). A histogram fitted to Gaussian distribution demonstrates a wider cytoplasmic gate of the mutant channel with a minimum distance of 3.93 ± 0.35 angstroms (mean $\pm$ SD), compared to 3.75 ± 0.31 angstroms (mean $\pm$ SD) of the WT SK2 channel (**Fig. 7d**). The N293A/V407F mutations induced a ~0.18 angstrom change in the cytoplasmic gate of the SK2 channel, which is much smaller than the difference between the nonconductive and conductive activated states of SK4 channels,³⁶ echoing the mere effect of these mutations on the apparent Ca^{2+} sensitivity.

Discussion

The SK channels are a unique group of potassium channels that are activated exclusively by intracellular Ca^{2+} .⁴⁰ This divalent cation binds to CaM that is constitutively associated with the SK channels and serves as the Ca^{2+} sensor.⁴⁰ Thus, understanding the apparent Ca^{2+} sensitivity of the complex of SK channel and CaM is crucial. Previously studies on the apparent Ca^{2+} sensitivity of the SK channels mostly focused on the CaMBD and its interaction with CaM.^{2,3,34} A R352H mutation in the CaMBD of SK4 channels was reported to increase apparent Ca^{2+} sensitivity of the channels.⁴¹ Our own previous studies identified a stretch of amino acid residues (E404-M412) in the proximal end of the CaMBD in SK2 channels as a critical determinant of apparent Ca^{2+} sensitivity. Mutating an aromatic residue F410 to alanine results in the hyposensitivity of SK2 channels to Ca^{2+} , whereas substituting a valine V407 to phenylalanine increased apparent Ca^{2+} sensitivity of SK2 channels drastically.³⁵ A crystal structure of the CaM/CaMBD complex^{38,39} was used as the model to guide such studies, until a recent cryo-EM study of the full-length SK4 channel subtype became available.³⁶ The cryo-EM structure lacks the CK2,³ PP2A⁴ and phosphatidylinositol biphosphate² that co-assemble together with the SK channels in physiological conditions. But this cryo-EM structure gave us an opportunity to investigate the hypersensitivity of the V-to-F mutant channel to Ca^{2+} .

The cryo-EM structure demonstrated the S4-S5 linker as another critical element in the SK4 channel to confer Ca^{2+} sensing by CaM to the channel gating. Ca^{2+} bound CaM, the HA and HB helices formed by CaMBD, and the S₄₅A and S₄₅B helices formed by the S4-S5 linker from a neighboring channel subunit, become a stable structural group right at the bottom of the pore-forming S6 transmembrane domain.³⁶ In the same report, functional experiments also established the important role of the binding interface between S₄₅A helix and CaM N-lobe in the channel activity. A thiol modification reagent, MTSES, adds negative charge to the S181C residue in the S₄₅A facing the CaM N-lobe and causes diminished SK4 channel activity. Mutation of this S181 to tryptophan or tyrosine causes hypersensitivity to Ca^{2+} .⁴² Mutation of the equivalent serine in SK3 channels to cysteine (S436C) also increases apparent Ca^{2+} sensitivity.⁴³

This CaM- S₄₅A interaction interface was then explored as potential binding pocket of small molecule modulators of the channels.⁴²

Now that we know that CaM interactions with both the CaMBD (HA and HB helices) and the S4-S5 linker (S₄₅A and S₄₅B helices) are crucial for the apparent Ca²⁺ sensitivity of the channels, then what about the interactions between the CaMBD and the S4-S5 linker? A homology model of the rat SK2 channels generated using this cryo-EM structure as a template showed similar interactions between HA helix from one channel subunit (green), the S₄₅A and S₄₅B helices from a neighboring channel subunit (yellow) and CaM (blue) (**Fig. 1a**). Similar to the SK4 structure, the S₄₅B helix is wedged between HA helix and the pore-forming transmembrane S6 domain, while the S₄₅A helix lies almost parallelly next to the proximal end of HA helix (**Fig. 1b**). Mutating hydrophobic or aromatic residues at the HA-S₄₅A-S₄₅B interface to alanine leads to hyposensitivity to Ca²⁺ (**Fig. 1c,d**), while mutating a polar residue at the HA-S₄₅A interface results in hypersensitivity to Ca²⁺ (**Fig. 2b,c**), addressing the hydrophobic nature of the HA-S₄₅A-S₄₅B interactions and their crucial role in the channel apparent Ca²⁺ sensitivity.

The V-to-F mutation induces hypersensitivity to Ca²⁺ in SK1, SK2 and SK3 channel subtypes.³⁵ The equivalent V298F mutation did not change the apparent Ca²⁺ sensitivity of the SK4 channel subtype (**Fig. 3**). This observation cannot be explained by the HA helix alone, because the four subtypes are 100% identical at the proximal end of the HA helix where equivalent residues to V407 are located (**Fig. 2a**). This can at least partially be explained by the HA-S₄₅A-S₄₅B interactions, as several differences exist between the amino acid sequences of SK4 and the other three channel subtype. Introduction of the V407F mutation into a SK2 chimera containing the S4-S5 linker of SK4 channels can no longer induce Ca²⁺ hypersensitivity (**Fig. 5**), while the equivalent V298F mutation induces Ca²⁺ hypersensitivity in a SK4 chimera containing the S4-S5 linker of SK2 channels (**Fig. 4**). These results further address the critical role of the hydrophobic HA-S₄₅A-S₄₅B interactions in the channel apparent Ca²⁺ sensitivity.

Enhancing the hydrophobicity of the HA (V407F) and the S₄₅A (N293A) helices at the same time gives birth to a SK2 channel that is most sensitive to Ca²⁺, with an EC₅₀ value of ~0.02 μM (**Fig. 7**). MD simulations revealed a larger minimum distance at the channel cytoplasmic gate (**Fig. 7**), suggesting more efficient coupling between CaM-mediated Ca²⁺ sensing to the channel opening as a result of the enhanced hydrophobic interactions at the HA-S₄₅A interface. The elevated apparent Ca²⁺ sensitivity of the N293A/V407F mutant channel might be attributed to this highly efficient coupling.

These studies on the apparent Ca²⁺ sensitivity of SK channels are physiologically relevant. The intracellular Ca²⁺ concentration is about ~0.1 μM in resting neurons,⁴⁴ which is not enough to activate the WT SK2 channel (EC₅₀ ~0.32 μM). However, the hyperactive N293A (EC₅₀ ~0.086 μM), V407F (EC₅₀ ~0.051 μM) and N293/V407F (EC₅₀ ~0.020 μM) mutants could be mostly in the activated state, barring other regulatory events (**Fig. 7a**). Our previous work demonstrated that the cognate V698F mutation *C. elegans* *kcnl-2* channel (equivalent to V407F in rat SK2) suppressed locomotion defects in a *C. elegans* ALS model (hSOD1G85R).³⁵ In excited neurons, the Ca²⁺ concentration can be elevated by Ca²⁺ influx from extracellular sources or Ca²⁺ release from intracellular stores. At Ca²⁺ concentration of 0.5 μM, the WT SK2 channel is mostly open, while the I289A (in the S₄₅A helix) mutant is almost completely in the closed state (**Fig. 1c**). Recently, an isoleucine to asparagine (I289N) mutation was reported to cause diminished SK2 channel current and the tremor phenotypes in the tremor dominant Kyoto (Trdk) rats.³⁷ Collectively, SK2 channels may be linked with movement disorders, which is being explored as a drug target for ataxia,^{13,14,21,45} spinal muscular atrophy,¹² and Parkinson's disease.⁴⁶ Given the potential of SK2 channels as a drug target for movement disorders, understanding the regulation of its apparent Ca²⁺ sensitivity is critical. Here, we focus on the HA-S₄₅A-S₄₅B binding interface as a key structural determinant in the regulation of SK2 channel apparent Ca²⁺ sensitivity. Future studies can take advantage of these results for the potential drug development for movement disorders.

Materials and Methods

Electrophysiology

In our studies, the effect of mutations on the apparent Ca^{2+} sensitivity of SK channels was investigated as previously described.^{2,39,47} Briefly, mutations were introduced to the rat SK2 or human SK4 channels using QuickChange II site-directed mutagenesis kit (Agilent). The chimeric channel cDNA constructs swapping the S4-S5 linkers between SK2 and SK4 channels were generated through gene synthesis (Genscript). The mutant channel cDNAs, along with CaM and GFP, at a ratio of 5:2.5:1 (weight), were transfected into HEK293 cells by the calcium-phosphate method. All of the mutant channels were expressed well and conducted K^+ current in patch-clamp recordings comparable to the WT channels. SK currents were recorded 1–2 days after transfection, with an Axon200B amplifier (Molecular Devices) at room temperature.

pClamp 10.5 (Molecular Devices) was used for data acquisition and analysis. The resistance of the patch electrodes ranged from 3–5 $\text{M}\Omega$. The pipette solution contained (in mM): 140 KCl, 10 Hepes (pH 7.4), 1 MgSO_4 . The bath solution containing (in mM): 140 KCl, 10 Hepes (pH 7.2), 1 EGTA, 0.1 Dibromo-BAPTA, and 1 HEDTA was mixed with Ca^{2+} to obtain the desired free Ca^{2+} concentrations, calculated using the software by Chris Patton of Stanford University (<https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm>). The Ca^{2+} concentrations were verified using a Ca^{2+} calibration buffer kit (Thermo Fisher Scientific). Briefly, a standard curve was generated using the Ca^{2+} buffers from the kit and a fluorescence Ca^{2+} indicator. Then the Ca^{2+} concentrations of our bath solutions were determined through interpolation on the standard curve.

Currents were recorded using an inside-out patch configuration. The apparent Ca^{2+} sensitivity of SK channels is regulated by phosphorylation.³ The EC_{50} values for Ca^{2+} determined in inside-out patches may deviate from the apparent Ca^{2+} sensitivity of SK channels in intact

cells. Nonetheless, the inside-out patch configuration allows us to measure the response of the channels to various Ca^{2+} concentrations from the same patch and effectively compare the apparent Ca^{2+} sensitivity of WT and mutant channels. The intracellular face was initially exposed to a zero- Ca^{2+} bath solution, and subsequently to bath solutions with a series of Ca^{2+} concentrations. Currents were recorded by repetitive 1-s-voltage ramps from -100 mV to $+100$ mV from a holding potential of 0 mV. One minute after switching of bath solutions, ten sweeps with a 1-s interval were recorded. The integrity of the patch was examined by switching the bath solution back to the zero- Ca^{2+} buffer. Data from patches, which did not show significant changes in the seal resistance after solution changes, were used for further analysis. To construct the concentration-dependent positive modulation of channel activities, the current amplitudes at -90 mV in response to various concentrations of Ca^{2+} were normalized to that obtained at maximal concentration of Ca^{2+} . The normalized currents were plotted as a function of the concentrations of Ca^{2+} . EC_{50} values and Hill coefficients were determined by fitting the data points to a standard concentration-response curve ($Y = 100 / (1 + (X/\text{EC}_{50})^{\text{Hill}})$). All data are presented in mean $\pm$ s.e.m. The data analysis was performed using pClamp 10.5 (Molecular Devices) in a blinded fashion. All data are shown as mean $\pm$ SEM unless otherwise indicated. One-way ANOVA and Tukey's post hoc tests were used for data comparison of three or more groups. The Student's *t*-test was used for data comparison if there were only two groups.

Molecular Dynamics (MD) simulations

The homology model of the rat SK2 channel was described in our previously report.³⁵ The WT or N293A/V407F mutant SK2 channels were immersed in an explicit lipid bilayer of POPC, POPE, POPS and cholesterol with molecular ratio of 25:5:5:1,⁴⁸ and a water box in $168.1\text{\AA} \times 165.6\text{\AA} \times 140.0\text{\AA}$ dimension by using the CHARMM-GUI Membrane Builder webserver (<http://www.charmm-gui.org/?doc=input/membrane>). 150mM KCl and extra neutralizing

counter ions were added into the systems. The total atoms of the systems were 293,068 for both the WT and N293A/V407F channels. The PMEMD.CUDA program in AMBER16 was used to conduct the MD simulations. The MD simulations were performed with periodic boundary conditions to produce isothermal-isobaric ensembles. Long range electrostatics were calculated using the particle mesh Ewald (PME) method ⁴⁹ with a 10 Å cutoff. Prior to production runs, energy minimization of the system was carried out. Subsequently, the system was heated from 0 K to 303K using Langevin dynamics with the collision frequency of 1 ps⁻¹. During the heating, the SK2 channel was position-restrained using an initial constant force of 520 kcal/mol/Å², and weakened to 10 kcal/mol/Å², allowing lipid and water molecules to move freely. Then, the system went through 5 ns equilibrium MD simulations. Finally, a total of 520 ns production MD simulation was conducted, and coordinates were saved every 100 ps for analysis. In total, 5,000 snapshot structures were collected during the MD simulations. The root mean square deviation (RMSD), the root mean square fluctuation (RMSF) of atomic positions, and minimum distances between atoms in the trajectories were analyzed using the built-in utility of GROMACS program.⁵⁰

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Author contributions: Y.W.N., A.V., E.H.M.M., K.A.Z., S.R., and K.P. undertook molecular biology and cell culture studies. Y.W.N., R.O., M.N., and M.Z. undertook electrophysiology studies. M.C. performed computational work. All authors contributed to the manuscript and the figures.

Conflict of interest: none.

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Legends to Figures

Fig. 1. Alanine substitution of the hydrophobic residues in the S4-S5 linker causes hyposensitivity of SK2 channels to Ca^{2+} . (a) A homology model of the rat SK2 channel was generated using the human SK4 channel cryo-EM structure with Ca^{2+} . (b) Ca^{2+} bound CaM (blue), HA and HB helices from one channel subunit (green), the S₄₅A and S₄₅B helices from a neighboring channel subunit (yellow) become a stable structural group right at the bottom of the pore-forming S6 transmembrane domain. (c) Concentration-dependent activation by Ca^{2+} of the WT and alanine mutant SK2 channels. (d) EC₅₀ values for activation by Ca^{2+} of the WT and alanine mutant channels. ‡ P < 0.001 compared with WT. No asterisk means no statistical significance compared with WT.

Fig. 2. Mutations of N293 in the S₄₅A helix change apparent Ca^{2+} sensitivity of SK2 channels. (a) Amino acid sequence alignment of human SK1 [GenBank: NP_002239.2], rat SK2 [Genbank: NP_062187.1], human SK3 [GenBank: NP_002240.3] and human SK4 [GenBank: NP_002241.1] at the S₄₅A and S₄₅B helices (highlighted in yellow) and the proximal end of HA helix (highlighted in green). (b) Activation by Ca^{2+} of the WT and channels carrying mutations at N293. (c) EC₅₀ values for activation by Ca^{2+} of the WT and mutant channels. * P < 0.05, ‡ P < 0.001 compared with WT. No asterisk means no statistical significance compared with WT.

Fig. 3. Mutations of asparagine in S₄₅A helix changes both SK2 and SK4 apparent Ca^{2+} sensitivity, while mutations of the valine in HA helix increases SK2 but not SK4 apparent Ca^{2+} sensitivity. (a) Concentration-dependent activation of the WT and N-to-A mutant SK2 and SK4 current by Ca^{2+} . (b) EC₅₀ values for the activation of the WT and N-to-A mutant SK channels by Ca^{2+} . (c) Concentration-dependent activation of the WT and V-to-F mutant SK2 and SK4 current by Ca^{2+} . (d) EC₅₀ values for the activation of the WT and V-to-F mutant SK channels by Ca^{2+} . ‡ P < 0.001 compared with respective WT channel subtype. No asterisk means no statistical significance compared with respective WT channel subtype.

Fig. 4. The introduction of V298F mutation leads to Ca^{2+} hypersensitivity of the SK4_{S4-S5-SK2} chimera. (a) A chimeric SK4 channel carrying the S4-S5 linker of SK2 channels (SK4_{S4-S5-SK2}), and a chimeric SK2 channel carrying the S4-S5 linker of SK4 channels (SK2_{S4-S5-SK4}) were generated. The S4-S5 linkers swapped between the two subtypes are shown in bold. The S4-S5 linker from SK2 channel is shown in black, while that of the SK4 channel is shown in blue font. Two critical differences in the S4-S5 linker between the subtypes are shown in red font. (b) Introduction of V298F mutation left-shifts the concentration-dependent activation of the SK4_{S4-S5-SK2} chimeric but not the WT SK4 channel. (c) EC_{50} values for the activation of the chimeric SK4 channels by Ca^{2+} . ‡ $P < 0.001$. No asterisk means no statistical significance.

Fig. 5. The introduction of V407F mutation fails to increase apparent Ca^{2+} sensitivity of the SK2_{S4-S5-SK4} chimera. (a) Introduction of V407F mutation left-shifts the concentration-dependent activation of the WT SK2 channels, but right-shifts the SK2_{S4-S5-SK4} chimeric channel. (b) EC_{50} values for the activation of the chimeric SK2 channels by Ca^{2+} . ‡ $P < 0.001$.

Fig. 6. An isoleucine I295 residue in the S4-S5 linker is required for V407F to induce Ca^{2+} hypersensitivity in SK2 channels. (a) An isoleucine (I295) from the S₄₅A helix (yellow) is in the vicinity of V407 from the HA helix (green), while a serine (S286) is beyond 5 angstroms. (b) I295V mutation right-shifts the Ca^{2+} dependent activation of the V407F mutant SK2 channel. (c) I295V mutation significantly compromised the Ca^{2+} hypersensitivity of the V407F mutant SK2 channel. † $P < 0.01$, ‡ $P < 0.001$. No asterisk means no statistical significance.

Fig. 7. N293A and V407F mutations additively increase apparent Ca^{2+} sensitivity in SK2 channels. (a) N293A/V407F double mutations further left-shifted the Ca^{2+} dependent activation of the SK2 channel. (b) N293A/V407F double mutations increase the apparent Ca^{2+}

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sensitivity of SK2 channels to $\sim 0.02 \mu\text{M}$. $\dagger P < 0.01$, $\ddagger P < 0.001$. (c) In MD simulations, the minimum distance at the cytoplasmic gate is shown as a function of the simulation time. (d) The distribution histogram of the minimum distance at the cytoplasmic gate shows a larger distance in the N293A/V407F double mutant channel.

Supplementary Figures

Fig. S1. The homology model of the SK2 channel. (a) The view of the channel from the extracellular side. (b) The view of the channel from the intracellular side. (c) The view of the channel from the intracellular side at the level of the HA helix and S4-S5 linker.

Fig. S2. Representative current traces of concentration-dependent activation by Ca^{2+} of the WT (a), V407F (b) and N293A/V407F (c) SK2 channels.

Fig. S3. The equivalent residue V298 in the HA helix is in the vicinity of V188 in the S₄₅A helix of the SK4 channel.

Fig. S4. The root mean square deviation (RMSD) curves of the MD simulations.

Fig. S5. The minimum distance at the cytoplasmic gate was defined by V391 residue in the transmembrane S6 domain of SK2 channels. (a) The side view shows the V391 residues from two opposite SK2 channel subunits. The top-down view shows the minimum distance at the cytoplasmic gate of WT (b) and N293A/V407F (c) mutant channels.

Fig. 1

(a)

SK1	247	LHSKIFTDASSRSIGAL	NK	ITFNTRFVMKTLMT	ICPGT	284	...	362	VAVVARKLELT	KA	EH	V	HN	FMM	383
SK2	276	LHSKLFTDASSRSIGAL	NK	INFNTRFVMKTLMT	ICPGT	313	...	391	VAVVARKLELT	KA	EH	V	HN	FMM	412
SK3	424	LHSKLFTDASSRSIGAL	NK	INFNTRFVMKTLMT	ICPGT	461	...	539	VAVVARKLELT	KA	EH	V	HN	FMM	560
SK4	169	LRSGVLLNASYRSIGAL	NQ	VRFRHWFWAKLYMNT	HPGR	206	...	282	VAVVARKLEFN	KA	EH	V	HN	FMM	303
		S4		S ₄₅ A		S ₄₅ B		S5		S6			HA

Fig. 2

Fig. 3

Fig. 5

Fig. 6

Fig. 7