Channelopathy-Causing Mutations in the S45A/S45B and HA/HB Helices of K\textsubscript{Ca}2.3 and K\textsubscript{Ca}3.1 Channels Alter Their Apparent Ca\textsuperscript{2+} Sensitivity

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Channelopathy-causing mutations in the S45A/S45B and HA/HB helices of KCa2.3 and KCa3.1 channels alter their apparent Ca2+ sensitivity

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Short title: Mutations altering Ca2+ sensitivity of KCa2.3 and KCa3.1 channels

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Abstract

Small- and intermediate-conductance Ca$^{2+}$-activated potassium (K$\text{Ca}_{2.3}$ and K$\text{Ca}_{3.1}$, also called SK and IK) channels are activated exclusively by a Ca$^{2+}$-calmodulin gating mechanism. Wild-type K$\text{Ca}_{2.3}$ channels have a Ca$^{2+}$ EC$_{50}$ value of $\sim$0.3 μM, while the apparent Ca$^{2+}$ sensitivity of wild-type K$\text{Ca}_{3.1}$ channels is $\sim$0.27 μM. Heterozygous genetic mutations of K$\text{Ca}_{2.3}$ channels have been associated with Zimmermann-Laband syndrome and idiopathic noncirrhotic portal hypertension, while K$\text{Ca}_{3.1}$ channel mutations were reported in hereditary xerocytosis patients. K$\text{Ca}_{2.3}$S436C and K$\text{Ca}_{2.3}$V450L channels with mutations in the S45A/S45B helices exhibited hypersensitivity to Ca$^{2+}$. The corresponding mutations in K$\text{Ca}_{3.1}$ channels also elevated the apparent Ca$^{2+}$ sensitivity. K$\text{Ca}_{3.1}$S314P, K$\text{Ca}_{3.1}$A322V and K$\text{Ca}_{3.1}$R352H channels with mutations in the HA/HB helices are hypersensitive to Ca$^{2+}$, whereas K$\text{Ca}_{2.3}$ channels with the equivalent mutations are not. The different effects of the equivalent mutations in the HA/HB helices on the apparent Ca$^{2+}$ sensitivity of K$\text{Ca}_{2.3}$ and K$\text{Ca}_{3.1}$ channels may imply distinct modulation of the two channel subtypes by the HA/HB helices. AP14145 reduced the apparent Ca$^{2+}$ sensitivity of the hypersensitive mutant K$\text{Ca}_{2.3}$ channels, suggesting the potential therapeutic usefulness of negative gating modulators.

Key words: HA/HB helices, S45A/S45B helices, K$\text{Ca}_{2.3}$ and K$\text{Ca}_{3.1}$ channels, negative gating modulator
Abbreviations

Asparagine-to-alanine (N-to-A)
Calcium (Ca\(^{2+}\))
Calmodulin (CaM)
Idiopathic noncirrhotic portal hypertension (INCPH)
Intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca3.1}\) or IK) channels
Small-conductance Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca2.x}\) or SK) channels
Valine-to-phenylalanine (V-to-F)
Wild-type (WT)
Zimmermann-Laband syndrome (ZLS)
Introduction

Small- and intermediate-conductance Ca\textsuperscript{2+}-activated potassium (K\textsubscript{Ca2.2} and K\textsubscript{Ca3.1}) channels are voltage independent. Mammalian KCNN genes encode three K\textsubscript{Ca2.2} channel subtypes, including K\textsubscript{Ca2.1} (SK1), K\textsubscript{Ca2.2} (SK2), K\textsubscript{Ca2.3} (SK3), and one K\textsubscript{Ca3.1} (IK or SK4) channel subtype. These channels are constitutively associated with the Ca\textsuperscript{2+}-binding protein calmodulin (CaM). Both K\textsubscript{Ca2.2} and K\textsubscript{Ca3.1} channels are activated by a unique Ca\textsuperscript{2+}-CaM gating mechanism.\textsuperscript{1}

Human heterozygous genetic mutations of K\textsubscript{Ca2.3} channels have been linked to Zimmermann-Laband syndrome (ZLS)\textsuperscript{2} and idiopathic noncirrhotic portal hypertension (INCPH)\textsuperscript{3}. The ZLS-related (K269E, G350D and S436C) and INCPH-related (V450L) mutant channels exhibited faster kinetics of current activation by Ca\textsuperscript{2+} upon break-in with whole-cell patch-clamp recordings, and thus were inferred to have elevated apparent Ca\textsuperscript{2+} sensitivity\textsuperscript{2}. The apparent Ca\textsuperscript{2+} sensitivity has not been determined quantitatively on any of these ZLS-related or INCPH-related mutant K\textsubscript{Ca2.3} channels. Meanwhile, heterozygous genetic mutations in K\textsubscript{Ca3.1}, which underlies the so-called Gardos channel in erythrocytes, lead to hereditary xerocytosis. The Gardos channelopathy-related (V282M/E\textsuperscript{4}, S314P\textsuperscript{5}, A322V\textsuperscript{6} and R352H\textsuperscript{7-9}) mutations were also speculated to cause hypersensitivity to Ca\textsuperscript{2+} of K\textsubscript{Ca3.1} channels, mostly based on more prominent responses of mutant channel current to an inhibitor TRAM-34\textsuperscript{5,7,8} or a positive modulator NS309\textsuperscript{5,6}. The apparent Ca\textsuperscript{2+} sensitivity has only been determined quantitatively on one of these Gardos channelopathy-related mutant channels, K\textsubscript{Ca3.1}_R352H\textsuperscript{8}.

A recent cryo-EM study revealed that the pore forming K\textsubscript{Ca3.1} channel subunits form a tetrameric structure, with the Ca\textsuperscript{2+} sensor protein CaM constitutively bound (Fig. 1A).\textsuperscript{10} In each channel subunit, there are six transmembrane α-helical domains that are denoted S1–S6. CaM interacts with the HA/HB helices, in addition to the linker between the S4 and S5 transmembrane domains (S4-S5 linker).\textsuperscript{10} The S4-S5 linker consists of two α-helices, S\textsubscript{45}A and S\textsubscript{45}B (Fig. 1B). The HA/HB helices from one channel subunit (green), the S\textsubscript{45}A and S\textsubscript{45}B helices from a neighboring channel subunit (yellow) and CaM (grey) closely interact with each other. Three of the Gardos channelopathy-related (S314P\textsuperscript{5}, A322V\textsuperscript{6} and R352H\textsuperscript{7-9}) missense mutations are located in the HA/HB helices,
while the fourth one (V282M/E4) is located in the transmembrane S5 domain close to the pore.

Utilizing this cryo-EM structure of human KCa3.1 (PDB code: 6CNN) as a template, we generated a homology model of the human KCa2.3 channel (Fig. 1C). The INCPH-related (V450L) and one of the ZLS-related (S436C) mutations are located in the S45A/S45B helices (Fig. 1D), while the other two ZLS-related (K269E and G350D) mutations are found in the N-terminus and transmembrane domains, respectively.

We previously identified a valine-to-phenylalanine (V-to-F) mutation in the HA helix that causes Ca²⁺ hypersensitivity in the KCa2.1, KCa2.2 and KCa2.3 subtypes¹¹, but the equivalent V-to-F mutation did not alter the apparent Ca²⁺ sensitivity in KCa3.1 channels.¹² In contrast, an asparagine-to-alanine (N-to-A) mutation in the S45A/S45B helices increased the apparent Ca²⁺ sensitivity in both KCa2.2a and KCa3.1 channel subtypes.¹² Thus, we speculate that the differential modulation of KCa2.3 and KCa3.1 channels may result more from effects mediated by the HA/HB helices than the S45A/S45B helices. Although KCa2.x and KCa3.1 channels are activated by the same Ca²⁺-CaM gating mechanism, they exhibit differences in their function, modulation and pharmacology.¹ The KCa3.1 structures¹⁰ and KCa2.x homology models¹²-¹⁵ offer an opportunity to perform structure-function studies and examine the differences between the channel subtypes, which might reveal structural information useful for the design of subtype-selective biophysical tools¹² and pharmacological tools¹⁶. To the best of our knowledge, Ca²⁺-hypersensitive human genetic mutations have only been reported in KCa2.3 and KCa3.1 channels, but not in KCa2.1 and KCa2.2 channels. Utilizing these disease-causing mutations in the HA/HB helices and the S45A/S45B helices as biophysical tools, we examined whether mutations in corresponding positions might influence KCa2.3 and KCa3.1 channels differentially.

Here, we quantified the elevated apparent Ca²⁺ sensitivity of KCa2.3 channels with the INCPH-related (V450L) and the ZLS-related (S436C) mutations in the S45A/S45B helices. Their equivalent mutations in the S45A/S45B helices of KCa3.1 channels also increased the apparent Ca²⁺ sensitivity. On the other hand, the Gardos channelopathy-related (S314P, A322V and R352H) mutations in the HA/HB helices elevated the
apparent Ca\(^{2+}\) sensitivity of KCa3.1 channels, whereas their equivalent mutations in KCa2.3 channels did not change the apparent Ca\(^{2+}\) sensitivity. A negative gating modulator, AP14145, reduced the apparent Ca\(^{2+}\) sensitivity of the INCPH- and ZLS-related mutant KCa2.3 channels, suggesting its potential therapeutic usefulness.
Results

Mutations in the S45A/S45B helices of KCa2.3 and KCa3.1 channels

We previously identified an asparagine-to-alanine (N-to-A) mutation in the S45A/S45B helices that increased the apparent Ca\(^{2+}\) sensitivity in both KCa2.2a and KCa3.1 channel subtypes.\(^{12}\) We first investigated the equivalent N441A mutation, together with the ZLS-related S436C and INCPH-related V450L mutations in the S45A/S45B helices of KCa2.3 channels (Fig. 2A). In the study first describing these disease-associated mutations\(^2\), the kinetics of current activation by Ca\(^{2+}\) upon break-in during whole-cell patch-clamp recordings was used to infer an increase in apparent Ca\(^{2+}\) sensitivity. In order to quantify this increase in apparent Ca\(^{2+}\) sensitivity, we used the inside-out patch-clamp to measure the Ca\(^{2+}\)-dependent activation of the mutant channels heterologously expressed in HEK293 cells (Fig. S1). With the inside-out patch configuration, we were able to measure the responses of the channels to various Ca\(^{2+}\) concentrations from the same patch and determine the apparent Ca\(^{2+}\) sensitivity of the mutant channels.

Compared with wild-type (WT) KCa2.3 channel (EC\(_{50}\) = 0.30 ± 0.025 \(\mu\)M, \(n = 8\)), the S436C, N441A and V450L mutations effectively elevated the KCa2.3 channel apparent Ca\(^{2+}\) sensitivity (Fig. 2B). EC\(_{50}\) values for Ca\(^{2+}\) were 0.087 ± 0.0058 \(\mu\)M (\(n = 6\), \(P < 0.0001\)), 0.064 ± 0.0098 \(\mu\)M (\(n = 6\), \(P < 0.0001\)), and 0.15 ± 0.013 \(\mu\)M (\(n = 6\), \(P < 0.0001\)), respectively (Fig. 2C and Table 1).

We then tested their equivalent mutations in KCa3.1 channels (Fig. S2). Three equivalent mutations S181C, N186A and V195L also increased apparent Ca\(^{2+}\) sensitivity of KCa3.1 channels (Fig. 3A). EC\(_{50}\) values for Ca\(^{2+}\) of the three mutants were 0.10 ± 0.011 \(\mu\)M (\(n = 7\), \(P < 0.0001\)), 0.10 ± 0.0055 \(\mu\)M (\(n = 5\), \(P < 0.0001\)), and 0.20 ± 0.020 \(\mu\)M (\(n = 5\), \(P < 0.01\)), compared with WT KCa3.1 channels (EC\(_{50}\) = 0.27 ± 0.012 \(\mu\)M, \(n = 8\), Fig. 3B and Table 1).

Mutations in the HA/HB helices of KCa2.3 and KCa3.1 channels

In our previous study, a valine-to-phenylalanine (V-to-F) mutation in the HA helix caused Ca\(^{2+}\) hypersensitivity in the KCa2.1, KCa2.2 and KCa2.3 subtypes\(^{11}\), but the equivalent V-to-F mutation did not alter the apparent Ca\(^{2+}\) sensitivity in KCa3.1
channels.\textsuperscript{12} Interestingly, a heterozygous V-to-F mutation in KC\textsubscript{a}2.3 (V555F) in a young child linked to ZLS was subsequently reported.\textsuperscript{17} We here investigated this newly reported ZLS-related mutation hypothesizing that it would display increased apparent Ca\textsuperscript{2+} sensitivity.

We investigated the KC\textsubscript{a}3.1\_V298F mutation, together with the Gardos channelopathy-related (S314P\textsuperscript{5}, A322V\textsuperscript{6} and R352H\textsuperscript{7-9}) missense mutations located in the HA/HB helices of KC\textsubscript{a}3.1 channels (\textbf{Fig. 4A} and \textbf{Fig. S3}). Three Gardos channelopathy-related S314P, A322V and R352H mutations increased the apparent Ca\textsuperscript{2+} sensitivity of KC\textsubscript{a}3.1 channels (\textbf{Fig. 4B}). The EC\textsubscript{50} values for Ca\textsuperscript{2+} of the three mutants were 0.064 ± 0.0033 μM (n = 9, P < 0.0001), 0.059 ± 0.0039 μM (n = 8, P < 0.0001), and 0.085 ± 0.0081 μM (n = 8, P < 0.0001), compared with WT KC\textsubscript{a}3.1 channels (EC\textsubscript{50} = 0.27 ± 0.012 μM, n = 8, \textbf{Fig. 4C} and \textbf{Table 1}). Consistent with our previous report\textsuperscript{12}, the V298F mutation did not change the apparent Ca\textsuperscript{2+} sensitivity compared with the WT KC\textsubscript{a}3.1 channels (\textbf{Fig. 4C} and \textbf{Table 1}).

We next tested the equivalent mutations in KC\textsubscript{a}2.3 channels (\textbf{Fig. S4}). Three equivalent mutations A571P, T579V and R612H did not alter the KC\textsubscript{a}2.3 channel apparent Ca\textsuperscript{2+} sensitivity (\textbf{Fig. 5A}). The EC\textsubscript{50} values for Ca\textsuperscript{2+} of the three mutants were 0.30 ± 0.027 μM (n = 8, P > 0.9999), 0.25 ± 0.032 μM (n = 6, P = 0.5666), and 0.27 ± 0.014 μM (n = 11, P = 0.8339), compared with WT KC\textsubscript{a}2.3 channels (EC\textsubscript{50} = 0.30 ± 0.025 μM, n = 8, \textbf{Fig. 5B} and \textbf{Table 1}). On the other hand, the V555F mutation increased the apparent Ca\textsuperscript{2+} sensitivity compared with the WT KC\textsubscript{a}2.3 channels (\textbf{Fig. 5B} and \textbf{Table 1}).

We then tested another two ZLS-related mutations, K269E in the N-terminus (\textbf{Fig. 6A}) and G350D in the transmembrane domains (\textbf{Fig. 6B}) of KC\textsubscript{a}2.3 channels. Both K269E and G350D mutations increased the apparent Ca\textsuperscript{2+} sensitivity of KC\textsubscript{a}2.3 channels, with EC\textsubscript{50} values of 0.086 ± 0.013 μM (n = 6, P < 0.0001) and 0.12 ± 0.013 μM (n = 8, P < 0.0001), respectively (\textbf{Table 1}).

\textbf{Effects of a negative gating modulator on the mutant KC\textsubscript{a}2.3 channels}

AP30663\textsuperscript{18}, a negative gating modulator of KC\textsubscript{a}2.3 channels, is currently in early clinical development for conversion of atrial fibrillation\textsuperscript{19}. AP30663 is not commercially
available. To explore potential pharmacological therapies for the diseases caused by mutant KCa2.3 channels, we tested the effects of a structurally related compound, AP14145\textsuperscript{20}, on the WT and mutant KCa2.3 channels (Fig. 6A-F). AP14145 (10 μM) right-shifted the Ca\textsuperscript{2+} concentration-response curves of all the mutant and WT channels, indicating its effectiveness as a negative gating modulator. AP14145 induced a \textasciitilde3.6-fold decrease in the apparent Ca\textsuperscript{2+} sensitivity of KCa2.3\_WT channels (Fig. 6F, Table 1). Its effects on the mutant channels were somewhat less prominent. AP14145 changed the EC\textsubscript{50} values for Ca\textsuperscript{2+} of KCa2.3\_K269E, KCa2.3\_G350D, KCa2.3\_S436C, KCa2.3\_V450L and KCa2.3\_V555F channels by \textasciitilde1.63, \textasciitilde2.25, \textasciitilde2.18, \textasciitilde2.07 and \textasciitilde2.09 folds, respectively (Fig. 6A-E, Table 1).
Discussion

\( \text{KCa}_2.x/\text{KCa}_3.1 \) channels are a unique group of potassium channels that are voltage-independent.\(^{21}\) Mutations in both \( \text{KCa}_2.3 \) and \( \text{KCa}_3.1 \) channels have been linked with genetic human disorders. We here quantified the effects of these mutations on the apparent \( \text{Ca}^{2+} \) sensitivity of the channels. The Gardos channelopathy-related \( \text{KCa}_3.1 \_\text{S314P}^5, \text{KCa}_3.1 \_\text{A322V}^6 \) and \( \text{KCa}_3.1 \_\text{R352H}^7-9 \) mutations in the HA/HB helices caused hypersensitivity to \( \text{Ca}^{2+} \), which could lead to increased \( \text{K}^+ \) outflow and erythrocyte dehydration \(^5,7,22,23\).

The ZLS- and INCPH-related mutations in the S45A/S45B helices increased the apparent \( \text{Ca}^{2+} \) sensitivity of \( \text{KCa}_2.3 \) channels. Exactly how the \( \text{Ca}^{2+} \) hypersensitivity of \( \text{KCa}_2.3 \) channels leads to ZLS and INCPH is unclear. Increased \( \text{KCa}_2.3 \) channel function in the central nervous system might be related to the defective cognitive function.\(^{24}\) Elevated \( \text{KCa}_2.3 \) channel activity in the vascular endothelium is speculated to be involved in distal digital hypoplasia\(^2\). A previous report\(^2\) demonstrated faster time courses of channel activation upon breakthrough of the cell membrane to the whole-cell configuration with pipette solution containing 1 \( \mu \text{M} \) of \( \text{Ca}^{2+} \), implying higher apparent \( \text{Ca}^{2+} \) sensitivity of the ZLS- and INCPH-related mutant \( \text{KCa}_2.3 \) channels. In this study, we quantitatively determined the \( \text{EC}_{50} \) values for \( \text{Ca}^{2+} \) of these mutant channels, using the inside-out configuration (Table 1). They are indeed hypersensitive to \( \text{Ca}^{2+} \), with the ZLS-related mutants being more sensitive to \( \text{Ca}^{2+} \) than the INCPH-related mutant channel. The reported ZLS\(^2\) and INCPH\(^3\) patients have heterozygous mutations in their \( \text{KCNN3} \) alleles. Their \( \text{KCa}_2.3 \) channels might be comprised of both WT and mutant subunits in the tetrameric channel architecture, which may be speculated to have apparent \( \text{Ca}^{2+} \) sensitivity higher than the WT but lower than the mutant channels reported in this study (Table 1).

The amino acid sequences of \( \text{KCa}_2.3 \) at the S45A and S45B helices shares \( \sim 59\% \) identity with the \( \text{KCa}_3.1 \) channel (highlighted in yellow, Fig. 2A). These two channel subtypes share \( \sim 60\% \) identity at the HA/HB helices (highlighted in green, Fig. 4A). When bound with \( \text{Ca}^{2+} \), CaM forms a stable structural group with the HA/HB helices, and the S45A/S45B helices from a neighboring \( \text{KCa}_3.1 \) channel subunit (Fig. 1A,B).\(^{10,A}\)
homology model of the human KCa2.3 channels showed similar interactions (Fig. 1C,D). Our previous study addressed the crucial role of the interactions between CaM, the HA/HB helices and the S45A/S45B helices for the apparent Ca\(^{2+}\) sensitivity of the channels.\(^{12}\)

In this study, we demonstrated that equivalent mutations in the S45A/S45B helices of KCa2.3 (KCa2.3_S436C, KCa2.3_N441A and KCa2.3_V450L) and KCa3.1 (KCa3.1_S181C, KCa3.1_N186A and KCa3.1_V195L) channels elevated the apparent Ca\(^{2+}\) sensitivity of both channel subtypes (Fig. 2 and Fig. 3). In contrast, mutations in the HA/HB helices of KCa3.1 (KCa3.1_S314P, KCa3.1_A322V and KCa2.3_R352H) channels caused Ca\(^{2+}\) hypersensitivity (Fig. 4), whereas their equivalent mutations in KCa2.3 (KCa2.3_A571P, KCa2.3_T579V and KCa2.3_R612H) did not alter the apparent Ca\(^{2+}\) sensitivity (Fig. 5). Furthermore, a mutation in the HA/HB helices of KCa2.3 (KCa2.3_V555F) channels caused Ca\(^{2+}\) hypersensitivity (Fig. 5), whereas its equivalent mutations in KCa3.1 (KCa3.1_V298F) did not change the apparent Ca\(^{2+}\) sensitivity (Fig. 4). As such, more synchronized changes in the apparent Ca\(^{2+}\) sensitivity of KCa2.3 and KCa3.1 channel subtypes were observed in response to equivalent mutations in the S45A/S45B helices. In contrast, these two channel subtypes often exhibited differential responses to equivalent mutations in the HA/HB helices. Given the involvement of KCa2.3 and KCa3.1 channels in genetic disorders, understanding the regulation of their apparent Ca\(^{2+}\) sensitivity is critical. With cryo-EM structures only available for KCa3.1 channels\(^{10}\), caution will be needed when performing structure-function studies of KCa2.x channel subtypes, especially for the HA/HB helices.

It was reported that the apparent Ca\(^{2+}\) sensitivity of KCa2.2 channels can be negatively regulated by the phosphorylation of CaM.\(^{25}\) Indeed, the apparent Ca\(^{2+}\) sensitivity of all KCa2.x/KCa3.1 channel subtypes can be reduced by the phosphorylation of CaM.\(^{26}\) As such, the EC\(_{50}\) values for Ca\(^{2+}\) determined in HEK293 cells may deviate from the apparent Ca\(^{2+}\) sensitivity of the channels expressed in other cell types (e.g. endothelial cells), as a result of the phosphorylation status.\(^{26}\) KCa3.1 is unique among these four subtypes in that its activation can be enhanced by the phosphorylation of a histidine residue (H358) in the HB helix\(^{27}\), possibly by antagonizing copper-mediated
inhibition of the channel. This unique regulation of KCa3.1 by the phosphorylation of H358 in the HB helix may also echo this notion that KCa3.1 channels are modulated differently by the HA/HB helices from KCa2.x channels.

The pharmacology for KCa3.1 channel subtype has been well developed. Senicapoc and TRAM-34 inhibit KCa3.1 channels with IC50 values of ~11 nM and ~20 nM, respectively, with selectivity (200-1000-fold) towards KCa3.1 channels over other channel subtypes. With excellent pharmacokinetic properties in humans, senicapoc has become a potential candidate for the treatment of Gardos channelopathy, a subset of hereditary xerocytosis. A clinical trial (ClinicalTrials.gov Identifier: NCT04372498) is studying senicapoc in patients with hereditary xerocytosis caused by KCa3.1 channel mutations. On the other hand, no pharmacological treatment has been explored for ZLS and INCPH caused by KCa2.3 channel mutations. AP14145, which is equipotent in modulating KCa2.2 and KCa2.3, with no effect on KCa3.1, has been reported to treat atrial fibrillation in pigs and goats. A structurally related compound, AP30663, is currently in early clinical development for conversion of atrial fibrillation. Our results indicated that AP14145 right-shifted the Ca2+ concentration-response curves (Fig. 6) and reduced the apparent Ca2+ sensitivity (Table 1) of the mutant KCa2.3 channels. The KCa2.3 V450L mutant channel with an EC50 value for Ca2+ of 0.15 ± 0.013 μM (Table 1) is associated with INCPH. This patient with KCa2.3 V450L mutation was not classified as ZLS. Among the ZLS-related mutant channels, KCa2.3 G350D is the least sensitive to Ca2+ with an EC50 value of 0.12 ± 0.013 μM (Table 1). As such, it can be inferred that mutations that elevate apparent Ca2+ sensitivity to < 0.12 μM can be associated with ZLS. AP14145 was able to reduce the apparent Ca2+ sensitivity of the ZLS-related mutant channels to 0.14 ± 0.011 μM (KCa2.3 K269E + AP14145), 0.27 ± 0.025 μM (KCa2.3 G350D + AP14145), 0.19 ± 0.026 μM (KCa2.3 S436C + AP14145) and 0.14 ± 0.015 μM (KCa2.3 V555F + AP14145, Table 1), implying the potential therapeutic usefulness of negative KCa2.x channel gating modulators. The KCNN3 gene encoding KCa2.3 channels has only very recently been associated with ZLS and INCPH. To the best of our knowledge, there are currently no transgenic mouse models for these disorders. Future development of animal models will enable preclinical studies for therapeutic options.
Materials and Methods

Electrophysiology

The effect of mutations on the apparent Ca\textsuperscript{2+} sensitivity of KCa2.3 and KCa3.1 channels was investigated as previously described.\textsuperscript{12,15,26} Briefly, mutations were introduced to human KCa2.3 and human KCa3.1 channels using QuickChange II site-directed mutagenesis kit (Agilent) or through molecular cloning services (Genscript). The WT and mutant channel cDNAs, constructed in the pIRES2-AcGFP1 vector (Clontech) were transfected into HEK293 cells by the calcium–phosphate method. AP14145 was purchased from Tocris Bioscience. KCa2.3 and KCa3.1 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 MΩ. The pipette solution contained (in mM): 140 KCl, 10 Hepes (pH 7.4), 1 MgSO\textsubscript{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\textsuperscript{2+} to obtain the desired free Ca\textsuperscript{2+} concentrations, calculated using the software by Chris Patton of Stanford University (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxc_S.htm). The Ca\textsuperscript{2+} concentrations were verified using a Ca\textsuperscript{2+} calibration buffer kit (Thermo Fisher Scientific). Briefly, a standard curve was generated using the Ca\textsuperscript{2+} buffers from the kit and a fluorescence Ca\textsuperscript{2+} indicator. Then the Ca\textsuperscript{2+} concentrations of our bath solutions were determined through interpolation on the standard curve.

Currents were recorded using an inside-out patch configuration. The inside-out patch configuration allows us to measure the response of the channels to various Ca\textsuperscript{2+} concentrations from the same patch and effectively compare the apparent Ca\textsuperscript{2+} sensitivity of WT and mutant channels. The intracellular face was initially exposed to a zero-Ca\textsuperscript{2+} bath solution, and subsequently to bath solutions with a series of Ca\textsuperscript{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\textsuperscript{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 \(Y = 100/(1 + (X/EC_{50})^\text{Hill})\). The data analysis was performed using pClamp 10.5 (Molecular Devices) in a blinded fashion. Concentration-response curves were analysed in GraphPad Prism 9.0.2 (GraphPad Software Inc.). All data are shown as mean ± 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.

**Computational modeling**

We built homology model for the human KC\(_{a}\)2.3 channel based on a cryo-EM structure of KC\(_{a}\)3.1 channel (PDB Code: 6CNN). Sequence alignment among KC\(_{a}\)2.3 and KC\(_{a}\)3.1 channels was generated by Clustal Omega server (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequence identity between KC\(_{a}\)2.3 and KC\(_{a}\)3.1 is 46.6%, which makes the KC\(_{a}\)3.1 Cryo-EM structure an excellent structural template to generate homology models for the KC\(_{a}\)2.3 channels. We used the MODELLER program \(^{34}\) to generate 10 initial homology models for KC\(_{a}\)2.3 channel based on the KC\(_{a}\)3.1 structural template, and selected the one with the best internal DOPE score from the program. Structural images were created with UCSF Chimera program.
Acknowledgments: We are grateful to Misa Nguyen, Young Hur, and Lucia Basilio for technical assistance. M.Z. was supported by a Scientist Development Grant 13SDG16150007 from American Heart Association, a YI-SCA grant from National Ataxia Foundation and a grant 4R33NS101182-03 from NIH.

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

Conflict of interest: none.
References


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Fig. 1. Channelopathy-causing mutations in the S45A/S45B helices and HA/HB helices of human K\textsubscript{Ca}2.3 and human K\textsubscript{Ca}3.1 channels. (A) The S45A/S45B helices and HA/HB helices of human K\textsubscript{Ca}3.1 channel cryo-EM structure with Ca\textsuperscript{2+} (PDB: 6cnn). Four CaM are shown in grey. Four channel subunits are shown in different colors (green, yellow, cyan, and magenta). (B) Ca\textsuperscript{2+} bound CaM (grey), 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. The Gardos channelopathy-related (S314P, A322V and R352H) mutations are shown as spheres (carbon atoms shown in red and nitrogen atoms shown in blue). (C) The S45A/S45B helices and HA/HB helices of a homology model of the human K\textsubscript{Ca}2.3 channel was generated using the human K\textsubscript{Ca}3.1 channel cryo-EM structure with Ca\textsuperscript{2+}. Four CaM are shown in grey. Four channel subunits are shown in different colors (green, yellow, cyan, and magenta). (D) The ZLS-related (S436C) and INCPH-related (V450L) mutations are shown as spheres (carbon atoms shown in red).
Fig. 2. The ZLS-related (S436C) and INCPH-related (V450L) mutations caused Ca\(^{2+}\) hypersensitivity of KCa2.3 channels. (A) Amino acid sequence alignment of human KCa2.3 [GenBank: NP_002240.3] and human KCa3.1 [GenBank: NP_002241.1] at the S45A and S45B helices (highlighted in yellow). Amino acid residues mutated are shown in red font. (B) Activation by Ca\(^{2+}\) of the WT and mutant KCa2.3 channels carrying mutations in the S45A/S45B helices. (C) EC\(_{50}\) values for activation by Ca\(^{2+}\) of the WT and mutant KCa2.3 channels. *** P < 0.001 compared with WT. No asterisk means no statistical significance compared with WT.
Fig. 3. The mutations equivalent to KCa2.3_S436C and KCa2.3_V450L caused Ca^{2+} hypersensitivity of KCa3.1 channels. (A) Concentration-dependent activation by Ca^{2+} of the WT and mutant KCa3.1 channels carrying mutations in the S45A/S45B helices. (B) EC_{50} values for the activation of the WT and mutant KCa3.1 channels by Ca^{2+}. ** P < 0.01, *** P < 0.001 compared with WT channels. No asterisk means no statistical significance compared with respective WT channels.
Fig. 4. The Gardos channelopathy-related (S314P, A322V and R352H) mutations elevated Ca\textsuperscript{2+} sensitivity of Kc\textsubscript{a}3.1 channels. (A) Amino acid sequence alignment of human Kc\textsubscript{a}2.3 [GenBank: NP_002240.3] and human Kc\textsubscript{a}3.1 [GenBank: NP_002241.1] at the HA and HB helices (highlighted in green). Amino acid residues mutated are shown in red font. (B) Concentration-dependent activation by Ca\textsuperscript{2+} of the WT and mutant Kc\textsubscript{a}3.1 channels carrying mutations in the HA/HB helices. (C) EC\textsubscript{50} values for the activation of the WT and mutant Kc\textsubscript{a}3.1 channels by Ca\textsuperscript{2+.} *** P < 0.001 compared with WT channels. No asterisk means no statistical significance compared with respective WT channels.
Fig. 5. The mutations equivalent to KCa3.1_S314P, KCa3.1_A322V and KCa3.1_R352H did not change apparent Ca²⁺ sensitivity of KCa2.3 channels. (A) Activation by Ca²⁺ of the WT and mutant KCa2.3 channels carrying mutations in the HA/HB helices. (B) EC₅₀ values for activation by Ca²⁺ of the WT and mutant KCa2.3 channels. *** P < 0.001 compared with WT. No asterisk means no statistical significance compared with WT.
Fig. 6. The effects of AP14145 on the Ca^{2+}-dependent activation of WT and mutant KCa2.3 channels. Activation by Ca^{2+} of the KCa2.3_K269E (A), KCa2.3_G350D (B), KCa2.3_S436C (C), KCa2.3_V450L (D), KCa2.3_V555F (E) and KCa2.3_WT (F) channels.
Supplementary Figures

Fig. S1. Representative current traces of concentration-dependent activation by Ca$^{2+}$ of the WT (A), S436C (B), N441A (C) and V450L (D) KCa2.3 channels.
Fig. S2. Representative current traces of concentration-dependent activation by Ca^{2+} of the WT (A), S181C (B), N186A (C) and V195L (D) KCa3.1 channels.
Fig. S3. Representative current traces of concentration-dependent activation by Ca$^{2+}$ of the V298F (A), S314P (B), A332V (C) and R352H (D) KCa3.1 channels.
Fig. S4. Representative current traces of concentration-dependent activation by Ca$^{2+}$ of the V555F (A), A571P (B), T579V(C) and R612H (D) KCa2.3 channels.
Table 1. Apparent Ca$^{2+}$ sensitivity of WT and mutant channels.

<table>
<thead>
<tr>
<th>K$_{Ca}$.2.3</th>
<th>EC$_{50}$ for Ca$^{2+}$ (μM)</th>
<th>Hill coefficient</th>
<th>Statistics</th>
<th>K$_{Ca}$.3.1</th>
<th>EC$_{50}$ for Ca$^{2+}$ (μM)</th>
<th>Hill coefficient</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_{Ca}$.2.3_WT</td>
<td>0.30 ± 0.025</td>
<td>3.88 ± 0.59</td>
<td>NA</td>
<td>K$_{Ca}$.3.1_WT</td>
<td>0.27 ± 0.012</td>
<td>2.87 ± 0.48</td>
<td>N/A</td>
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<td>K$_{Ca}$.2.3_WT + AP14145</td>
<td>1.08 ± 0.10</td>
<td>1.34 ± 0.14</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_K269E</td>
<td>0.086 ± 0.013</td>
<td>3.45 ± 0.46</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_K269E + AP14145</td>
<td>0.14 ± 0.011</td>
<td>3.15 ± 0.72</td>
<td>P &lt; 0.05 vs K$_{Ca}$.2.3_K269E</td>
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<tr>
<td>K$_{Ca}$.2.3_G350D</td>
<td>0.12 ± 0.013</td>
<td>4.19 ± 0.43</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_G350D + AP14145</td>
<td>0.27 ± 0.025</td>
<td>3.86 ± 1.09</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_G350D</td>
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<tr>
<td>K$_{Ca}$.2.3_S436C</td>
<td>0.087 ± 0.0058</td>
<td>4.37 ± 0.85</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_S181C</td>
<td>0.10 ± 0.011</td>
<td>2.78 ± 0.25</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.3.1_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_S436C + AP14145</td>
<td>0.19 ± 0.026</td>
<td>2.28 ± 0.45</td>
<td>P &lt; 0.01 vs K$_{Ca}$.2.3_S436C</td>
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<tr>
<td>K$_{Ca}$.2.3_N441A</td>
<td>0.064 ± 0.0098</td>
<td>4.21 ± 1.09</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_N186A</td>
<td>0.10 ± 0.0055</td>
<td>2.66 ± 0.45</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.3.1_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_V450L</td>
<td>0.15 ± 0.013</td>
<td>4.15 ± 0.58</td>
<td>P &lt; 0.001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_V195L</td>
<td>0.20 ± 0.020</td>
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<td>P &lt; 0.01 vs K$_{Ca}$.3.1_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_V450L + AP14145</td>
<td>0.31 ± 0.061</td>
<td>3.01 ± 0.62</td>
<td>P &lt; 0.05 vs K$_{Ca}$.2.3_V450L</td>
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<tr>
<td>K$_{Ca}$.2.3_V555F</td>
<td>0.067 ± 0.0047</td>
<td>3.77 ± 0.20</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_V298F</td>
<td>0.32 ± 0.032</td>
<td>3.39 ± 0.43</td>
<td>P &gt; 0.05 vs K$_{Ca}$.3.1_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_V555F + AP14145</td>
<td>0.14 ± 0.015</td>
<td>2.81 ± 0.33</td>
<td>P &lt; 0.001 vs K$_{Ca}$.2.3_V555F</td>
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<tr>
<td>K$_{Ca}$.2.3_A571P</td>
<td>0.30 ± 0.027</td>
<td>2.75 ± 0.25</td>
<td>P &gt; 0.9999 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_S314P</td>
<td>0.064 ± 0.0033</td>
<td>4.07 ± 0.41</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.3.1_WT</td>
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<td>K$_{Ca}$.2.3_T579V</td>
<td>0.25 ± 0.032</td>
<td>2.79 ± 0.47</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_A322V</td>
<td>0.059 ± 0.0039</td>
<td>3.94 ± 0.29</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.3.1_WT</td>
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<tr>
<td>K$_{Ca}$.2.3_R612H</td>
<td>0.27 ± 0.014</td>
<td>2.78 ± 0.23</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.2.3_WT</td>
<td>K$_{Ca}$.3.1_R352H</td>
<td>0.085 ± 0.0081</td>
<td>3.66 ± 0.55</td>
<td>P &lt; 0.0001 vs K$_{Ca}$.3.1_WT</td>
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</table>