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COMMENTARY

Heteromeric wild-type/mutant potassium channel subunit composition as a major determinant of channelopathy phenotype in heterozygous patients

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Channelopathies, diseases associated with changes in ion channel function due to genetic or acquired defects, have long been at the center of drug discovery efforts. It seems the more we learn, the more complex the picture of potential pathways of ion channel malfunction becomes. However, only through in-depth studies can we fully comprehend the state of disrepair and develop rational therapies to help patients. A recent study by Geng et al. (2023) in the *Journal of General Physiology* provides novel insight into the impact of channel assembly from a mixture of wild-type (WT) and neurological disorder–associated mutation-carrying subunits on the properties of BK potassium channels as they are expected to appear in heterozygous patients.

BK potassium channels, also known as Slo1 and KCa1.1, are synergistically activated by Ca^{2+} and membrane depolarization (Barrett et al., 1982; Latorre et al., 1982). Therefore, opening of BK channels provides a negative feedback loop that ensures membrane repolarization in response to increased intracellular Ca^{2+} levels and membrane depolarization (Lancaster and Nicoll, 1987; Storm, 1987). In addition to the dual activation mechanism, BK (for big K^+) channels have the largest single-channel conductance of all potassium selective channels (Pallotta et al., 1981; Marty, 1981). The large conductance leads to a rapid outward potassium current that hyperpolarizes the cells once BK channels open. Due to their unique characteristics and abundant expression in excitable cells, BK channels are key players in numerous physiological processes and, not surprisingly, genetic mutations in BK channels are linked to severe channelopathies (Latorre et al., 2017; Sancho and Kyle, 2021; Miller et al., 2021). G375R mutation is one of the recently identified de novo mutations in BK channels associated with a severe developmental delay and facial dysmorphism (Liang et al., 2019). Initially, this mutation was classified as a complete loss-of-function (LOF) mutation (Liang et al., 2019). However, the study by Geng et al. (2023) shows that the effect of the mutation is far more nuanced with a strong gain-of-function (GOF) effect, accompanied by a smaller decrease-of-function (DOF) component.

Similar to other potassium channels, BK channels are assembled from four subunits, arranged around a centrally located pore (Fig. 1). Each subunit contains seven transmembrane segments (S0–S6) and two intracellular C-terminal regulators of conductance of K^+ (RCK1 and RCK2) domains that contain Ca^{2+} binding sites (Schreiber and Salkoff, 1997; Xia et al., 2002; Zeng et al., 2005; Tao and MacKinnon, 2019). The segments S0–S4 form the voltage-sensors (VS) of the channel, while the segments S5–S6 from all subunits form the pore domain (PD; Tao and MacKinnon, 2019; Koval et al., 2007). The tetrameric assembly of potassium channels leads to the potential homomeric as well as heteromeric channel assemblies that can fine tune channel properties. Unlike potassium channels, voltage-dependent sodium and calcium channels are each formed from a single pore-forming subunit, each containing four homologous repeats (Zheng and Trudeau, 2015). Therefore, added complexity arising from heteromeric subunit assembly is unique to potassium channels.

Dual effect of the G375R mutation on BK channel gating

Gly^{375} is positioned in the S6 pore-lining helix with the side chain facing away from the pore lumen (Fig. 1). The glycine introduces a noticeable bend in S6 and has been proposed to function as a "gating hinge" (Tao and MacKinnon, 2019). In addition, Gly^{375} is located in the "deep pore" region that has been proposed to undergo structural rearrangements during the voltage-dependent channel gating (Chen et al., 2014). Therefore, substitution of Gly^{375} with a larger and positively charged arginine would be expected to affect channel gating. Indeed, Geng et al. (2023) found that co-expression of WT and G375R mutant subunits at 1:1 ratio in *Xenopus laevis* oocytes shifted the half-maximal activation voltage ($V_{1/2}$) of BK channels in the hyperpolarized direction by a staggering 120 mV, based on analysis of

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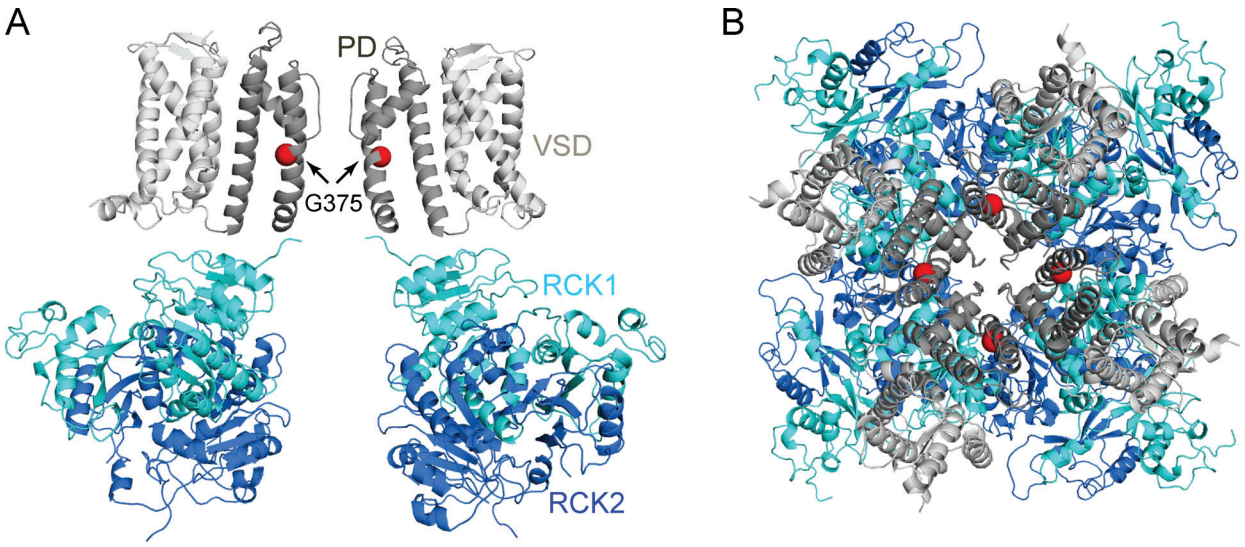

Figure 1. **Location of the G375 residue in the context of the BK channel structure. (A and B)** Ribbon representation of the full-length structure of a human BK channel (PDB accession no. 6V3G) viewed from the side (A) and down the pore (B) from the extracellular side of the channel. In A, only transmembrane domains from two opposing subunits are shown for clarity, while the cytosolic domains reflect the RCK-containing C-terminal domains from the other two opposing subunits. In B, all four subunits are shown to highlight the tetrameric symmetry in the channel. The transmembrane segments forming VSD and PD are shown in light and dark grey, respectively. RCK1 and RCK2 domains are shown in light and dark blue, respectively. The glycine residue at the position 375 in human BK channels (corresponding to G310 in the BK channel structure determined for a transcript that was missing the first 65 amino acids, and G310 in the frequently used mouse BK channels) is shown in red spheres for each of the subunits.

the macroscopic currents recorded from excised inside-out membrane patches. A similar effect was observed for whole-cell currents. This profound GOF would cause opening of mutant BK channels at much more negative potentials than WT channels, underpinning the molecular mechanism of the severe phenotype observed in patients with a de novo G375R mutation.

Since the macroscopic current analysis indicated that the G375R mutation causes a large left-shift in $V_{1/2}$, Geng et al. (2023) used single-channel current analysis to further investigate the functional consequences of the G375R mutation on the single channel open probability (Po) and conductance. Consistent with the results from the macroscopic current analysis, single channel current analysis of homomeric WT and mutant channels indicated that the G375R mutation left-shifted the voltage dependence of Po with an observed shift in $V_{1/2}$ of >180 mV.

In addition to the profound shift in voltage dependence of Po, the homomeric G375R channels exhibited an ∼40% decrease in the single-channel conductance compared to WT channels. As proposed by Geng et al. (2023), this DOF could be caused by the decrease in the pore vestibule volume due to the substitution of Gly^{375} with a larger arginine and/or by an electrostatic repulsion of the permeant K^+ ions introduced by the substitution. As the macroscopic current analysis indicates, for the averaged cellular response this respectable DOF is thwarted by the GOF effect of the G375R mutation. Importantly, in native cells BK channels are also regulated by a variety of additional factors, including alternative splicing, regulatory β and γ subunits, intracellular Mg^{2+}, and cholesterol (Cui, 2021; Gonzalez-Perez et al., 2014; Shipston and Tian, 2016; Bukiya and Dopico, 2019). Additional studies are necessary to investigate how the GOF and DOF effects of the G375R mutation integrate with the common pathways of BK channel regulation.

Mechanism of Po regulation by Gly^{375}

While it is clear that Gly^{375} is an important residue for BK channel gating, the mechanism of the Po regulation by Gly^{375} is not clear. Is it the structural flexibility introduced by the glycine residue, and/or size and charge at the 375 position that regulates Po? It has been previously reported that G375D substitution shifted the $V_{1/2}$ of BK channel activation to the hyperpolarized potentials by 55 mV, based on the analysis of macroscopic currents recorded in the inside-out patch-clamp configuration (Chen et al., 2014). This shift is less than the one caused by the G375R mutation; however, overall it is in the same direction despite the opposite charges of the substituted residues. Still, it is premature to rule out the possible contribution of a charge at the 375 position without the single-channel current analysis as it is possible the G375D mutation causes opposite effects on Po and single-channel conductance than G375R and this response is masked in the macroscopic currents. Alternatively, the charge substitutions could affect BK channel gating by changing the rotamer preferences of ionized side chains located in the vicinity of the 375 position, as it was shown for the effect of residue substitutions on the ion selectivity of pentameric ligand-gated ion channels (Cymes and Grosman, 2016).

The G375R mutation left-shifted the $V_{1/2}$ of BK channel activation in the absence and also presence of intracellular Ca^{2+}, although, the shift was seemingly larger in the presence of Ca^{2+}, based on the macroscopic currents recorded from excised inside-out membrane patches. Interestingly, for the G375D mutation the left-shift in $V_{1/2}$ was smaller in the presence of Ca^{2+}

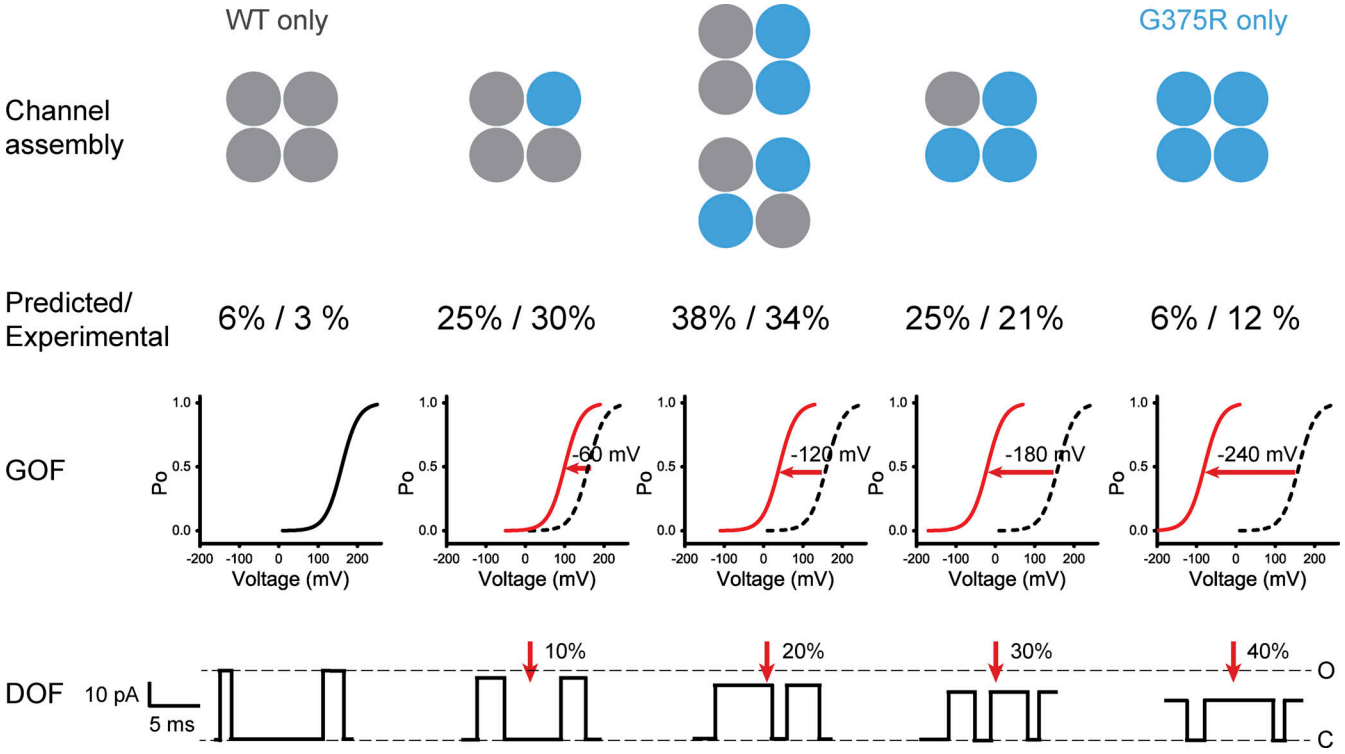

Figure 2. **Linear dependence of the GOF and DOF effects of the G375 mutation on the number of mutant subunits in the BK channel tetramer.** In the top row, cartoons depicting the six potential subunit arrangements for BK channel tetramers from WT (grey) and mutant (blue) subunits, with the corresponding percentage populations of the assembled channels based on the theoretically predicted and experimentally suggested values given below. The next two rows depict, respectively, the expected voltage-dependence of Po and the idealized single-channel currents recorded at +100 mV for each of the six assembled channels. The Po-versus-voltage plots for homomeric WT channels are black and mutant channels of a given stoichiometry are in red. The plots in dashed black lines correspond to the Po-versus-voltage plots for homomeric WT channels shown to illustrate the shift in $V_{1/2}$ due to the incorporation of mutant subunits. The horizontal red arrows illustrate the shift of approximately −60 mV in $V_{1/2}$ each time a mutant subunit is exchanged for a WT subunit and vertical red arrows illustrate the decrease of ∼10% in the single-channel conductance each time a WT subunit is exchanged for a mutant subunit.

than in the absence (Chen et al., 2014). Single-channel based studies involving substitutions of different size and charge residues at the position 375, investigated over the range of Ca^{2+} concentrations, are needed to investigate if and how Ca^{2+}-dependent gating and effects of Gly^{375} are allosterically coupled.

Heteromeric rather than homomeric channel assembly likely dominates in heterozygous patients

Many diseases are manifested in heterozygous patients that carry both a WT and a mutant allele. For channelopathies associated with potassium channels this would lead to the assembly of heteromeric as well as homomeric channels from WT and mutation-carrying subunits. Geng et al. (2023) used the binomial equation to predict BK channel subunit assembly from WT and mutant subunits, assuming a random subunit assembly and an even production of WT and mutant subunits. They point out that the most populous channel assembly would be a heteromer with two mutant and two WT subunits, accounting for 38% of the assembled channels, followed by a heteromer with either three mutant and one WT subunits, or vice-versa, each accounting for 25% of the assembled channels (Fig. 2). Homomeric WT and mutant channels would then each account for a meager 6% of the assembled channels. To test this random assembly scenario, Geng et al. (2023) mimicked BK channel assembly in heterozygous patients by injecting equal amounts of cRNA encoding the WT and G375R mutant subunits into *Xenopus* oocytes. The analysis of histograms of the $V_{1/2}$ values for single-channel recordings from 33 assembled channels indicated that a heteromer with 2 mutant and 2 WT subunits was indeed the most populous channel, accounting for 34% of the assembled channels, followed by a heteromer with 3 WT and 1 mutant subunits, and a heteromer with 1 WT and 3 mutant subunits, accounting for 30 and 21% of the assembled channels, respectively (Fig. 2). Homomeric WT and mutant channels accounted for only 3 and 12% of the assembled channels, respectively. Overall the experimentally suggested channel stoichiometry distribution was consistent with the theoretical predictions. Simulations of 1 million groups of 33 assembled channels suggested that the differences between the experimentally suggested and theoretically predicted populations are most likely insignificant, although, some tendency towards preferential assembly and/or uneven subunit expression can't be excluded. Nevertheless, the main experimental finding is loud and clear: in heterozygous patients with equal expression of WT and mutant subunits, 85% of the assembled channels are expected to be heteromers.

The functional changes due to the GOF and DOF effects of the G375R mutation showed a linear dependence on the number of

mutant subunits present in a tetramer. Substitution of each WT subunit with a mutant one resulted in a ∼60 mV leftward shift in $V_{1/2}$ and ∼10% decrease in the single-channel conductance (Fig. 2). Such a linear dependence suggests that there is little subunit cooperativity in the GOF and DOF effects. Interestingly, the GOF and DOF effects for channels with two WT and mutant subunits assembled in adjacent and diagonal arrangement were not clearly distinct. It is possible that analysis of a larger number of channels could reveal some differences for the two arrangements.

Physiological implications of the G375R mutation

The experimental results suggest that in heterozygous individuals over 95% of the assembled channels will have at least one mutant subunit incorporated and, therefore, will display an assembly-mediated dominant GOF phenotype, even though only 50% of all expressed channel subunits carry the mutation. Moreover, due to the huge leftward shift introduced by the G375R mutation and population advantage, the heteromeric and homomeric mutant channels would completely functionally dominate homomeric WT channels under physiological conditions. For instance, assuming a normal neuronal resting potential of approximately −70 mV, the first to activate would be the homomeric mutant channels with the averaged $V_{1/2}$ of approximately −80 mV and some heteromeric channels assembled from one WT and three mutant subunits with the averaged $V_{1/2}$ of approximately −20 mV. Activation of the channels with these two assemblies would hyperpolarize the membrane potential below the normal resting potential and up to the K^+ reversal potential, making it harder to activate BK channels with other subunit assemblies. Essentially, this would make homomeric WT channels and even channels with only one G375R subunit functionally irrelevant. Not only at rest but also during a strong excitation, e.g., large amplitude postsynaptic potentials, activation of BK channels assembled from predominantly mutant subunits at the onset of depolarization would prevent membrane potential from reaching values necessary for the activation of channels assembled from predominantly WT subunits.

Because of the activation at resting membrane potential, the homomeric mutant channels would have substantial impact on cellular excitability, despite accounting for only ∼10% of the assembled BK channels. The hyperpolarization caused by the opening of homomeric mutant channels could prevent action potential initiation. The physiological effect of the homomeric channels in native cells would also have to be assessed within the context of the regulatory subunits, intracellular Ca^{2+} levels and other BK channel regulatory pathways, that could shape the physiological impact of G375R mutation in a cell type–specific manner.

General implications for potassium channel channelopathies

The study by Geng et al. (2023) drives home the important message that in heterozygous patients, assuming equal expression of both WT and mutant subunits, over 95% of disease-associated potassium channels in the membrane will contain at least one mutant subunit. This suggests a potential therapeutic strategy: a compound that inhibits (for GOF mutations) or activates (for DOF mutations) mutant subunit-containing channels with higher affinity than WT channels would increase the relative contribution of channels composed exclusively of WT subunits, potentially rescuing the WT phenotype. The benefit of such a strategy would have to be weighed against whether enough predominantly WT channels might be available to sustain normal physiological function.

Taken together, the work of Geng et al. (2023) highlights that the subunit composition and phenotype of a mutation have major implications for preclinical studies of potassium channel channelopathies and must be investigated in detail to better assess potential physiological impact of the genetic mutation.

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