The unique turret region of Kv3 channels governs the mechanism of action of highly specific positive allosteric modulators.

Manuel Covarrubias (✉ Manuel.Covarrubias@jefferson.edu )
Thomas Jefferson University

Qiansheng Liang
Thomas Jefferson University

Lianteng Zhi
Thomas Jefferson University

Leonardo Cirqueira
University of Brasilia

Nadia Pilati
Auitofny Srl

Agostino Marasco
Auitofny Srl

Martin Gunthorpe
Auitifony Therapeutics Ltd  https://orcid.org/0000-0002-0216-1735

Giuseppe Alvaro
Auitifony Srl

Charles Large
Auitifony Thereapeutics, Ltd

Werner Treptow
Universidade de Brasilia

Article

Keywords:

Posted Date: April 11th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2798797/v1

License: ☑️  This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Small-molecule modulators of diverse voltage-gated K+ (Kv) channels may help treat severe neurological disorders. However, the development of selective modulators requires an understanding of their mechanism-of-action (MoA). We applied an orthogonal approach to elucidate the MoA of an imidazolidinedione derivative (AUT5), which is a highly specific positive allosteric modulator (PAM) of Kv3.1 and Kv3.2 channels. AUT5 modulation involves positive cooperativity and preferential stabilization of the open state. Critically, we found that the unique and highly conserved extracellular turret region of Kv3.1 and Kv3.2 essentially governs AUT5 modulation. Furthermore, leveraging on the cryo-EM structure of Kv3.1a, atomistic blind docking calculations revealed four equivalent AUT5 binding sites near the turrets and between the voltage-sensing and pore domains of the channel's tetrameric assembly. Therefore, the unique Kv3 turret emerges as a novel structural correlate of the selective MoA of a new class of Kv3 channel PAMs with a therapeutic potential.

Introduction

Voltage-gated K (Kv) channels play diverse critical roles as regulators of active electrical signaling in excitable tissues, such as the brain, heart, and muscle. For instance, Kv channels shape the repolarization of action potentials, determine the latency to the first spike in a train of action potentials and the frequency of repetitive spiking \(^{1,2}\). The molecular basis of this functional diversity resides in 40 Kv channel genes classified in 12 sub-families and many additional genes encoding ancillary beta subunits, which are differentially expressed in relevant tissues and parts of the brain and different subcellular compartments \(^{3–6}\). This diversity has stimulated a special interest in developing small-molecules and peptides that could selectively modulate aspects of function in excitable tissues and, therefore, could have potential as novel medicinal agents \(^{7–12}\). The search for Kv channel openers or positive allosteric modulators (PAMs) has gained most of the attention because they could help treat common hyperexcitability disorders (epilepsy, neuropathic pain, tinnitus, cardiac arrhythmias, etc.) and develop new anesthetics. In contrast, negative modulation of Kv channels may help treat conduction disorders, which are characterized by hypoexcitability. Presently, there is only a limited number of potentially useful Kv channel PAMs. For example, retigabine, one of the best characterized Kv channel PAMs, that targets Kv7.2/Kv7.3 complexes was developed as an anticonvulsant \(^{8,13–16}\). Here, we elucidated the structural basis of the positive modulation of Kv3 channels by AUT5, an imidazolidinedinedione derivative from a novel class of PAMs \(^{17}\).

Kv3 channels are high-voltage activated Kv channels that belong to the superfamily of Shaker-related Kv channels \(^3\). As such, Kv3 channels are domain-swapped tetrameric assemblies, in which each subunit is characterized by three conserved regions, the regulatory cytoplasmic N-terminal T1 domain, and two membrane-spanning regions, including the voltage sensing domain (VSD) and the pore domain (PD) \(^{18}\). The VSD is composed of four segments (S1-S4), and the PD includes the selective filter flanked by segments S5 and S6 \(^{18}\). Whereas the VSD is mainly responsible for sensing the transmembrane voltage
and gating, the PD determines K⁺ selectivity, permeation, and gating. All members of the Kv3 sub-family (Kv3.1, Kv3.2, Kv3.3 and Kv3.4) are mainly expressed in axons and nerve terminals of neurons in the neocortex, the hippocampus, the basal ganglia, the thalamus, the cerebellum, and the brain stem 19,20. Therein, the fast spiking phenotype of neurons depends on the expression and specialized biophysical properties of Kv3 channels 19. Also, Kv3 channels are major determinants of action potential repolarization in neurons with diverse electrophysiological phenotypes, including the neuromuscular junction 19,21–27. A growing number of recently discovered pathogenic Kv3 gene variants have been linked to idiopathic developmental epileptic encephalopathies (DEE), progressive myoclonus epilepsy (PME-7), intellectual disability and ataxia have been recently discovered, which is driving a keen search for effective therapeutic interventions based on small molecules targeting these channels 28.

We and others have previously characterized the biophysical and pharmacological properties of the novel imidazolidinedione derivatives and have found that they are relatively selective PAMs of Kv3 channels 29–32. Positive modulation of Kv3 channels has a significant impact on the neuron's ability to generate fast spiking, with potential to be beneficial in disorders of the auditory system, and disorders associated with cognitive deficits due to dysfunction of corticolimbic circuits where high frequency gamma power and network synchronization is impaired 30,33–37. Recent studies suggest potentially beneficial effects of these compounds in progressive myoclonus epilepsy and psychotic disorders 38,39. Therefore, a greater understanding of the MoA of these novel compounds may help to optimize their use across different disorders. Here, we used an orthogonal approach to determine the biophysical and structural basis of the selective positive modulation of Kv3.1 and Kv3.2 by AUT5. The main results show that AUT5 with an EC₅₀ of 3.2 µM induces cooperative positive modulation by preferentially stabilizing the open state and that the structural determinants of this action are in the Kv3-specific sequence that forms the external post-S5 linker also known as the turret region that is clearly resolved in the new cryo-EM structure of Kv3.1a 40. With further support from atomistic blind docking calculations, we conclude that the unique turret of Kv3.1 and Kv3.2 determines nearby binding of AUT5 at an inter-subunit site between the VSD and the PD and the transduction mechanism that underlies positive modulation. The insights gained here may enable further development of Kv3 modulators to treat a range of neurological and psychiatric disorders more effectively.

Results

Highly selective positive modulation of Kv3.1 and Kv3.2 by AUT5.

AUT5 is a potent PAM of Kv3 channels. However, the selectivity of this modulation among diverse Kv channels has not been established. To address this, we tested several Kv channels representing phylogenetically related subfamilies upon heterologous expression in Xenopus oocytes and characterization of the expressed currents using TEVC before and after bath application of 2 µM AUT5 (Materials and Methods; Fig. 1). Whereas the peak conductance – voltage (Gᵥ-Vᵥ) relations of Kv1.2, Kv2.1, K-Shaw2, Kv3.4 and Kv4.2 were not significantly affected, those of Kv3.1 and Kv3.2 exhibited
significant changes (Fig. 1, Supplementary Materials, Fig. S1). The G_p-V_c relation of Kv3.1 was leftward shifted (ΔV_{0.5} = -11.2 ± 1.0 mV, n = 13, P = 7x10^{-8}), the equivalent gating charge was slightly reduced (Δz = -0.15 ± 0.05 e_0, P = 0.011) and the G_{max} was modestly increased (ΔG_{max} = 8.7 ± 1.9%, P = 0.003). The G_p-V_c relation of Kv3.2 was also leftward shifted (ΔV_{0.5} = -26.5 ± 0.9 mV, n = 74, P = 3x10^{-42}), and the equivalent gating charge was also modestly reduced (Δz = -0.84 ± 0.06 e_0, P = 3x10^{-22}). The G_{max}, however, was also modestly reduced, albeit the change was quite variable (ΔG_{max} = -6.0 ± 1.1%, P = 6x10^{-6}). Generally, these effects were reversible upon washout of the compound (Supplementary Materials, Fig. S2). Observing qualitatively similar effects of AUT5 on Kv3.1 and Kv3.2, and the lack of a significant effect on Kv3.4 (a close mammalian homolog) and K-Shaw2 (a Drosophila melanogaster, Kv3 ortholog) was especially surprising and potentially significant from the mechanistic and physiological perspectives. In agreement with differential modulation of Kv3 channels by AUT5, when Kv3.1 and Kv3.4 were co-expressed to promote the assembly of Kv3.1/Kv3.4 heteromultimers that may natively exist in certain neurons \(^1\), we found that the presence of Kv3.4 subunits dampened positive modulation by AUT5 (Supplementary Materials, Fig. S3).

**Preferential stabilization of the open state and positive cooperativity underlie the positive modulation of Kv3.2 by AUT5.**

Since Kv3.2 exhibits the largest AUT5-induced hyperpolarizing shift of the G_p-V_c relation, we pursued in-depth biophysical and structural characterizations of this modulation to elucidate the MoA. To determine the biophysical basis of the hyperpolarizing shift, we first characterized the voltage dependence of the gating kinetics before and after bath application of 2 µM AUT5. Deactivation and activation kinetics were quantified by measuring the time constants of macroscopic tail current relaxations over a range of repolarizing voltages and the activation trajectories of depolarization-evoked macroscopic currents, respectively (Materials and Methods). Inspection of the currents suggested a substantial slowing of the tail currents at hyperpolarized voltages (Fig. 2A and 2B). A plot of the voltage dependence of the time constants of deactivation and activation yielded the expected bell-shaped curve with a left arm corresponding to the voltage dependence of deactivation and a right arm corresponding to the voltage dependence of activation, and a maximum that approximately aligns with the G_p-V_c V_{0.5} (Materials and Methods; Fig. 2E). Consistent with the analysis of G_p-V_c relations, the time constant vs. voltage curve exhibits a substantial AUT5-induced hyperpolarizing shift that is mainly caused by preferentially increasing the time constants of deactivation (i.e., slower tail current relaxations) (Fig. 2E). For instance, the deactivation time constants at -70 mV were 1.3 ± 1.0 ms and 11.8 ± 4.0 ms in the absence and presence of 2 µM AUT5, respectively (n = 9, P = 0.03). By contrast, the time constants of current activation at voltages >10 mV are unaffected by AUT5 (Fig. 2E). Therefore, the AUT5-induced hyperpolarizing shift of the G_p-V_c relation results from slowed deactivation, which indicates preferential stabilization of the Kv3.2 open conformation. Then, to quantify the concentration dependence of the positive modulation of Kv3.2 by AUT5, we created an aggregate plot of the AUT5-induced ΔV_{0.5} of the G_p-V_c relation vs. [AUT5] and characterized it empirically by assuming a logistic equation (Materials and Methods) (Fig. 2F). The
best fit of the logistic equation yielded \( EC_{50} = 3.2 \, \mu M \) and \( n_H = 1.9 \). This result additionally suggests that positive cooperativity involving multiple interacting binding sites underlies the preferential stabilization of the open state by AUT5.

The unique turret region of Kv3 channels determines the positive modulation by AUT5.

We hypothesized that the structural correlates of the highly specific positive modulation of Kv3.1 and Kv3.2 by AUT5 may concern discrete and specific structural differences. Thus, we compared the sequences of Kv3 that exhibit robust positive modulation by AUT5 (Kv3.1 and Kv3.2) and Kv3 channels that lack this property (Kv3.4 and K-Shaw2) and found potentially significant differences in the cytoplasmic T1 domain, the extracellular S1-S2 linker and the extracellular S5-S6 linker (upstream of the pore helix and the selectivity filter), also known as the ‘turret’ region (Supplementary Materials, Fig. S4). Thus, to test potential contributions of these regions, we created the following constructs: deletion of the T1 domain (ΔT1-Kv3.2), exchange of the S1-S2 linker of Kv3.4 for that of Kv3.2 (3.4x3.2 S1S2), and deletion of the turret (ΔTurret-Kv3.2) (Materials and Methods; Table S1). Under basal conditions, ΔT1-Kv3.2, 3.4x3.2 S1S2 and ΔTurret-Kv3.2 caused modest rightward shifts of the \( G_p - V_c \) relation (<15 mV; Supplementary Materials, Figs. S5 and S6). However, whereas ΔT1-Kv3.2, retained intact positive modulation by 2 μM AUT5, the 3.4x3.2 S1S2 chimera exhibited modestly decreased modulation (32% less than wild type Kv3.2) and the ΔTurret-Kv3.2 exhibited no modulation (Figs. 3; Supplementary Materials, Fig. S5 and S6). This suggests that the S1-S2 linker may play a limited role as a determinant of the positive modulation by AUT5; however, the presence of the turret is most critical. This is a remarkable result because Kv3.1 and Kv3.2 have nearly identical turret regions (Fig. 4 and Fig. S4), and Kv channels that lack a homologous turret sequence, such as Kv1.2, Kv2.1, Kv4.2 and the Drosophila Kv3 ortholog K-Shaw2 (Fig. 4; Supplementary Materials, Fig. S4), exhibit no modulation by 2 μM AUT5 (Fig. 1; Supplementary Materials, Fig. S1). Also, compared to Kv3.1 and Kv3.2, Kv3.4 has a turret with eight potentially significant differences (Fig. 4). To determine whether the presence of a specific turret sequence can explain the differential modulation of Kv3.2 and Kv3.4 by AUT5, we created a Kv3.2 chimera in which we exchanged the turret of Kv3.4 for that of Kv3.2 (Materials and Methods; 3.4x3.2/Turret; Table S1) and characterized the \( G_p - V_c \) relation of this chimera before and after bath application of 2 μM AUT5. Supporting a contribution of the turret to gating under basal conditions, the 3.4x3.2/Turret exchange caused a 11.8 mV depolarizing shift of the \( V_{0.5} \) (\( P < 0.001 \); Supplementary Materials, Fig. S6). More significantly, the 3.4x3.2/Turret exchange nearly eliminated the modulation by 2 μM AUT5, closely recapitulating the effect of the Kv3.2 turret deletion (Fig. 3), which strongly supports the role of the Kv3.2 turret as a critical determinant of the positive modulation by AUT5.

To conclusively demonstrate that the turret is necessary and sufficient to determine the positive modulation by AUT5, we must also show that conversely the presence of the Kv3.2 turret in Kv3.4 is sufficient to confer a significant AUT5-induced hyperpolarizing shift of the \( G_p - V_c \) relation. Thus, we created a Kv3.4 chimera in which we exchanged the Kv3.2 turret for that of Kv3.4 (Materials and Methods; 3.2x3.4/Turret; Table S1) and, as above, characterized the \( G_p - V_c \) relation before and after bath
application of 2 µM AUT5 (Fig. 5). The \( G_p - V_c \) relation of the 3.2x3.4/Turret chimera under basal conditions was leftward shifted relative to that of the wild-type Kv3.4 (7.8 mV; Supplementary Materials; Fig. S7), consistent again with a contribution of the turret to voltage dependent gating. Further supporting this contribution, turret deletion in Kv3.4 produced a similar leftward shift (11 mV; Supplementary Materials; Fig. S7). Upon bath application of 2 µM AUT5 to oocytes expressing the 3.2x3.4/Turret chimera, the conductance starts to increase slightly at more negative voltages relative to the control indicating a hint of positive modulation (Fig. 5). Also, the \( G_p - V_c \) relation in the presence of AUT5 crosses the control \( G_p - V_c \) relation and displays a modest decrease of the \( G_{\text{max}} \) (Fig. 5). Considering these effects, we hypothesized that the presence of Kv3.4 fast open-state inactivation truncated the conductance at the most depolarized voltages, which partially obscured the expected AUT5-induced hyperpolarizing shift of the \( G_p - V_c \) relation.

Considering that Kv3.4 undergoes fast inactivation and is relatively insensitive to AUT5 modulation, and that Kv3.1 and Kv3.2 undergo little to no inactivation and exhibit robust positive modulation by AUT5, we conducted experiments to determine how fast Kv3.4 inactivation may have obscured the positive modulation by AUT5. Accordingly, we leveraged previous studies that demonstrated quick elimination of Kv3.4 fast inactivation by protein kinase C (PKC) – dependent phosphorylation of the channel’s N-terminal inactivation domain \(^{41}\). Replicating those results, bath application of a phorbol ester that activates PKC (50 nM phorbol 12-myristate 13-acetate, PMA) to oocytes expressing the fast-inactivating Kv3.4 current induced time-dependent elimination of fast inactivation over a period of \( \sim 10 \) min (Supplementary Materials, Fig. S8). This modulation, however, did not significantly change the low sensitivity of the wild type Kv3.4 to modulation by 2 µM AUT5, demonstrating that fast inactivation per se is not masking positive modulation of the wild type Kv3.4 by AUT5 (Fig. 5, Supplementary Materials, Fig. S8). Based on the results from the 3.4x3.2/Turret chimera (Fig. 3), it is more likely that the turret is the culprit, and that fast inactivation partially obscured the expected positive modulation of the 3.2x3.4/Turret chimera by AUT5. To test this hypothesis, we exposed oocytes expressing the 3.2x3.4/Turret chimera to 50 nM PMA and, once fast inactivation was eliminated, we characterized the \( G_p - V_c \) relations before and after bath application of 2 µM AUT5, while keeping PMA in the bath (Fig. 5). Consistent with the hypothesis, once fast inactivation was eliminated, the 3.2x3.4/Turret chimera displayed a significant AUT5-induced hyperpolarizing shift of the \( G_p - V_c \) relation (\( \Delta V_{0.5} = -15.7 \pm 1.0 \) mV; Fig. 5), which phenocopies \( \sim 60\% \) of the positive modulation observed with wild-type Kv3.2 (\( \Delta V_{0.5} = -26.5 \pm 0.9 \) mV; Figs. 1 and 5).

The highly specific positive modulation of Kv3 channels by AUT5 depends on discrete differences within the turret region.

There are eight differences (numbered 1–8) between the putative turret sequences of Kv3.2 and Kv3.4 (Fig. 4). According to the cryo-EM structure of human Kv3.1a \(^{40}\), however, four differences are located within the segment of the extracellular linker that shapes the turret, including N3, S4, A5 and S6 in Kv3.1 and Kv3.2, and the corresponding S3, R4, G5 and N6 of Kv3.4 (Fig. 4). The SAS triad is especially
interesting because it faces the VSD (especially the S1-S2 linker) at the inter-subunit interface in the cryo-EM structure of Kv3.1. Therefore, to determine whether specific discrete differences within the turret are responsible for the differential sensitivities of these Kv3 channels to modulation by AUT5, we exchanged individual Kv3.4 residue differences for those at the equivalent positions in Kv3.2 (N3S, S4R, A5G and S6N) and characterized the $G_p-V_c$ relations before and after exposing the oocytes expressing these mutants to 2 µM AUT5. Consistent with the results from the Kv3.2 turret deletion and turret chimera under basal conditions, single Kv3.2 turret mutations caused slight depolarizing shifts of the $G_p-V_c$ relation relative to the voltage dependence of wild type Kv3.2 (Supplemental Materials, Fig. S6). More significantly, however, these mutations had significant effects on how AUT5 modulated voltage-dependent gating (Fig. 6A; Supplementary Materials, Fig. S9). Compared to wild type Kv3.2, both N3S and A5G inhibited the AUT5-induced hyperpolarizing shift, albeit the neutralizing effect of the latter was slightly greater ($\Delta V_{0.5} = -14.2 \pm 0.6$ mV and $-11.3 \pm 1.0$ mV, respectively) (Fig. 6). By contrast, S4R increased the $\Delta V_{0.5}$ (-42.2 ± 2.7 mV) and S6N had no effect (-25.9 ± 1.2 mV) (Fig. 6). Since no individual mutation completely neutralized the positive modulation by AUT5 and the effects were qualitatively diverse (decrease, increase and no effect), we explored the effects of combined mutations. Thus, we created three new Kv3.2 mutant constructs that combined N3S with substitutions at each position of the SAS triad (N3S/S4R, N3S/A5G, N3S/S6N), and a triple mutation that exchanged the RGN triad of Kv3.4 for the SAS triad of Kv3.2 (S4R/A5G/S6N). These mutations under basal conditions also caused slight-modest depolarizing shifts of the $G_p-V_c$ relations relative to the voltage dependence of wild type Kv3.2 (Supplementary Materials, Fig. S6). In response to AUT5 application, double mutations similarly neutralized the AUT5-induced hyperpolarizing shift, albeit the inhibition of PAM was partial (Fig. 6A; Supplementary Materials, Fig. S9). The triple triad mutation, in contrast, was more effective at neutralizing the AUT5-induced hyperpolarizing shift (Fig. 6A; Supplementary Materials, Fig. S9), an effect that nearly matched the complete neutralization produced by a complete exchange of the Kv3.4 turret for the Kv3.2 turret (Fig. 3). These results demonstrate that discrete Kv3.2 turret substitutions that exchange Kv3.4 residues for Kv3.2 residues (single, double, and triple) are sufficient to neutralize the positive modulation by AUT5, albeit these effects are generally partial and do not suggest simple additivity.

If discrete Kv3.2 turret substitutions can neutralize the positive modulation by AUT5 and are specific critical determinants of this modulation, discrete reciprocal substitutions in Kv3.4 should be able to confer positive modulation by AUT5. To test this hypothesis, we created the following Kv3.4 turret mutations (numbered as explained above): S3N, R4S, G5A, N6S and S3N/N6S (Fig. 4). We then assessed their function and modulation by AUT5 following exposure to PMA as described above (Fig. 6). In the presence of PMA, these mutants displayed voltage-dependence that was slightly hyperpolarized compared to the voltage dependence of wild type Kv3.4 (Supplementary Materials; Fig. S7). Supporting the important role of specific Kv3.2 turret residues, all mutations conferred similar AUT5-induced positive
modulation that is greater than what is observed with wild type Kv3.4 in the presence of PMA (Fig. 7; Supplementary Materials, Fig. S10). Most significantly, the double Kv3.4 mutation S3N/N6S was sufficient to match the AUT5-induced positive modulation of the Kv3.4 chimera with a turret fully converted to that of Kv3.2 (Fig. 6B). Overall, a combination of mutational (including reciprocal chimeras and discrete reciprocal substitutions between Kv3.2 and Kv3.4) and biophysical analyses strongly suggests that discrete structural differences between the turret regions of Kv3 channels account for the highly specific positive modulation of Kv3.1 and Kv3.2 channels by AUT5.

Docking calculations reveal the AUT5 site of action in Kv3.1 and Kv3.2 channels.

Although the results so far strongly suggest that the Kv3 turret governs the positive modulation by AUT5, we do not know the extent to which the turret itself may shape the compound’s binding site. To elucidate this problem, we leveraged the recently solved cryo-EM structure of the Kv3.1a channel (40) and used blind docking calculations to determine the most likely location of the AUT5 binding site (Materials and Methods). These calculations and clustering analysis suggested 17 independent binding sites (Fig. 7A), distributed over the transmembrane region of Kv3.1a with binding energies ranging from −8.5 to -7.5 kcal/mol (Supplementary Materials; Fig. S11A). Across the space of docking solutions, only site 4 was located near the turret, mainly occupying the inter-subunit interface between S4 and S5 (Fig. 7B). In site 4, the AUT5 imidazolidine moiety faces polar amino acids and the benzofuran group is buried within the protein-membrane interface, making close amino acid contacts with G308, R311, V312, F315, M362, Y365, A366, R368 and I369 (Supplementary Materials, Fig. S11B).

Given that AUT5 acts similarly on the Kv3.1 and Kv3.2, we also investigated the interaction of this compound with a structural ColabFold 42 generated model of Kv3.2. The same docking and analysis protocols suggested 28 transmembrane sites with binding energies ranging from −8.7 to -7.3 kcal/mol (Supplementary Materials; Fig. S11A). AUT5 was found to occupy a unique site near the turret of Kv3.2, making contacts with R348, V349, F352, I355, M399, Y402 and V406. With a root mean square deviation of 5.86 Å, including rotation and translation of the binder within the binding site, docking solutions of AUT5 were found to be structurally similar between Kv3.1 and Kv3.2, demonstrating that the binding pocket located near the turret is a key player in the AUT5 MoA (Fig. 7).

Considering the notable selectivity of the compound (Fig. 1), we also conducted blind docking calculations with AUT5-insensitive Kv channels with known structures, including Kv1.2, the Kv1.2-Kv2.1 chimera, and Kv4.2 43–47. AUT5 binding sites near the turret were not detected for the AUT5-insensitive channels (Supplementary Materials; Fig. S11). This result provides strong computational support to the experimental observations demonstrating a highly specific modulation of Kv3.1 and Kv3.2 by AUT5 that is dictated by the structural features conferred by their specific and highly conserved turret sequences.

Discussion
Toward elucidating the MoA of AUT5, a potent PAM of Kv3 channels, we have demonstrated here that 1) among several Kv channels, this compound selectively potentiates the function of Kv3.1 and Kv3.2 by inducing a parallel hyperpolarizing shift of the $G_p$-$V_c$ relation; 2) this potentiation results from a preferential stabilization of each channel's open state involving positive cooperativity; 3) deletion of the extracellular turret loop between transmembrane segments S5 and S6 in Kv3.2 eliminates the positive modulation; 4) exchange of the turret loop between Kv3.2 and Kv3.4, which exhibit differential sensitivities to 2 µM AUT5, can reciprocally eliminate and confer positive modulation; 5) moreover, discrete single, double and triple substitutions that partially convert the Kv3.2 turret into the Kv3.4 turret and vice versa are sufficient to significantly reduce and confer positive modulation; 6) leveraging on the cryo-EM structure of the human Kv3.1a, blind docking calculations demonstrate a specific inter-subunit AUT5 binding site near the turret and between the S4 segment of VSD and S5 segment of the PD; and 7) additional blind docking calculations applied to the crystal and cryo-EM structures AUT5-insensitive Kv channels (Kv1.2 and Kv4.2) revealed no AUT5 binding near divergent turret regions. Based on these results, we propose a model to explain how the unique turret of Kv3.1 and Kv3.2 confers highly specific positive modulation by AUT5 and reveals novel inter-subunit interactions that may determine the specialized gating properties of these Kv channels.

Among representative members of four major Kv channel subfamilies with similar structural properties (Kv1, Kv2, Kv3 and Kv4), including a cytoplasmic T1 domain and a tetrameric assembly with a domain swapped architecture, we found that Kv3.2 exhibits the largest PAM at 2 µM AUT5, closely followed by Kv3.1. Surprisingly, Kv3.4 and the Drosophila melanogaster Kv3 ortholog K-Shaw2 displayed no significant PAM when expressed in Xenopus oocytes. Kv3.4, however, was not completely insensitive to AUT5. Upon testing Kv3.4 stably expressed in HEK293 cells, we found a slight albeit not significant PAM at 2 µM AUT5 ($\Delta V_{0.5} = -6.7 \pm 3.3$ mV; $P = 0.11, n = 5$) and dual positive (with small depolarizations) and negative (with strong depolarizations) modulations at 10 µM AUT5 (Supplemental Materials; Fig. S12). These results suggest that ancillary cellular factors may also play a role determining the sensitivities of Kv3 channels to imidazolidinedione compounds. Follow up experiments outside the scope of this study would be necessary to discover these factors. At any rate, leveraging on the sharp difference in AUT5 sensitivities between Kv3.2 and Kv3.4 expressed in Xenopus oocytes and their distinct turret sequences, we were able to establish that the Kv3 turret is a critical intrinsic structural determinant of AUT5-induced PAM.

The turret of K$^+$ channels is a variable extracellular loop between the first transmembrane helix of the PD (S5) and the pore helix that helps shape the ion selectivity filter (Fig. 4). Considering its appearance in the crystal structure of the bacterial KcsA channel, Doyle et al. named it the extracellular turret region. Subsequent studies discovered that this region is also apparent in diverse eukaryotic Kv channel structures, where, in addition to shaping the selectivity filter, it 1) helps determine binding of inhibitory toxins and synthetic compounds that occlude the outer mouth of the central pore; 2) contributes to voltage-dependent gating rearrangements; 3) interacts with an ancillary protein to modulate binding of polyunsaturated fatty acids; and 4) is a structural determinant of C-type inactivation. However, to
the best of our knowledge, there is no study that demonstrates the novel allosteric role of the turret in the mechanism of action of highly specific and potent Kv channel PAMs, such as AUT5, the imidazolidinedione derivative investigated here.

The results from turret swapping and discrete turret substitutions between Kv3.2 and Kv3.4 demonstrate that the turret helps tailor the specific voltage-dependent properties of Kv3 channels, consistent with studies of other Kv channels (Supplementary Materials, Figs. S6 and S7) 52,53. Moreover, the results from these experiments provide compelling evidence to conclude that the turret of Kv3 channels governs their differential sensitivities to AUT5 (Figs. 3, 5 and 6). As expected, reciprocal conversions of the turrets between Kv3.2 and Kv3.4 eliminated and conferred positive modulation by AUT5. However, considering that the effects of combined mutations did not suggest additivity (Figs. 3, 5 and 6), the turret itself may not contribute significant binding energy to the interaction of Kv3.2 with AUT5. Rather, the mutations that convert the Kv3.2 turret into that of Kv3.4 and vice versa probably change the turret conformation, which may have significant structural consequences. The turret conformation may regulate access to a deeper inter-subunit binding site, and, through allosteric interactions, it may also regulate the stability of AUT5 binding in the pocket between the PD and the VSD of neighboring subunits.

Supporting the results from the mutational and biophysical analyses, blind docking calculations based on the cryo-EM structure of Kv3.1a revealed the preferred AUT5 binding sites near the turrets of the channel’s tetrameric assembly. The positive cooperativity suggested by the analysis of the ΔV0.5 vs. [AU5] plot (Fig. 2E) is consistent with the presence of multiple interacting AUT5 sites. However, the binding pocket is not directly lined by the turret. Rather, it is located deeper between the PD and VSD, where the apolar moiety of AUT5 makes contacts with various hydrophobic amino acid side chains, and the polar moiety of AUT5 makes contacts with more superficial polar groups. Surprisingly, the contact residues are highly conserved among Kv3 variants (Fig. 7; Supplementary Materials, Fig. S4) and, therefore, the differential AUT5 sensitivities of Kv3.1, Kv3.2 and Kv3.4 may not be determined by pocket contacts. Alternatively, as suggested above, we propose that only the turrets of AUT5-sensitive Kv3 channels can adopt a permissive conformation dictated by their specific sequences and this, in turn, shapes a deeper pocket and VSD-PD interactions necessary for the transduction mechanism that ultimately dictates positive modulation. Supporting this possibility, the identified binding site is in the vicinity the S1-S2 and S3-S4 loops of the VSD.

It has been previously suggested that the turret of the PD in Kv1.2 channels undergoes opening rearrangements that may change its relationship with the S1-S2 loop of the VSD 52. The turret may thereby indirectly affect the conformation of the S3-S4 loop and, as a result, influence voltage-dependent gating. There is ample evidence demonstrating that the S3-S4 loop of Kv channels undergoes gating rearrangements resulting from the voltage-dependent movements of the S4 voltage sensor that govern gating 56–58. We propose that AUT5 binding in the inter-subunit pocket induces turret rearrangements that are coupled to the S1-S2 and S3-S4 loops. A significant, albeit partial, neutralizing effect of exchanging the S1-S2 linker of Kv3.4 for that of Kv3.2 supports this model (Supplementary Materials, Fig. S5). Consequently, upon AUT5 binding, the voltage sensors are immobilized in the activated conformation and
the open state of the channel is stabilized, resulting in positive modulation. However, this is not a general property of Kv channels. Our data strongly suggest that this coupling depends strictly on the conformation of the turret or its propensity to undergo a necessary conformational shift. Whereas the Kv3.2 and Kv3.1 turrets have permissive conformations that support VSD-PD coupling and positive modulation by AUT5, the turrets of Kv channels that are relatively insensitive to AUT5 lack this property.

Although this study did not fully map the conformations and interactions proposed above, it establishes that the unique Kv3 turret plays an essential governing role in the specific positive modulation of Kv3.1 and Kv3.2 channels by AUT5 and concretely suggests the structural basis of a MoA for related imidazolidinedione derivatives. This knowledge opens opportunities to develop novel small molecule modulators with therapeutic potential to restore or finetune Kv3 channel activity. Follow up structural studies to directly visualize the binding of imidazolidinedione derivatives (e.g., cryo-EM) may sharpen our views of how these compounds direct the turret to modulate the interactions between the S1-S2 and S3-S4 linkers in the VSD. The MoA for imidazolidinedione derivatives involving the turret region and neighboring extracellular linkers is entirely different from that recently suggested for a different class of Kv3 channel PAMs that bind at an internal site.

Materials And Methods

Experimental design.

To elucidate the MoA of AUT5, we designed an orthogonal approach that combined 1) electrophysiological analysis to assess specificity among Kv channels and identify the biophysical basis of the MoA; 2) reciprocal mutational and functional analyses to identify the critical molecular determinants of the MoA; and 3) leveraging on the recently solved cryo-EM structure of the Kv3.1a channel, computational analysis (blind docking) to identify the AUT5 binding site and other potentially relevant interactions.

cDNAs and site-directed mutagenesis.

All human Kv3.2a, Kv3.4a, and rat Kv1.2, Kv2.1, Kv3.1, Kv4.2, as well as Drosophila K-Shaw-F335A cDNAs encoding the investigated Kv channels were maintained in appropriate expression vectors suitable for in vitro transcription as previously reported. Site-directed mutagenesis was conducted according to the QuickChange protocol (Stratagene, La Jolla, CA). All chimeras were created by phusion based PCR (Thermofisher Scientific), and primers used are listed in Supplementary Materials, Table S2. All mutations were verified by automated DNA sequencing (Genewiz, South Plainfield, NJ). The mRNAs were synthesized with an in vitro RNA transcription kit (mMESSAGE mMACHINE by Ambion, ThermoFisher Scientific) followed by purification before heterologous expression in Xenopus oocytes.

Reagents.
AUT5 ((5R)-5-ethyl-3-[6-(spiro[1-benzofuran-3,1'-cyclopropan]-4-yloxy)-3-pyridinyl]-2,4-imidazolidinedione; PubChem CID: 57410333) was synthesized and purified by Autifyon Therapeutics, LTD (Stevenage, UK). Lyophilized AUT5 was dissolved in DMSO to make a 10 mM stock, which was kept at 4°C and was stable for up to 3 months. Immediately before use, AUT5 was diluted to the desired working concentrations in ND96 containing (in mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, 2.5 sodium pyruvate, adjusted to pH 7.4 with NaOH. Collagenase A was purchased from Sigma (St. Louis, MO), Leibovitz’s medium was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA), PMA, DMSO and all other standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO) (Supplementary Materials, Table S3).

**Heterologous expression of Kv channels.**

*Xenopus laevis* surgeries were performed according to a protocol approved by the Thomas Jefferson University IACUC. As previously described, a standard collagenase-based dissociation technique was used to harvest mature stage V-VI oocytes suitable for heterologous expression and electrophysiological recording. Ovaries were digested with collagenase A in calcium-free ND96 (in mM: 96 NaCl, 2 KCl, 1 MgCl$_2$, 5 HEPES, 2.5 sodium pyruvate, adjusted to pH 7.4 with NaOH) for 1.5 h. To improve yield, this step was repeated once with fresh collagenase A. Upon completion of the dissociation steps, the oocytes were washed at least 3 times with regular ND96, and at least 3 times additionally with Leibovitz’s L-15 medium (500 mL Leibovitz’s L-15 medium plus 220 mL H$_2$O, supplemented with 10 mM HEPES and 0.01 mg/mL gentamicin, and titrated with NaOH, pH 7.4). Isolated oocytes were then transferred to a 19°C incubator and maintained in 35 mm Petri dishes containing Leibovitz’s L-15. Mature oocytes lacking the follicular layer of cells (stage V-VI) were then selected for mRNA injection with a nanoliter microinjector using 3-000-203-G/X glass needles (Drummond Scientific Company). Typically, 46–92 nL of mRNA were injected per oocyte. The mRNA concentration was adjusted to obtain expression levels that are appropriate for TEVC (e.g., 2–7 µA at +50 mV, see below). Injected oocytes were maintained at 19°C in Leibovitz’s L-15 medium until they were transferred to the TEVC chamber for the recording of the expressed currents 24–72 h post-injection.

**Two-electrode voltage-clamping (TEVC)**

Oocytes were transferred to a recording chamber containing ND96 (RC-3Z; Warner Instruments) and whole-oocyte currents were recorded at room temperature (21–23°C) under TEVC conditions (OC-725C, Warner Instrument, Hamden, CT) according to established procedures. The electrodes were filled with 3 M KCl and all recordings were conducted with ND96 in the bath. Freshly made AUT5 at the desired concentration was delivered into the recording chamber by means of a gravity-driven perfusion system with an exchange time of about 1 s and, during recording, the bath was continuously perfused. Data acquisition was performed using the Digidata 1440A and pClamp 9.2 and 10.3 (Molecular Devices, Sunnyvale, CA). Passive leak and capacitance transients were subtracted on-line by means of a standard p/4 protocol. Specific voltage clamping protocols are described in the pertinent figures and figure legends.
Data processing and analysis

Data processing, analysis, plotting and curve-fitting were performed in Clampfit 10.3. (Molecular Devices, Sunnyvale, CA) and Origin 9.1 Pro (OriginLab, Northampton, MA). Assuming the independently determined reversal potential ($V_r = -95$ mV), the peak chord conductance ($G_p = I_p / |V_c - V_r|$) was determined to characterize the voltage dependence of the expressed Kv channels. $I_p$ is the peak current evoked by the command voltage $V_c$. The activation parameters were derived from the best fit to the $G_p - V_c$ relation assuming the following equation:

$$G_p(V_c) = \frac{G_{p\text{max}}}{1 + e^{(V_c - V_{0.5}) / k}} \text{ Eq. 1}$$

Where $G_{p\text{max}}$ is the maximum peak conductance, $V_{0.5}$ is the midpoint voltage, and $k$ is the slope factor. Assuming $T = 22.5$ °C, the equivalent gating charge $z$ was estimated as follows: $z = 25.5 / k$. To compare results from different oocytes and across different experiments, we calculated the relative $G_p$ ($G_p / G_{p\text{max}}$). $G_{p\text{max}}$ was estimated from the best-fit Boltzmann equation. All $G_p - V_c$ data were obtained from paired sets (same oocyte, before and after application of AUT5). Measurement of the control $G_p - V_c$ relation was followed by determination of a stable response to AUT5 (current evoked by a depolarizing step to $+60$ mV, before and after bath application of AUT5) and subsequent measurement of the $G_p - V_c$ relation in the presence of AUT5. To assess modulation by AUT5, we quantified the changes of the $V_{0.5}$ ($\Delta V_{0.5}$), $z$ ($\Delta z$) and $G_{p\text{max}}$ ($%G_{p\text{max}}$).

To determine the time constants of current activation at various command voltages, we obtained the best-fit to the rising phase of the currents (excluding the short initial current lag) assuming a 1st-order exponential equation or a sum of exponential terms:

$$I(t) = A_1 e^{-t / \tau_1} + A_2 e^{-t / \tau_2} + \cdots + A_n e^{-t / \tau_n} \text{ Eq. 2}$$

Where $A_1$, $A_2$ and $A_n$ are $\tau_1$, $\tau_2$ and $\tau_n$ are the amplitudes and time constants of the corresponding terms in the equation, respectively. Typically, no more than three terms were necessary to obtain a satisfactory fit. A similar approach was used to obtain the time constants of current deactivation at various tail command voltages. The weighted averages of the time constants ($\tau_W$, Eq. 4) were computed whenever two or more terms were necessary to obtain the best empirical description of the current trajectories, which was often the case at depolarized voltages.

$$\tau_W = \frac{A_1 \tau_1 + A_2 \tau_2 + \cdots + A_n \tau_n}{A_1 + A_2 + \cdots + A_n} \text{ Eq. 3}$$

The $\tau_W$ was then plotted against the deactivation and activation command voltages to characterize the voltage dependence.
The concentration dependence of the AUT5-induced positive modulation (hyperpolarizing shift of the $G_p$-$V_c$ relation) was quantified by plotting $\Delta V_{0.5}$ vs. [AUT5]. Then, the EC$_{50}$ and Hill coefficient ($n_H$) were determined from the best-fit of Eq. 4 shown below ($X = [AUT5]$).

$$Y(X) = Y_{MAX} \frac{X^{n_H}}{EC_{50} + X^{n_H}} \text{ Eq. 4}$$

Where $Y_{MAX}$ is the maximal effect on the measured parameter. The fit was weighted assuming $w_i = 1/y_i$.

**Statistical analysis.**

All experiments were repeated with at least three independent batches of oocytes. For descriptive purposes within the main text numerical results are expressed as mean±SEM, unless indicated otherwise. The paired Student t-test was used to evaluate $V_{0.5}$, $z$ and $G_{p_{max}}$ changes induced by AUT5 (comparing before vs. after application of AUT5). Since aggregated data from wild-type and mutants across different batches of oocytes were often not normally distributed and had unequal variances (based on the Levene's test), the non-parametric Kruskal-Wallis test was used to evaluate activation parameter changes induced by AUT5 ($\Delta V_{0.5}$, $\Delta z$ and $\%G_{p_{max}}$). When evaluating changes caused by mutations, individual comparisons were made relative to the corresponding wild type.

**Blind docking calculations.**

Blind docking calculations were performed as described elsewhere (Stock et al., 2018). AUT5 structure file was obtained from PubChem (PubChem ID: 57410333) and converted to a pdb file using OpenBabel 3.0.0. A set of Kv channel structures was used as input for docking calculations. High-resolution structures for the AUT5-sensitive channel Kv3.1a were obtained directly from Protein Data Bank (IDs: 7PHH, 7PHI, 7PHK and 7PHL). Three-dimensional structures for Kv3.2 were generated using ColabFold, with default parameters (msa_mode: MMseqs2 (UniRef + Environmental); pair_mode: unpaired + paired; model_type: auto). AUT5-insensitive channel structures were obtained from PDB: Kv1-Kv2 Chimera (IDs: 2R9R, 6EBK, 7SIT) – Kv1.2 (ID: 2A79) and Kv4.2 (ID: 7E84). For blind docking calculation, all Kv channel had their intracellular domains removed.

Docking was performed with Autodock Vina. Docking solutions were resolved with an exhaustiveness parameter of 350, by searching a box volume of 120 x 120 x 90 Å$^3$ containing the transmembrane domain of the protein receptor. AUT5 was allowed to have flexible bonds for all calculations. Clustering of docking solutions was carried out following a maximum neighborhood approach, with a proximity criterion (RMSD < 0.5 Å). When clustered, the solution contacts were merged, and the lowest energy solution was adopted as the bound ligand position. Among all suggested sites in blind docking procedure, the site that had the most percentage of turret residues in its composition was selected as the AUT5 binding site.

**Declarations**
Acknowledgments

We thank present and past members of the Covarrubias lab and the Jefferson Synaptic Biology Center on their helpful feedback during the execution of this work. All experiments were conducted in the Covarrubias lab and blind docking calculations were conducted in the Treptow lab.

Funding

This work was supported by a grant from Autifony Therapeutics, Ltd. (M.C.) and intramural funds from the Jefferson Synaptic Biology Center (M.C.); and grants from the National Council for Scientific and Technological Development CNPq (grants 02089/2019-5 and 200114/2020-4) (W.T.).

Authors contributions

Conceptualization: C.H.L., G.A., M.G., and M.C.


Visualization: Q.L., L.C., W.T., and M.C.

Funding acquisition: C.H.L., and M.C.

Project Administration: C.H.L., M.G., and M.C.

Supervision: C.H.L., M.G., and M.C.

Writing (original draft): M.C.


Competing interests

Authors declare that they have no competing interests.

Data and materials availability

All data needed to evaluate the conclusions of this work are included in the paper and/or the Supplementary Materials.

References


**Figures**
AUT5 is a selective PAM of Kv3.1 and Kv3.2 channels. (A)-(C) and (E)-(H) Representative families of whole-oocyte currents before (black) and after (red) bath application of 2 µM AUT5, and the corresponding $G_p$-$V_c$ curves (Materials and Methods). Scale bars represent 1 µA and 100 ms. (D) The voltage pulse protocol used to evoke the currents. (I) Scatter plots of the AUT5-induced changes in $V_{0.5}$, $z$,
and $G_{\text{max}}$. Each symbol represents a measurement from an individual oocyte. Short vertical bars indicate the mean values. P values evaluate differences relative to Kv3.2 (Kruskal-Wallis).

Figure 2

**Preferentially stabilization of the open state and positive cooperativity underlie the PAM of Kv3.2 by AUT5.** (A) Representative families of tail currents before (black) and after (red) bath application of 2 µM AUT5. Scale bars represent 0.5 µA and 50 ms. These currents were evoked by the voltage pulse protocol shown on the right-hand side of this panel. To accurately measure slow time constants in the presence of AUT5, the tail current portion of the protocol was 1000 ms. (B) Overlay of tail currents relaxations at -70 mV in the absence (black) and presence (red) of 2 µM AUT5. (C) Overlay of currents evoked by a step depolarization from -100 to +20 mV the absence (black) and presence (red) of 2 µM AUT5. Dashed lines in panels (A)-(C) represent the zero current level. (D) Voltage dependence of the time constants of current deactivation (filled symbols) and current activation (hollow symbols) before (black) and after (red) bath application of the absence (black) and presence (red) of 2 µM AUT5 ($n = 9$ oocytes). Asterisks in (D) indicate $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***) (paired Student t-test before and after bath application...
The effect of [AUT5] on the $\Delta V_{0.5}$ (n = 4-72 oocytes). Each filled gray symbol represents a measurement from an individual oocyte. The solid line is the best weighted fit of the logistic equation with the parameters shown on the graph (Materials and Methods).

**Figure 3**

The turret region is necessary for the positive modulation of Kv3.2 by AUT5. (A) and (B) Representative families of Kv3.2 DTurret and 3.4x3.2 Turret currents (left) before (black) and after (red) bath application of 2 mM AUT5, and the aggregate of $G_p$-$V_c$ curves with their corresponding analysis of $V_{0.5}$, $G_{max}$ and $z$ (right). Representative currents were evoked by the voltage protocol shown on Fig. 1, and the solid lines across the symbols of the $G_p$-$V_c$ curves are the best-fits to the 1st-order Boltzmann equation (Materials and Methods). Scale bars represent 1 µA and 100 ms. (C) Aggregate scatter graphs of the AUT5-induced changes in $V_{0.5}$, $G_{max}$ and $z$ from individual oocytes. Short vertical bars indicate the mean values. The sample sizes of the wild type groups are as indicated in Fig. 1. The indicated P values evaluate differences relative to wild type Kv3.2 (Kruskal-Wallis). The results from Kv3.2ΔTurret relative to wild type Kv3.4 are indistinguishable. Each symbol represents a measurement from an individual oocyte.
The unique extracellular turret motif of Kv3 channels. (A) Sequence alignment comparing transmembrane segment S5, turret linker, pore helix (PH), selectivity filter (SF) and transmembrane segment S6 from homologous VDKCs. Note that the turrets of hKv4.2, hKv2.1, hKv1.2 and dShaw2 are shorter compared to those of hKv3.1, hKv3.2 and hKv3.4. (B) Detailed comparison of amino acid sequences in the turrets of Kv3 channels (dShaw is a Drosophila homolog). Note that, except for the flanking residues at the numbered positions 1 and 8, Kv3.1 and Kv3.2 have identical turret regions. (C) Topology of a single Kv3.1α subunit depicting the major domains: cytoplasmic T1 domain (T1D), voltage-sensing domain (VSD=S1-S4) and pore domain (PD=S5-S6). Arrows indicate the extracellular S1-S2 loop and turret region.
Figure 5

The Kv3.2 turret region confers positive modulation by AUT5 in Kv3.4 upon elimination of fast inactivation. A) and B) Representative families of 3.2x3.4Turret currents (control and post PMA, left, top and bottom, respectively) before (black) and after (red) bath application of 2 µM AUT5 and the aggregate of $G_p$-$V_c$ curves with their corresponding analysis of $V_{0.5}$, $G_{max}$ and $z$ (right). Representative currents were evoked by the voltage protocol shown on Fig. 1, and the solid lines across the symbols of the $G_p$-$V_c$ curves are the best-fits of the 1st-order Boltzmann equation (Materials and Methods). Blue traces and symbols are from oocytes exposed to 50 nM PMA before applying AUT5. PMA remained in the chamber until the end of the experiment. (C) Aggregate scatter graphs of the AUT5-induced changes in $V_{0.5}$, $G_{max}$ and $z$ from individual oocytes. Short vertical bars indicate the mean values. The Kv3.2 WT results are replotted here as a reference. The sample sizes of the wild type groups are as indicated in Fig. 1 and Fig. S9. The indicated P values evaluate differences relative to wild type Kv3.4 with/without in the presence of PMA (Kruskal-Wallis). Each symbol represents a measurement from an individual oocyte.
Figure 6

Discrete turret mutations reciprocally eliminate and confer positive modulation by AUT5 in Kv3.2 and Kv3.4, respectively. (A) and (B) Scatter plot of $V_{0.5}$ changes induced by 2 µM AUT5. The red and blue dashed lines are shown as the reference value of the $V_{0.5}$ of Kv3.2 WT and Kv3.4 WT, respectively. The location of the mutations is according to the key shown on this figure and Fig. 4 and Table S1, which indicates eight turret differences between Kv3.2 and Kv3.4. Modulation of Kv3.4 WT and Kv3.4 mutants by 2 µM AUT5 was tested in the presence of 50 nM PMA (blue bracket) to eliminate fast inactivation as shown in Fig. 5. Short vertical bars indicate the mean values. The indicated P values evaluate differences relative to wild type Kv3.2 in (A) and wild type Kv3.4 in the presence of PMA in (B) (Kruskal-Wallis). The sample sizes (number of oocytes) in (A) are: N3S, n=15; S4R, n=9; A5G, n=12; S6N, n=12; N3S/A5G, n=6; N3S/S4R, n=5; N3S/S6N, n=4; S4R/A5G, n=5; S4R/A5G/S6N, n=12. The sample sizes (number of oocytes) in (B) are: S3N, n=9; R4S, n=7; G5A, n=9; N6S, n=10; S3N/N6S, n=10. Each symbol represents a measurement from an individual oocyte.
Figure 7

Blind docking calculations reveal the putative interfacial AUT5 binding site near the turret region of Kv3.1a and Kv3.2a. (A) Molecular structures of AUT5, Kv3.1a (white) and docking solutions (red) across the search grid volume. (B) Surface representation of Kv3.1a, Kv3.2a showing the AUT5 bound molecule at a site near the turret. Inset details the bound molecule and highly conserved amino acids at the binding site. (C) Sequence alignment of S4-S5 regions of Kv3.1a and Kv3.2a channels. Analysis and images were generated using VMD 67.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Liangetal.NCsupplementarymaterialsmss041023.docx