

Pharmacological Exploration of Calcium-activated Potassium Channels Using Computational and
Electrophysiological Methods

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To my parents, both biological and academic, for always propelling me forward despite my inherently unambitious nature.

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Abstract

Potassium channels are a critical regulatory component of numerous vital cellular processes, being centrally involved in calcium signaling, membrane potential, volume regulation and action potential firing to name a few. And as such, they represent an incredibly attractive avenue for therapeutic intervention in a multitude of different diseases. KCa3.1, a calcium-activated potassium channel found throughout the peripheral tissue, serves mainly as a calcium-signaling regulator, allowing influxes of calcium through inward-rectifier calcium channels by generating a counterbalancing potassium-efflux and inducing hyperpolarizations. This calcium signaling is essential for cellular activation, production of various cytokines and the proliferation of immune cells and fibroblasts. KCa3.1 also plays an additional role in erythrocyte hydration (one of the channel's first claims to fame) and vasodilation through endothelium-derived hyperpolarization. The related small-conductance KCa2.x channels (KCa2.1, 2.2, and 2.3) can be found alongside KCa3.1 within the peripheral tissue, but also are highly expressed throughout the CNS. KCa2.x channels help in regulating the firing rate of excitable cells, underlying the medium afterhyperpolarization of neuronal action potentials, in addition to having a prominent role in atrial repolarization. Over the course of this dissertation, we will discuss our exploration of binding sites, mechanisms of action, and selectivity of both biologics and small molecule channel modulators targeting these calcium-dependent potassium channels. By advancing our understanding of these complex interactions between protein and modulator, we hope to open up new possibilities for the design of more effective and precise therapeutic agents targeting these crucial ion channels.

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CHAPTER 1: Introduction and Literature Review

Potassium has the distinction of being not only the most prevalent cytosolic cation within the human body, but also the most permeable at resting membrane potential (Hille, 2001). This combination of features enables potassium to almost single-handedly set the negative membrane voltage at which our cells rest, preventing spontaneous action potentials and highlighting the necessity of proper potassium homeostasis (Pohl et al., 2013). The Na^+/K^+ ATPase, also known as the sodium-potassium pump, is found in the membrane of all animal cells, and it has the burdensome role of maintaining the potassium (and sodium) gradient of our cells (Gadsby, 2009). This pump transfers three sodium ions out of the cell while simultaneously bringing in two potassium ions, both against their natural gradient, all at the cost of an ATP molecule being dephosphorylated (Morth et al., 2007). The Na^+/K^+ ATPase plays a crucial in maintaining the extracellular fluid potassium concentration of approximately 3.5-5 mmol/L, in comparison to the 140 mmol/L concentration found in the cytosol (Boron et al., 2017). In direct contrast to this hard-working pump, ion channels flow with, rather than against, the natural gradient of their permeating ion. They conduct much more quickly, by several orders of magnitude, and rather than maintaining the gradient, they spend it to correct for large changes in membrane potential, or depolarizations. Potassium channels serve as the other half of the complex ionic homeostasis system of our cells. They are indispensable, being the most widely distributed type of ion channel in essentially all living organisms, and they represent an attractive therapeutic target of interest in both industry and academia. Throughout this introduction, we will be focusing on calcium-activated potassium channels in particular, and discuss where they are found, how they function, why they are important, and how they can be modulated to potentially treat a number of different disease pathologies.

Calcium-activated Potassium Channels



Figure 1.1, The two groups of the KCa channel family; Adapted from Wei 2005

Calcium-activated potassium channels form a unique family of 6 (formerly 8) potassium channels, all of which rely to varying extents on cytosolic calcium to function. However, they can be further divided into two groups based on their unique activation mechanisms. On one side of the family are the “slo” channels KCa1.1 (slo1, also known as the BK channel) and KCa5.1 (slo3). These two channels can be distinguished from the rest of the KCa family by their alternate gating mechanisms, which are independent of changes in intracellular calcium concentrations. KCa1.1, for instance, is both calcium and voltage sensitive, still possessing a voltage-sensing domain on its S4 transmembrane segment (Lee et al., 2010). KCa5.1 is alternately gated by changes in pH, while remaining relatively insensitive to changes in cytosolic calcium concentration (Brenker et al., 2014). This side of the family also previously included KCa4.1 (slo2.2) and KCa4.2 (slo2.1), also known as Slack and Slick respectively, before these channels were correctly reclassified as KNa channels given their complete reliance on changes in intracellular sodium and chloride concentrations, and total insensitivity to intracellular calcium (Kaczmarek, 2013). The other side of the KCa family tree is comprised of the small- and intermediate-conductance calcium-activated potassium channels KCa2.1, KCa2.2, KCa2.3 and KCa3.1. These channels rely entirely on changes in cytosolic calcium levels for activation, driven by a calmodulin subunit constitutively bound to the channel’s C-terminus (Wei et al., 2005). These calcium-sensitive channels are the primary focus of this dissertation.

KCa Channel Identification

Calcium-dependent potassium channels were first discovered to exist in 1958 by the Hungarian biochemist György Gárdos, after he noticed that altering calcium levels within the cytosol of erythrocytes resulted in a rapid efflux of potassium (Gárdos, 1958). Of course, it would not be until nearly 40 years later that some of these channels were finally cloned, opening the door for much more in depth exploration of both their structures and functional mechanisms. The first of the KCa channels to be genetically identified and cloned were the small-conductance calcium-activated channels (KCa2.1, KCa2.2 and KCa2.3). This was accomplished by the Adelman group in 1996, using a probe containing a known potassium channel identifier (the classic T-X-G-Y-G-D motif, a highly conserved pore sequence forming the selectivity filter

of all selective potassium channels) to screen both rat and human brain tissue, wherein these channels are highly expressed (Köhler et al., 1996). Aside from their heightened expression throughout the CNS, this subset of KCa channels can also be distinguished from KCa3.1 in both their smaller single channel conductances (on the order of ~10 pS) as well as their distinct sensitivity to the bee venom peptide Apamin. The cloning of this Apamin-insensitive intermediate-conductance calcium-activated potassium channel KCa3.1 would take place the following year in 1997, where it was identified via a BLAST search of the Database of Expressed Sequence Tags (dbEST) using the SK channel sequence as a reference (Ishii et al., 1997), and cloned from the human pancreas and lymphoid tissue (Logsdon et al., 1997 and Jensen et al., 1998).

KCa Channel Structure

Unsurprisingly, the small-conductance calcium-activated potassium channels, KCa2.1, KCa2.2 and KCa2.3 share almost 90 percent sequence homology within their transmembrane domains, with the only significant differences being found along their lengthy N and C termini. Contrarily, the intermediate-conductance calcium-activated potassium channel, KCa3.1, possesses only 40 percent sequence homology when compared with its' cousin channels (Brown et al., 2020). Despite this relative lack of homology between KCa3.1 and the KCa2.x channels, both groups share a patently similar structural layout consisting of 6 transmembrane segments flanked by N and C termini hanging within the cytosol, a potassium selective filter between transmembrane segments 5 and 6 containing the classic G-Y-G motif, a shortage of positively charged residues along their S4 transmembrane segments, and a calmodulin binding domain located on their C-terminus (Xia et al., 1998 & Fanger et al., 1999). Additionally, these channels require a tetrameric conformation of subunits in order to function.

Interestingly, while both the small- and intermediate-conductance calcium-activated potassium channels are gated via an identical constitutively bound calmodulin subunit, they still possess significantly different calcium sensitivities. The small-conductance KCa2.x channels display a calcium EC₅₀ ranging

from 300 to 900 nM; considerably higher than the 95-300 nM EC₅₀ range exhibited by the intermediate-conductance channel KCa3.1. This could be due, at least in part, to differential phosphorylation of the two channel subtypes, specifically involving casein kinase 2 (CK2) and its role in phosphorylation of calmodulin residue T79 for the small-conductance KCa channels. Phosphorylation at this position has been shown to significantly inhibit interactions between KCa2.x and phosphatidylinositol 4,5-bisphosphate (PIP2), which ordinarily binds in the nearby calmodulin-SK channel interface and has been shown to play an important role in calcium-dependent channel activation (Zhang et al., 2014).

KCa Channel Expression

The small- and intermediate-conductance calcium activated potassium channels share patently similar structures and gating mechanisms, but they are quite distinct in their tissue distribution. KCa2 channels are widely distributed throughout both the periphery and the central nervous system. Through clever use of selective blockers such as Apamin, KCa2.x channels have been found to be present in several different regions of the brain. KCa2.1 and KCa2.2 have been found to be expressed in the hippocampus, neocortex, and reticularis thalami, while KCa2.3 channels have been found in the basal ganglia and the thalamus (Stocker et al., 2000, Sailer et al., 2002 and 2004). Additionally, KCa2 channels are expressed in dopaminergic pacemaker neurons of the midbrain and cerebellum, where they are postulated to regulate firing critical for muscle coordination and movement (Kshatri et al., 2018). In the periphery, KCa2 channels are widely dispersed throughout the heart, liver, prostate, skeletal muscle, smooth muscle, adrenal glands, genital tissues, and vascular endothelium (Xu et al., 2003). KCa2.3 represents the vast majority of peripheral KCa2 expression, especially in the endothelium, although this peripheral KCa2 expression is dwarfed significantly by KCa3.1 (Chen et al., 2004).

In contrast to the KCa2 channels, KCa3.1 is found almost exclusively in peripheral tissue, although channel expression has also been observed in Purkinje neurons of the cerebellar cortex (Engbers et al., 2012). In addition, the channel is known to be highly expressed in activated microglia within the brain

under the right neuroinflammatory conditions (Lu et al., 2019). In the periphery, KCa3.1 is expressed primarily in erythrocytes, endothelial cells, immune cells (including T cells, B cells, macrophages, mast cells and microglia), and secretory epithelial cells lining the lung and GI tract (Van et al., 2024). And of course, within each distinct cell type, the physiological channel function can differ consequently.

KCa Channel Function

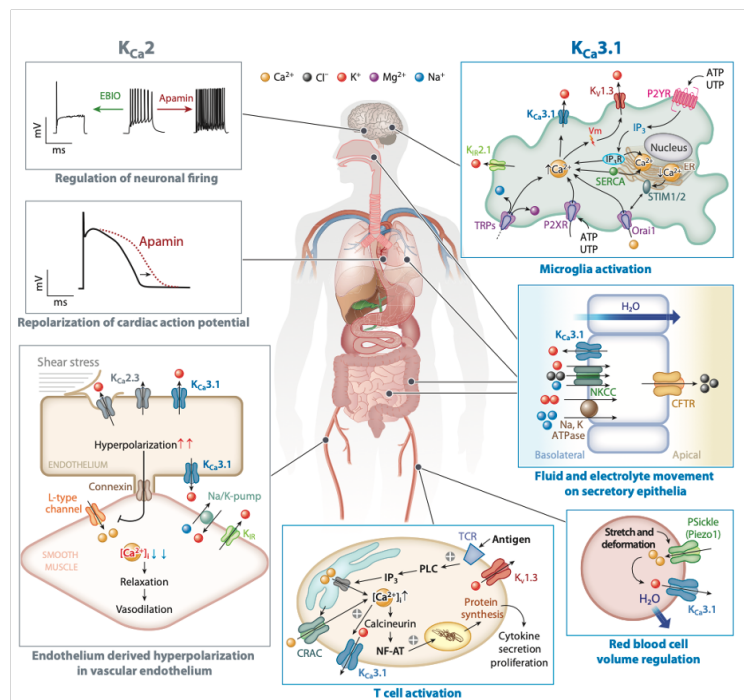


Figure 2.2, Physiological role of KCa channels throughout the human body, (Brown et al., 2020)

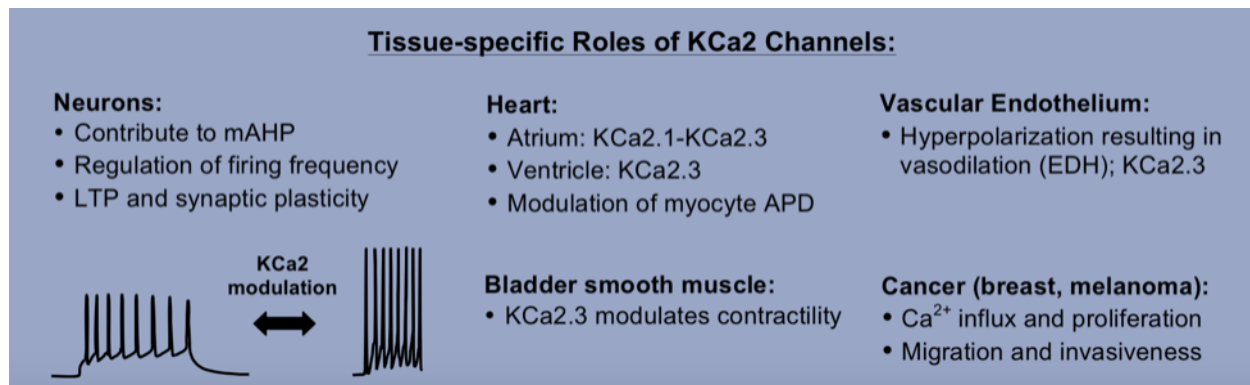
Given the diverse expression of KCa channels, their function varies accordingly depending on the tissue in which the channel is found. KCa3.1, expressed throughout the peripheral tissue, typically serves as a calcium-signaling regulator, allowing influx of calcium through inward-rectifier calcium channels by generating a counterbalancing potassium-efflux and inducing hyperpolarizations. This calcium signaling is essential for cellular activation, migration, cytokine production, and proliferation of immune cells and fibroblasts (Sforza et al., 2018). In erythrocytes, the KCa3.1 channel plays an additional role in volume regulation, where potassium efflux is followed by cytosolic dehydration and cellular shrinkage in an effort

to maintain homeostatic osmolarity (Fermo et al., 2020 and Petkova-Kirova et al., 2024). KCa3.1 expressed in secretory epithelial cells of the lungs and GI tract aids in salt and fluid secretion (Valero et al., 2019 and Orfali et al., 2023). And in vascular endothelial cells, KCa3.1 is the largest contributor of what is known as endothelium-derived hyperpolarization (or EDH), a phenomenon where channel activation within the endothelium can lead to hyperpolarization of neighboring smooth muscles cells within the vasculature, either directly via gap junctions or through release of local vasodilators such as nitric oxide and prostacyclin (Wölflle et al., 2009).

<u>Tissue-specific Down-stream Effects of KCa3.1 Activation:</u>			
Erythrocytes: • Volume decrease	T cells: • Ca^{2+} influx • NFAT translocation • Proliferation • Cytokine secretion	Dedifferentiated VSMC, fibroblast, cancer cell: • Ca^{2+} influx • Proliferation • Migration	Macrophages/microglia: • Migration • Oxidative burst
Vascular Endothelium: • Hyperpolarization resulting in vasodilation (EDHF)	Secretory Epithelia (gut/lung): • Salt and fluid secretion		

The KCa2.x channels are far more prominent in the CNS than KCa3.1, but as mentioned previously, they do retain some peripheral expression. In the vascular endothelium, the KCa2.3 channel plays a similar role to KCa3.1 in contributing towards EDH and promoting vasodilation. Knock-out of either channel in rodents was shown to significantly elevate arterial blood pressure and impair vasodilation, although the two channels do seem to operate through distinct vasodilatory mechanisms given how they respond differently to acetylcholine and nitric oxide induction (Brähler et al., 2009). In the bladder, KCa2.x channels are expressed in smooth muscle cells and play an important role in modulating urinary smooth muscle contraction (Herrera et al., 2005). In the heart, KCa2.x channels expressed in cardiac myocytes and pacemaking cells play an essential role in regulating cardiac excitability via their repolarizing effect, with KCa2.2 being the primary regulatory force (Jeevaratnam et al., 2018). This role is far more prominent in the atrium, but KCa2.x channels are still expressed sparsely in the ventricles and are prone to upregulation

following instances of heart failure (Zhang et al., 2021). And lastly in the CNS, KCa2.x expression underlies what is known as the medium afterhyperpolarization (mAHP) phase of neuronal action potentials, driving depolarized neurons into a brief state of hyperpolarization and thereby preventing premature follow-up action potentials (Stocker et al., 1999). This mAHP effect has an obvious influence on neuronal firing frequency, but it also affects synaptic plasticity, synaptic transmission and even long-term potentiation (Martina et al., 2007 and Sun et al., 2020).



KCa Channelopathy and Therapeutic Indications

There are several known KCa channel mutations which have been associated with various disease phenotypes, ranging from blood disorders to neurological conditions. Gain-of-function mutations within the KCNN4/KCa3.1 gene, for instance, have been shown to cause erythrocyte dehydration similar to mutant PIEZO1-driven hereditary xerocytosis (Fermo et al., 2020). These mutant variants include V282E/M and S314P, with both susceptible residues notably being located near the activation gate of the channel's S6 transmembrane segment. Numerous partial or total loss-of-function mutations within the KCNN2/KCa2.2 gene have been linked to neurological disorders affecting motor, language, and intellectual development. These known KCa2.2 mutations include Y160*, Y267*, I288S, L321del, I359M, Y361C, G362S, L388V, and L432P. Most of these variants are non-hereditary *de novo* mutations, some decreasing channel conductance, others introducing stop codons and causing premature protein truncation, but all result in

similarly undesirable neurodevelopmental phenotypes of varying severities (Mochel et al., 2020). And finally, certain gain-of-function mutations within the KCNN3 gene (KCa2.3) have been directly linked to a very rare genetic disorder known as Zimmermann-Laband syndrome (ZLS), best described as a developmental disorder affecting both cognitive and physical development. Mutant KCa2.3 variants known to cause ZLS are as follows: K269E, A287S, G350D, S436C, A536T, V539del and V555F (Bauer et al., 2019).

This plethora of disease conditions associated with KCa channel dysfunction demonstrates a clear need for promising new modulators targeting these channels. However, therapeutic indications for targeting these channels are not limited to just these naturally occurring channelopathies. Senicapoc, one of the more prominent KCa3.1 inhibitors, was the focal point of a clinical trial aimed at treating sickle cell anemia in 2009-2011, and while it did end up failing in phase 3, it still significantly improved several hematological properties, including a reduction in hemolysis (Ataga et al., 2011). More recently, this same inhibitor was investigated for use in treating COVID-19 complications and Alzheimer's disease in two separate clinical trials, the latter of which is still currently in progress at the University of California, Davis (Jin et al., 2019, Cummings et al., 2023, and Granfeldt et al., 2022). And this therapeutic potential extends to other neuroinflammatory conditions such as stroke reperfusion injury and multiple sclerosis (MS) (Chen et al., 2016).

Positive gating modulators of KCa3.1 have been pitched as potential antihypertensives, and show promise in reducing the symptoms of cystic fibrosis by stimulating chloride secretion in bronchial epithelial cells (Devor et al., 2022). KCa2.2 inhibitors have been shown to prolong cardiac action potentials, and as such, are being carefully scrutinized for use in treating atrial fibrillation (Li et al., 2009 and Burashnikov et al., 2020). There is already a KCa2.x activator on the market for treating muscle spasms in the form of Chlorzoxazone, although the exact central mechanism underlying the drug effect is still up for debate (Cao et al., 2001). Chlorzoxazone has even shown promise in treating alcohol dependence in rodents, providing yet another potential therapeutic avenue for KCa channel modulators

(Hopf et al., 2011). Regardless, the KCa channel family undisputedly represents a promising, multifaceted therapeutic target for several different disease states, and a need remains for new channel-modulating pharmacophores.

KCa Channel Pharmacology

Inhibitors

Diverse in form and function, inhibitors of the KCa channels are comprised of both peptides and small molecules, with several known binding pockets having already been defined across our four channels of interest (KCa2.1, 2.2, 2.3, and 3.1). Many of these binding sites are shared amongst the KCa family, which can make the engineering of compound selectivity an issue. However, selectivity does exist, especially between the small- and intermediate-conductance calcium-activated potassium channels, and often in the form of a naturally occurring venom peptide.

Most ubiquitous and well-known amongst KCa channel selective peptides is the relatively small neurotoxin Apamin. This 2000 dalton, 18-amino acid long peptide found in honeybee venom is incredibly selective for the KCa2 channels, with IC_{50} 's of 4.1 nM (95% CI 3.3-5.0 nM), 87.7 pM (95% CI 74.2-103.3 pM), and 2.3 nM (95% CI 1.8-2.9 nM) for the KCa2.1, KCa2.2 and KCa2.3 channels respectively. And despite the KCa channels being highly homologous, the peptide demonstrates no effect on KCa3.1, or on any other known channel for that matter (Kuzmenkov et al., 2022). Through mutagenesis studies and as-of-yet unpublished cryogenic electron microscopy work from the Zhang laboratory at Chapman University, Apamin is believed to bind extracellularly, sitting directly above the pore and making contacts with residues of the S3-S4 loop. In fact, a single serine substitution at position T216 of KCa2.1's S3-S4 extracellular loop was shown to shift the IC_{50} of apamin on KCa2.1 from 4.1 nM to 167 pM, noticeably similar to the 87 pM IC_{50} reported for apamin on the KCa2.2 channel (of which serine is the endogenous residue at that particular position) (Nolting et al., 2007).

Several scorpion venom peptides also display comparable potency and selectivity for the KCa2 channels. The most potent of which even share apamin's RXCX epitope motif; a motif seemingly required for high KCa2.x potency (Wulff et al., 2008). The 31-amino acid long leiurotoxin I, derived from deathstalker scorpion (*Leiurus quinquestriatus*) venom, is one such peptide, possessing an RMCQ motif on its binding surface and inhibiting KCa2.2 and KCa2.3 with IC₅₀'s mirroring that of apamin. Leiurotoxin I also inhibits KCa2.1, albeit with significantly lower potency, exhibiting an IC₅₀ in the high nanomolar range (Shakkottai et al., 2001). It was later modified to be even more selective by replacing the methionine of its RXCQ motif with a positively charged unnatural amino acid diaminobutanoic acid (Dab). The resulting synthetic peptide Lei-Dab7 was found to inhibit KCa2.2 almost exclusively, requiring over 600 times the concentration to exert the same effect on KCa2.1 and 2.3, making it both quite a useful tool for selective targeting of KCa2.2 in addition to highlighting the importance of the residues within that short 4 amino acid motif (Shakkottai et al., 2001).

PO5, purified from the venom of the fat-tailed scorpion (*Adroctonus mauretanicus mauretanicus*), is another 31-amino acid long high potency KCa2 channel-targeting venom peptide genetically similar to leiurotoxin I, although sharing apamin's exact RRCQ motif. And unlike apamin and leiurotoxin I, it inhibits KCa2.2 and 2.3 with equal potency (IC₅₀ ~ 25 nM) while remaining completely inert on KCa2.1 (Zerrouk et al., 1993). Tamapin, derived from the Indian red scorpion (*Mesobuthus tamulus*), represents the last of the leiurotoxin I-like peptides. This peptide, also 31-amino acids in length, possesses an RRCE motif on its binding surface and inhibits KCa2.1 and KCa2.3 with low nanomolar affinity. But it also has the unique distinction of being the most potent known peptide inhibitor of KCa2.2, with a reported 24 pM IC₅₀ (Pedarzani et al., 2002). Given their genetic similarity, these three peptides (leiurotoxin I, PO5 and tamapin) are classified jointly as the short-chain scorpion toxin subfamily 5, and together with apamin, represent the most potent available peptides targeting the KCa2 channels.

Although equally potent, peptides targeting KCa3.1 are typically far less selective than those targeting the KCa2 channels. Maurotoxin (MTX), a 34 amino acid long peptide found in Tunisian chactoid

scorpion venom (*Scorpio maurus palmatus*), is currently the most potent inhibitor of KCa3.1, with an IC_{50} of 1 nM. However, this peptide has an even higher affinity for the voltage-gated potassium channel Kv1.2, with a reported IC_{50} of 100 pM (Castle et al., 2003). Charybdotoxin (ChTX), a 37-amino acid long peptide produced by the same deathstalker scorpion species responsible for leiurotoxin I, inhibits KCa3.1 activity with comparable potency to MTX ($IC_{50} = 5$ nM), but likewise has higher affinity for a voltage-gated potassium channel; Kv1.3 in this case (Rauer et al., 2000). The 39-amino acid long peptide Margatoxin, found within the venom of the central American bark scorpion (*Centruroides margaritatus*), follows this trend of Kv channel selectivity over KCa3.1, blocking Kv1.3 in the low picomolar range (Garcia-Calvo et al., 1993). However, it remains a relatively potent blocker of KCa3.1 with an IC_{50} of 459 nM (Jensen et al., 1998). There are also venom peptides derived from different sea anemone species which display that same predilection for Kv channels over KCa3.1. Stichodactyla toxin (ShK), purified from the sun anemone (*Stichodactyla helianthus*) displays 1000-fold higher selectivity for Kv1.1 and Kv1.3 over KCa3.1, exhibiting IC_{50} 's of 10 pM, 16 pM, and 30 nM, respectively (Rauer et al., 1999 and Pennington et al., 2015). This selectivity was even explored and improved upon by George Chandy and company in 2005, resulting in the synthetic peptide ShK-186, which was found to be 100-fold more selective for Kv1.3 even over Kv1.1 (Beeton et al., 2005). BgK, another sea anemone toxin derived from the *Bunodosoma granulifera* species, is both less potent and less selective than ShK, but still inhibits Kv1.3 and KCa3.1 in the low and medium nanomolar range (Rauer et al., 1999).

Based on both site-directed mutagenesis and x-ray crystallography experiments conducted by the Chandy lab in 1999 and the MacKinnon lab in 2013, these promiscuous KCa3.1-targeting peptides seem to sit on top of the selectivity filter, acting as a traditional pore blockers. But unlike the KCa2-selective peptides, which interact with residues of the S3-S4 extracellular loop, KCa3.1-targeting peptides like ChTX and ShK instead interact directly with residues in the outer vestibule of the channel, situating themselves right above the central pore and inserting their own lysine residue into the selectivity filter for especially tight contacts with the channel's pore facing residues (Rauer et al., 1999 and Banerjee et al.,

2013). Given the high degree of homology in that pore region between KCa3.1 and the Kv channels, this observed peptide promiscuity is not terribly surprising. Despite the lack of ideal selectivity, these KCa3.1-targeting peptides still remain useful tool compounds, some of which are even used as such within the later chapters of this dissertation.

Many small molecule inhibitors have also been found to target KCa channels, with some achieving comparable potency to the peptide modulators. Dequalinium, a quaternary ammonium cation commonly used as an antiseptic agent, inhibits KCa2.x channels with nanomolar affinity, exhibiting IC₅₀'s of 444 nM for KCa2.1, 162 nM for KCa2.2, and 136 nM for KCa2.3 (Strøbaek et al., 2000 & 2006). This small molecule was later used as the backbone of an SAR study by the University College of London, resulting in two derivative compounds with even higher potency. The first of which, UCL 1684, is capable of blocking KCa2.x channels at picomolar or near-picomolar concentrations, with reported IC₅₀'s of 762 pM for KCa2.1, 364 pM for KCa2.2, and 9.5 nM for KCa2.3 (Strøbaek et al., 2000 & Fanger et al., 2001). The second dequalinium derivative, UCL 1884, possesses similar affinities, inhibiting KCa2.1, 2.2, and 2.3 with 1 nM, 110 pM and 2.1 nM IC₅₀'s respectively (Shah and Haylett, 2000 and Hosseini et al., 2001).

The pyrimidine (-)-B-TPMF, developed by NeuroSearch alongside the structurally similar positive gating modulator (-)-CM-TPMF (which I'll be discussing later), is another low nanomolar KCa channel inhibitor, but this compound is much more selective than the previously mentioned small molecules. (-)-B-TPMF inhibits KCa2.1 in the low nanomolar range, with an IC₅₀ of 31 nM, while requiring micromolar concentrations to effect either KCa2.2 or KCa2.3 (Hougaard et al., 2012). Especially unique for a KCa channel inhibitor, this compound binds in a pocket initially believed to accommodate only positive gating modulators, located between the S5 and S6 transmembrane segments and away from the central pore. It was shown to interact with a serine at position 293 which is exclusive to KCa2.1, explaining the compound's compelling selectivity. This serine 293 also happens to be the same residue known to interact with the two positive gating modulators GW542573X and (-)-CM-TPMF, making this binding pocket a very interesting site overall (Hougaard et al., 2012). The benzimidazole derivative NS8593 is equally

strange, seemingly able to shift calcium sensitivity of KCa2 channels rather than directly blocking channel conductance. This compound shifts the calcium EC₅₀ from 420 nM to 1.6 μ M, and itself possesses an IC₅₀ of 420, 600 and 730 nM for KCa2.1, 2.2. and 2.3 when in the presence of 500 nM free intracellular Ca²⁺ (Strøbaek et al., 2006). And against expectations, despite having such a different mechanism of action, it still seems to bind within the inner pore, not unlike the other more traditional KCa channel blockers (Jenkins et al., 2011).

Clotrimazole represents one of the first classically used KCa3.1 blockers, with reported IC₅₀ values ranging from 50 to 100 nM (Alvarez et al., 1992 and Stocker et al., 2003). It was even investigated for use in treating sickle cell anemia by reducing erythrocyte dehydration, however the compound also happens to be a potent inhibitor of cytochrome P450, preventing its use long term (Brugnara et al., 1996). It is worth mentioning that this P450 activity was used as the pharmacological basis for eventual FDA approval of the drug as a topical antifungal in 2004, for which it is still used today (Sawyer et al., 1975 and FDA/HHS, 2002). Clotrimazole would also be used as the basis for several SAR studies, both in industry and academia, leading to the discovery of several newer, more pharmacologically favorable compounds such as TRAM-34 and Senicapoc.

First synthesized by the Chandy lab in 2000, TRAM-34 is a triarylmethane derivative of clotrimazole which selectively inhibits KCa3.1 at low nanomolar concentrations (IC₅₀ = 20 nM) (Wulff et al., 2000). Like clotrimazole, TRAM-34 sits in the water-filled cavity directly under the selectivity filter, interacting with a threonine and valine at positions 250 and 275 respectively (Wulff et al., 2001). However, TRAM-34 exhibits no activity as a P450 inhibitor, due to its lack of an imidazole ring. Despite its selectivity, the compound is easily metabolized and has a short half, making it an ideal tool compound but lacking the properties necessary for direct development as a drug (Chen et al., 2011). Senicapoc, also known as ICA-17043, represents another clotrimazole derivative, discovered by Icagen in 2006, and inhibiting KCa3.1 with an IC₅₀ of 11 nM (Stocker et al., 2003). This compound exhibits more ideal pharmacokinetic properties than TRAM-34 and was even part of a clinical trial for treating sickle cell disease. But while the

compound was well tolerated by patients and measurably improved several hematological parameters, it failed to prevent sickle cell crises (Ataga et al., 2011). Despite this, senicapoc remains one of the most potent small molecule blockers of KCa3.1 and is used robustly as a tool compound for studying potassium channels in academia.

And lastly, developed by NeuroSearch in 2013, NS6180 is one of the more recent small molecule additions to the world of KCa-channel pharmacology. Possessing a novel benzothiazinone scaffold, this compound is unique among KCa-targeting small molecule inhibitors. However, pharmacologically speaking, NS6180 behaves near-identically to TRAM-34. Not only does NS6180 display equivalent potency and selectivity for KCa3.1 ($IC_{50} = 9 \text{ nM}$), but it also interacts with the same T250 and V275 residues (Strøbæk et al., 2013). As such, NS6180 has largely been confined to the shadows when it comes to commonly used KCa3.1-selective tool compounds.

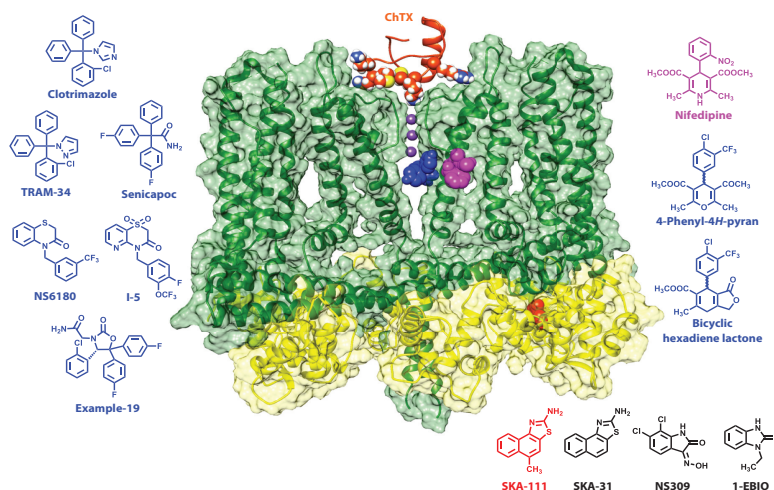


Figure 3.3, Pharmacology of the Intermediate-conductance calcium-activated K^+ Channel KCa3.1

Positive Allosteric Modulators

While the inhibitors targeting KCa channels generally possess significant selectivity between the KCa2.x and KCa3.1 channel subtypes, positive allosteric modulators targeting these channels are typically less selective. Riluzole is an extreme example of this. Approved for treating ALS in 1995, riluzole was first recognized for its neuroprotective properties, although the mechanism behind this neuroprotection is still somewhat clouded by the compound's promiscuity. Riluzole acts as a micromolar inhibitor of multiple different voltage and ligand gated channels, including the N- and P/Q-type calcium channels, the kainite and NMDA receptors, and voltage-gated sodium and potassium channels found in myelinated nerve fibers (Benoit and Escande, 1991, Debono et al., 1993, and Huang et al., 1997). And though possibly not the main contributor of its therapeutic effects, the compound also happens to be a non-selective positive gating modulator of the KCa2.x and 3.1 channels, with EC_{50} values ranging from 2 -8 μ M across the board. In any case, riluzole was eventually used as the structural backbone of multiple SAR studies, leading to the synthesis of several related compounds offering much higher potency and selectivity for KCa3.1, with slightly lower affinity for KCa2.1, 2.2 and 2.3 channels. These derivatives include SKA-31, SKA-111, and SKA-121.

SKA-31, first described by the Wulff lab in 2009, was selected out of a library of 41 benzothiazole derivatives as one of the more promising KCa-channel modulators (Sankaranarayanan et al., 2009). This compound exhibited favorable pharmacokinetic properties, with a half-life of around 12 hours. It also was shown to distribute more readily into plasma than tissue, which would be ideal for targeting endothelium-expressed KCa channels to treat something like hypertension. And more importantly, it demonstrated some selectivity for KCa3.1 (EC_{50} = 260 nM) over the small-conductance calcium activated potassium channels (EC_{50} = 1.9-2.9 μ M) (Sankaranarayanan et al., 2009). This potency and selectivity for KCa3.1 was further improved upon by the Wulff lab in 2014 with the synthesis of two additional benzothiazole derivatives, SKA-111 and SKA-121. Both compounds possess a similar low micromolar affinity for the small-conductance calcium-activated potassium channels, ranging in EC_{50} from 4.4-13.7 μ M, but they each activate KCa3.1 in the low nanomolar range (EC_{50} = 109-111 nM) (Coleman et al., 2014). Also of note is

the 200 to 400-fold selectivity over the Kv, Nav and Cav channels known to be “off-target” binders of riluzole. These benzothiazole compounds are believed to bind in the interface between the N-lobe of calmodulin and the intracellular S-4-S5 loop of the channel, although only the SKA-111 binding has been confirmed via site-directed mutagenesis up to this point (Shim et al., 2019). Additionally, SKA-111 does have higher brain penetrance than SKA-121, a feature which led to a drastic reduction in heart rate in mice (likely through cardiac and neuronal KCa2.x channel activation). As a result, SKA-121 might be the better therapeutic option for selective targeting of the peripherally expressed KCa3.1 channel.

Like riluzole, chlorzoxazone is another low affinity KCa channel modulator approved for therapeutic use despite a murky mechanism of action. Since its FDA approval in 1958, no significant steps have been made to determine how this muscle relaxant exerts its effect pharmacologically, mainly due to the existence of more effective therapeutics like diazepam removing the need for such knowledge. But it is known to act on KCa2.2 and KCa3.1 at high micromolar concentrations, with reported EC₅₀'s of 87 and 98 μ M respectively (Cao et al., 2001 and Pedarzani & Stocker, 2008). And this could certainly contribute to its pharmacodynamic effect.

Even less potent than chlorzoxazone but still deserving of a place in the history of KCa-channel pharmacology is the benzimidazolone 1-EBIO. This was in fact the first positive gating modulator of KCa3.1 to be discovered (Devor et al., 1996). Initially investigated for its' observed inhibitory effect on Cl⁻ secretion in T84 cells, this compound was eventually found to work via its affinity for KCa3.1, activating the channel with an EC₅₀ of 12- 84 μ M (Singh et al., 2001; Wulff and Köhler, 2013). 1-EBIO also modulates KCa2.1, KCa2.2 and KCa2.3 in even higher micromolar concentrations, with reported EC₅₀'s ranging from 80-650 μ M (Wulff and Köhler, 2013). Given the lack of especially head-turning potency, an SAR study was conducted in 2001 using 1-EBIO as the template, leading to the development of the dichlorinated analogue DC-EBIO. This new derivative touted a much more impressive 840 nM EC₅₀ for KCa3.1 while still activating KCa2 channels with low micromolar affinity (EC₅₀ = 16-27 μ M) (Singh et al., 2001; Pedarzani et al., 2007; Hougaard et al., 2007).

NeuroSearch developed their own dichlorinated KCa-channel modulator, NS309 (6,7-dichloro-1*H*-indole-2,3-dione 3-oxime), a few years later in 2004 (Strøbaek et al., 2004). Structurally similar to DC-EBIO, NS309 is significantly more potent, activating KCa3.1 with an EC₅₀ sitting in the low nanomolar range (10-90 nM) (Pedarzani and Stocker, 2008). This compound is also quite selective between KCa-channels, requiring concentrations about 10-20 times higher to achieve the same effect on KCa2.1, KCa2.2 and KCa2.3 (Hougaard et al., 2007; Pedarzani and Stocker, 2008). However, this compound also has affinity for the hERG channel which, in combination with somewhat poor pharmacokinetic properties, does pigeonhole the compound's utility outside of research (Strøbaek et al., 2004; Brown et al., 2020). Similar to both 1-EBIO and the benzothiazole derivatives such as SKA-121, NS309 produces robust 30-70 fold increases in KCa-channel current when applied at saturating concentrations in low calcium conditions (250 free Ca²⁺) (Brown et al., 2017). Additionally, all three compound types are capable of superagonism, increasing KCa-channel current amplitude even in saturating calcium conditions (10 uM free Ca²⁺). Taken altogether, this hints at a shared binding site, within the aforementioned interface between the N-lobe of Calmodulin and the intracellular S4-S5 linker, which we will explore further in Chapter 2.

Between 2007 and 2012, NeuroSearch also developed a handful of KCa2.x selective compounds, illuminating two additional binding pockets capable of accommodating positive gating modulators. The first of which is CyPPA (cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine), followed by its' more potent derivative NS13001. CyPPA positively modulates KCa2.2 and KCa2.3 selectively with low micromolar affinity (5.6-14 uM EC₅₀), while remaining inactive on both KCa2.1 and KCa3.1 (Hougaard et al., 2007). NS13001 follows suit with its' selectivity, remaining inactive on both KCa2.1 and KCa3.1, but displaying significantly increased potency on the remaining small-conductance calcium-activated potassium channels, especially KCa2.3 (EC₅₀ = 1.8 uM for KCa2.2 and 140 nM for KCa2.3) (Kasumu et al., 2012). Both compounds seem to exert their effect by causing a shift in the channel's calcium sensitivity, with saturating concentrations reportedly shifting the calcium EC₅₀ of the KCa2.x channels from the mid to low nanomolar concentrations (Hougaard et al., 2007; Kasumu et al.,

2012). It would later be confirmed through mutagenesis that CyPPA (and most likely NS13001 as well) is sitting in a novel binding pocket located between the C-lobe of calmodulin and the HA and HB helices which form the channel's calmodulin binding domain, with a single KCa3.1 substitution from arginine to the KCa2.2-equivalent lysine of its HB helix imparting the previously inactive CyPPA with KCa3.1 activity (Nam et al., 2022).

In direct contrast to CyPPA and NS13001 are the KCa2.1 selective positive gating modulators GW542573X and (-)-CM-TPMF. GW542573X, first described by NeuroSearch in 2009, was the first KCa2.1 selective compound to be developed (Hougaard et al., 2009). This compound activates KCa2.1 at low micromolar concentrations ($EC_{50} = 8.2 \mu M$), while requiring 10-100 fold increases in concentration to achieve the same effect on KCa2.2, 2.3 and 3.1 (Hougaard et al., 2009). In an attempt to increase potency, GW542573X was later modified in 2012, and the resulting compound (-)-CM-TPMF was shown to have much more robust affinity for KCa2.1 ($EC_{50} = 24 \text{ nM}$) while maintaining the same overall 10-100 fold selectivity over the remaining KCa channels (Hougaard et al., 2012). Aside from their rare KCa2.1 selectivity, these compounds are also unique with respect to their binding site, sitting in yet another novel pocket that accommodates the negative gating modulator (-)-B-TPMF. Like (-)-B-TPMF, both GW542573X and (-)-CM-TPMF bind adjacent to the selectivity filter between transmembrane segments S5 and S6, and all seem to rely on a critical interaction with a serine residue at position 293 (Hougaard et al., 2012). Equally noteworthy, both compounds not only act as superagonists (similar to NS309 and the benzothiazole “SKA” derivatives) but also are capable of activating the channel in the absence of calcium, proving themselves to be true KCa channel “openers” rather than the more ubiquitous calcium-sensitizers/stabilizers (Hougaard et al., 2009 & 2012).

Conclusion

Potassium channel pharmacology is an ever growing field, and new modulators are continuously being discovered, with many already on the drug market. Dofetilide and Ibutilide, both delayed rectifier

potassium channel (I_{Kr}) blockers, have been approved to treat persistent atrial fibrillation since the 90's (Kowey et al., 1997). Pinacidil and Cromakalim, ATP-sensitive potassium channel openers, have been used as vasodilators for even longer (Longman et al., 1988 and Friedel et al., 1990). Most widely used are still sulfonylureas such as tolbutamide and carbutamide, having been prescribed for treating type-II diabetes mellitus since the mid 1950's (Brown et al., 1956). And this represents merely a fraction of all currently FDA-approved potassium channel modulators. Despite this relative success, we believe there are many potassium channels still requiring further in-depth exploration as therapeutic targets, including the small- and intermediate-conductance calcium activated potassium channels $KCa2.1$, $KCa2.2$, 2.3 , and $KCa3.1$. By more thoroughly investigating how known modulators of these channels exert their effects, both spatially and mechanistically, we believe it is possible to further enrich our knowledge of not only how best to search for new modulators, but also how to design them to more selectively address unmet medical needs.

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Chapter 2: NS309 Functions as a Superagonist for the Calcium-Activated Potassium Channel

KCa3.1

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Abstract

NS309 (6,7-dichloro-1*H*-indole-2,3-dione-3-oxime) is widely used as a pharmacological tool to increase the activity of small- and intermediate-conductance calcium-activated potassium channels. NS309 is assumed to function as a positive allosteric gating modulator (PAM). However, its binding site and the molecular details of its action remained unknown. Here, we show that NS309 has a profound effect on the calcium dependent gating of K_{Ca}3.1. In inside-out experiments, 10 mM NS309 shifted the calcium EC₅₀ from 430 to 31 nM. In whole-cell experiments, changing free intracellular calcium from 250 nM to 3 mM decreased the EC₅₀ of NS309 from 74 nM to 8.6 nM. We further observed that NS309 could elicit greater responses than saturating calcium making it a “superagonist”. Molecular modeling suggested two possible binding sites for NS309 in K_{Ca}3.1 which we probed by mutagenesis and determined that NS309 is binding in the interface between the S₄₅A segment of the intracellular S4-S5 linker and the N-lobe of the channel associated calmodulin. Molecular dynamic simulations revealed that NS309 pushes several water molecules out of the interface pocket and establishes stable contacts with S181 and L185 in the S₄₅A segment of K_{Ca}3.1 and E54 in calmodulin and promotes longer sustained widening of the inner gate of K_{Ca}3.1 at V282 in the S6 segment. Hydrophilic substitutions of the hydrophobic gating residues V282 and A279 resulted in constitutively open channels that could not be further potentiated by NS309 suggesting that NS309 produces its agonistic effects by increasing the open probability of the inner gate of K_{Ca}3.1.

Significance Statement

Our study identifies the binding site of the K_{Ca} channel activator NS309 between the S4-S5 linker of $K_{Ca}3.1$ and the N-lobe of the channel associated calmodulin. NS309 acts as a stabilizing force within the gating interface of this calcium-activated potassium channel and increases the open probability of the inner hydrophobic gate. NS309 can induce greater responses than saturating calcium concentrations making it a “superagonist”.

Nonstandard abbreviations used: CaM, calmodulin; CaM-BD, calmodulin binding domain; CI, confidence interval; DMSO, dimethyl sulfoxide; EBIO, 1-ethylbenzimidazolin-2-one; HEK, human embryonic kidney; K_{Ca} , Ca^{2+} -activated K^+ channel; $K_{Ca}3.1$, intermediate-conductance Ca^{2+} -activated K^+ channel; K_V , voltage-gated K^+ channel; MD, molecular dynamics; NS309, 6,7-dichloro-1*H*-indole-2,3-dione-3-oxime; PAM, positive allosteric modulator; pdb, Protein data bank; SK, small-conductance K_{Ca} channel; SKA-111, 5-methylnaphtho[1,2-*d*]thiazol-2-amine.

Introduction

Small- and intermediate-conductance calcium-activated K^+ channels are voltage-insensitive, ligand-gated ion channels (Kaczmarek et al. 2017), that mediate membrane hyperpolarization when activated by their endogenous ligand: calcium. Structurally, the K_{Ca2} ($K_{Ca2.1}$, $K_{Ca2.2}$, $K_{Ca2.3}$) and $K_{Ca3.1}$ channels resemble voltage-gated K^+ channels (Alexander et al. 2023). They are tetramers of alpha subunits, each containing six transmembrane (TM) domains, with a pore located between domains S5 and S6. However, in contrast to Kv channels, the 4TM voltage sensor domain is not functional, and the channels are instead gated by calmodulin which is constitutively bound to the calmodulin binding domain (CaM-BD) in the C-terminus of each alpha subunit. A complete channel consists of eight subunits, four alpha subunits forming the membrane embedded part of the channel and four calmodulin molecules located on the intracellular side of the channel, where they can “sense” increases in free intracellular Ca^{2+} in the immediate surroundings of the channel. K_{Ca} channels open and mediate K^+ efflux with calcium EC_{50} values of ~ 250 nM for $K_{Ca3.1}$ and ~ 600 nM for the K_{Ca2} channels and can hyperpolarize cells toward the K^+ equilibrium potential in response to calcium influx or calcium release events in many important physiological processes (Kaczmarek et al. 2017). For example, in neurons K_{Ca2} channels underly the medium afterhyperpolarization and regulate firing frequency by preventing premature action potential generation (Adelman, Maylie and Sah 2012). In the vascular endothelium, $K_{Ca3.1}$ and $K_{Ca2.3}$ channels mediate the endothelium derived hyperpolarization response that can relax the underlying smooth muscle (Edwards, Feletou and Weston 2010). In erythrocytes $K_{Ca3.1}$ plays an important role in calcium-dependent volume regulation and in the immune system $K_{Ca3.1}$ modulates the activation of T cells, B cells, mast cells, microglia, and macrophages (Feske, Wulff and Skolnik 2015). $K_{Ca2/3}$ channels accordingly constitute potential drug targets for a wide range of neurological, cardiovascular, and immunological diseases (Brown et al. 2020).

How exactly calcium mediates K_{Ca} channel gating has been a matter of debate. An X-ray structure of the CaM-BD of $K_{Ca2.2}$ in complex with calmodulin solved in 2001 showed an anti-parallel dimer of two $K_{Ca2.2}$ C-terminal fragments connected by two calmodulins leading to the hypothesis that calmodulin

mediated CaM-BD dimerization would gate K_{Ca2} channels (Schumacher et al. 2001). Subsequently two groups independently regenerated this dimeric crystal and showed by crystallography and NMR that K_{Ca} channel activators like NS309 are binding in the interface between the calmodulin N-lobe and the CaM-BD when soaked into this dimeric crystal (Cho et al. 2018; Zhang et al. 2012). However, a long-standing conundrum in the field was how this dimer of dimers with its 2-fold symmetry could be fitted under, and then gate the 4-fold symmetric pore (Halling et al. 2014). With the publication of the $K_{Ca3.1}$ cryo-EM structure in both its closed and open conformations in 2018 this mystery was solved (Lee and MacKinnon 2018). In the closed conformation, the C-lobes of the calmodulin molecules are bound to the CaM-BDs of the alpha subunits, while the calmodulin N-lobes remained flexible and therefore were not visible in the structure. However, in the presence of calcium, the calmodulin N-lobes “swing” over and bind to the S4-S5 linker of the adjacent alpha subunit. The S4-S5 linkers are pulled downward, which shifts the S6 helices and triggers opening of the gate located at the pore-lining S6 residue V282 (Lee and MacKinnon 2018).

The $K_{Ca3.1}$ cryo-EM structures beautifully explained the calmodulin mediated gating mechanism, but also suggested a reinterpretation of the NS309 binding site. NS309 (6,7-dichloro-1*H*-indole-2,3-dione-3-oxime), the currently most widely used positive allosteric modulator (PAM), was first described in 2004 in a rapid report by scientists at the Danish company NeuroSearch (Strobaek et al. 2004). Subsequently, more detailed studies showed that NS309 increases the apparent calcium sensitivity of K_{Ca} channels (Hougaard et al. 2007). In inside-out recordings, NS309 was found to left-shift the calcium concentration response of $K_{Ca2.3}$ in a concentration-dependent fashion and to reduce the calcium EC_{50} from 450 nM in the absence of NS309 to as low as 25 nM in the presence of 30 mM of NS309. However, unlike the openers of large-conductance calcium-activated $K_{Ca1.1}$ (a.k.a. BK) channels that can activate currents in the absence of calcium, NS309 is a gating modulator and requires at least some calcium to be present (Hougaard et al. 2007). We here took a closer look at the effects of NS309 on $K_{Ca3.1}$, the K_{Ca} channel it activates most potently but on which it has not been well studied. NS309 not only increases the calcium sensitivity of $K_{Ca3.1}$, but, *vice versa*, calcium increases the potency of NS309. We also observed “superagonism”,

meaning that NS309 can elicit responses that are greater than the responses of K_{Ca}3.1 to saturating calcium. Based on *in silico* modeling and mutagenesis experiments NS309 exerts its superagonistic effects by binding between S181 and L185 in the S₄₅A segment of the S4-S5 linker and the N-lobe of calmodulin and thus stabilizes the hydrophobic gate at V282 of K_{Ca}3.1 in the open state.

Methods

In-silico Protein Preparation and Molecular Modeling

Cryo-EM structures (Lee and MacKinnon 2018) of the full length human K_{Ca}3.1 channel in open state 1 (PDB: 6CNN) and open state 2 (PDB: 6CNO) were used as templates for all molecular modeling described. Prior to either ligand docking or molecular dynamics simulations, both structures were first refined using the cryo-EM refinement protocol of the Rosetta software suite (Wang et al. 2016). Using Rosetta software and the REF2015 energy function, loop modeling was performed to fill in the 19 missing residues of the S3-S4 extracellular loop which are not resolved in the cryo-EM structures (Alford et al. 2017). For this loop modeling protocol, the Rosetta remodel algorithm was used to generate the basic structure of the loop. Subsequently, the Rosetta kinematic closure algorithm was used to repack the loop into a more stable configuration (Bender et al. 2016). This was followed by a round of Rosetta fast relax. 10,000 loop configurations were generated, and the lowest scoring conformations were evaluated by eye. The final loop position was selected as the lowest scoring loop configuration that did not clash with the lipid membrane as determined by the Orientations of Proteins in Membranes database (Lomize et al. 2012).

For potential binding site identification, the refined 6CNN pdb file was uploaded into Schrödinger's molecular modeling suite (Schrödinger, LLC, New York, NY, 2023) and preprocessed, before being submitted to the SiteMap program (Halgren 2007) using all default parameters. Each binding site required the presence of a minimum of 50 so-called site points (coordinates set 1 Å apart and forming a grid which is used to define a potential pocket capable of accommodating a small molecule ligand). Schrödinger's ligand docking platform, Glide (Friesner et al. 2004), was then used to dock NS309 into each of the

predicted binding sites using the standard precision docking mode, featuring Glide's proprietary "Emodel" scoring system, which most heavily emphasizes predicted coulomb/van der Waals interactions between ligand and receptor (Friesner et al. 2004). In this standard precision docking mode, compounds are first run through a conformer generation stage, followed by a rough scoring protocol to determine the most favorable poses of the ligand within a rigid receptor pocket. Top ranked poses are then refined using the OPLS3 forcefield before being minimized within the field of the receptor (Harder et al. 2016). For all Glide docking experiments, we used an inner box size of 8 Å and an outer box size of 16 Å, with all other ligand docking parameters set to the default options.

For RosettaLigand docking (Davis and Baker 2009), conformers of NS309 were first generated with Open Eye OMEGA software version 4.2.2.0 (OpenEye Scientific Software, Santa Fe, NM; <http://www.eyesopen.com>) and then placed randomly into the potential binding pocket and merged with the Rosetta-refined 6CNN pdb using UCSF Chimera software (Resource for Biocomputing, Visualization, and Informatics, San Francisco, CA). That merged pdb file was then taken through three stages of RosettaLigand docking as described in (Shim et al. 2019), with the top 1000 models filtered by lowest total energy score, and the top ten of those filtered via interface energy for comparison.

Molecular Dynamics Simulation and Analysis

A single protein chain of the K_{Ca}3.1 Rosetta model based on 6CNO (open state 2) was copied four-fold to create a symmetric protein model using UCSF ChimeraX (Meng et al. 2023). Systems were built with either zero, two (placed on opposite protein chains), or four NS309 molecules. The initial position of NS309 was determined from RosettaLigand docking as described above (see Figure 3). General CHARMM forcefield (CGenFF) parameters (Vanommeslaeghe et al. 2010) for the NS3909 molecule were generated by CGenFF web server (cgenff.com). CHARMM-GUI membrane builder was used to generate the MD simulation systems using the CHARMM36m protein (Huang et al. 2018), C36 lipid force field (Klauda et

al. 2010), standard CHARMM ion force field parameters (Beglov and Roux 1994), atom pair-specific Lennard-Jones parameters (NBFI) for aromatic chlorine – protein interactions (Lin and MacKerell 2018), and TIP3P water model (Jorgensen et al. 1983). The K_{Ca}3.1 channel was embedded in a lipid bilayer composed of POPC, POPS, POPE, and cholesterol in a 25:5:5:1 ratio as was done in a previous simulation of K_{Ca}2 channels (Nam et al. 2022). The system was solvated with 0.15 M KCl aqueous solution. Four potassium ions were placed in the selectivity filter of the K_{Ca}3.1 channel and three calcium ions were placed in the calcium-binding sites of each calmodulin using their positions in the cryo-EM structure (6CNO). Amber20 PMEMD was the MD engine used to perform the simulations. The standard minimization protocol followed by a six-step staged equilibration from the CHARMM-GUI generated Amber input files was used for the MD simulation systems (Jo et al. 2008; Lee et al. 2016; Wu et al. 2014). The equilibration was then extended for an additional 10 ns with a 1 kcal/mol/Å² harmonic restraint placed on all backbone protein atoms. Subsequently, all restraints were removed, and the production MD simulation was run for at least 500 ns. Three replicate simulations were run for each condition, totaling 4.5 μs of production MD simulation.

Trajectories were analyzed using custom Python scripts making use of the MDAnalysis Python library (Michaud-Agrawal et al. 2011). The pore opening was assessed based on the minimum distance between any of the hydrogen atoms on V282 residues of opposite chains. The equilibration procedure was designed to maintain a hydrated pore and prevent sudden closing of the S6 helical gate. If the intracellular gate distance fell below a critical value of 7 Å within 20 nanoseconds of starting the production MD run, the simulation was discarded. This critical value represents the approximate size of a hydrated K⁺ ion, narrower pores are not able to conduct hydrated ions (Moldenhauer et al. 2016). This rapid closing and dehydration of the pore occurred in roughly half of the simulations and was more likely to occur if there were no NS309 molecules in the system. Specifically, five of eight no-ligand simulations, five of eight two-ligand simulations and two out of five four-ligand simulations exhibited this rapid closing and were discarded. Closure of the pore below 7 Å also lead to a loss of hydration of the channel pore. Pore hydration

was analyzed using the Visual Molecular Dynamics (VMD) program (Humphrey, Dalke and Schulten 1996) to evaluate whether there was a continuous chain of water molecules connecting the bulk water on the intracellular side of the channel to the bottom of the selectivity filter. In assessing the significance of the pore widening effect of NS309, the simulation trajectories were binned into segments of 180 ns. The average of the gate distance across this 180 ns bin was taken as one observation. The autocorrelation time of the simulation was calculated to be on the order of 100 ns such that the separate bins can be considered independent observations of the pore diameter. Accordingly, an ANOVA test was performed and there was found to be a significant difference between average values for different simulation conditions. A Tukey's Honestly Significant Difference Test (Tukey's HSD, implemented in R) was used to determine p-values. Hydrogen bond contacts were defined as interactions between NS309 and protein residues with donor-acceptor distances less than 3.5 Å and donor-acceptor angles of at least 100°. Hydrophobic van der Waals contacts were taken as non-bonded interactions with heavy-atom distances less than 4 Å.

Molecular Biology

All mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and verified by Sanger sequencing. The human K_{Ca}3.1 gene *KCNN4* (NCBI reference sequence: NP_002241.1) inserted into a pEGFP-C1 expression vector was used as the template for all K_{Ca}3.1 mutations (Wulff et al., 2001). For K_{Ca}2.2 mutations, the rat K_{Ca}2.2 gene *KCNN2* (NCBI reference sequence: NP_051353.1) subcloned into a pcDNA3.1+ vector served as the mutagenesis template.

The following primers were used to generate our hK_{Ca}3.1 and rK_{Ca}2.2 mutants:

hKCa3.1-N201F forward 5-TACATGTTTACGCACCCTGGCCGCCTG-3;

hKCa3.1-N201F reverse 3-CACCGGTTCGAAATGTACAAGTGCGTG-5;

hKCa3.1-N201A forward 5-TACATGGCCACGCACCCTGGCCGCCTG-3;

hKCa3.1-N201A reverse 3-CACCGGTTCGAAATGTACCGGTGCGTG-5;

hKCa3.1-R287A forward 5-GTGGCCGCGAAGCTGGAGTTTAACAAG-3;

hKCa3.1 R287A reverse 3-GACCACCGGCACCACCGGCGCTTTCGAC-5;

hKCa3.1-L185A forward 5-GGCGCTGCCAATCAAGTCCGCTTCCGC-3;

hKCa3.1 L185A reverse 3-ATGGCGTCGTAGCCGCGACCGGTTAGTT-5;

hKCa3.1-S181A-L185A forward 5-TACCGCGCCATCGGCGCTGCCAATCAA-3;

hKCa3.1-S181A-L185A reverse 3-GAGTTGCGAAGGATGGCGCGGTAGCCG-5;

hKCa3.1-V282G forward 5-ACAGCCCTGCTGGGGGCCGTGGTGGCC-3;

hKCa3.1-V282G reverse 3-TGTCGGGACGACCCCCGGCACCACCGG-5;

hKCa3.1-A279G forward 5-TGCACAGGGCCTGCTGGTGGCCGTGGTG-3;

hKCa3.1-A279G reverse 3-TACCCACAGACGACGTGTCCGGACGAC-5;

rKCa2.2-S288A forward 5-TCTAGAGGCCATTGGGGCACTTAATAAG-3;

rKCa2.2-S288A reverse 3-TGGCTACGGAGGAGATCTCGGTAACCC-5;

rKCa2.2-S288A-L292A forward 5-GGGGCAGGCTAATAAGATAAACTTCAAT-3;

rKCa2.2-S288A-L292A reverse 3-AGATCTCGGTAACCCCGTCGATTATTC-5.

Mutant channels were transfected into HEK-293 cells (ATCC, product #CRL-1573) using the Eugene 6 transfection reagent, according to Promega's suggested protocol (<https://www.promega.com/resources/protocols>). Opti-MEM reduced serum media (Life Technologies, Benicia, CA) was used during the transfection to ensure high transfection efficiency. For K_{Ca}2.2 mutagenesis a pEGFP-C1 expression vector was co-transfected along with the mutant channel at a ratio of 1:3 respectively. After 24 hours cells were trypsinized and prepared for the manual patch-clamp experiments described below.

Chemicals and Reagents

Laboratory chemicals used for preparation of internal and external electrophysiology solutions were purchased at the purest available grade from commercial vendors. NS309 was purchased from Sigma-Aldrich (St. Louis, MO). Charybdotoxin (ChTx) was purchased from Alomone Labs (Jerusalem, Israel). Compound stock solutions were prepared in dimethyl sulfoxide (DMSO), at 10 mM for NS309 and 100 mM for Charybdotoxin. Dilutions were prepared immediately prior to perfusion in sodium ringer external solution.

Electrophysiology

All electrophysiology experiments were performed in the whole-cell or inside-out configuration on transiently transfected HEK-293 cells, with Green Fluorescent Protein (GFP) either co-transfected or already tagged to the plasmid of interest. Cells were cultured in a high glucose Dulbecco's Modified Eagle

Medium (Gibco, Long Island, NY) containing 110 mg/mL sodium pyruvate, 4.5 g/L D-Glucose and L-glutamine, and supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin and 100 ug/mL streptomycin. For whole-cell experiments, cells were transfected in 12-well plates for 24 hours before being detached with 75 mL TrypLE Express (Gibco, Long Island, NY), resuspended in cell culture medium, and plated on coverslips precoated with poly-L-lysine (Gibco, Long Island NY). Cells were allowed to adhere to the coverslips for a minimum of 1 hour before whole-cell patch-clamp experiments. For inside-out experiments, cells were transfected directly on their glass coverslips for 24-48 hours. Coverslips were then shattered as needed and placed in the microscope bath chamber to circumvent the need for cell detachment.

An inverted Olympus XL-70 microscope (Olympus, Tokyo, Japan) equipped with a pE-300lite illumination system (CoolLED, Andover, NY) was used to visualize and select transfected cells expressing GFP. Whole-cell patch-clamp experiments were conducted with an EPC-10 amplifier (HEKA, Lambrecht/Pfalz, Germany), with a holding potential of -80 mV applied between ramp protocols. Pipettes were pulled from soda lime glass (micro-hematocrit tubes; Kimble Chase, Rochester, NY), and had resistances which ranged from 1.5 to 2.5 M Ω . Solutions of NS309 in Na⁺ Ringer were freshly prepared immediately before perfusion from 10 mM stock solutions in DMSO, with the final DMSO concentration never exceeding 0.1%. For current measurements, we used an internal pipette solution containing 154 mM KCl, 1.75 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, and either 6 mM CaCl₂ (250 nM free Ca²⁺), 9.45 mM (3 μ M free Ca²⁺), or 9.85 mM (10 mM free Ca²⁺), with a pH of 7.2 and an osmolarity of ~ 300 mOsm. A potassium fluoride solution containing 160 mM KF, 2 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, a pH of 7.2, and an osmolarity of ~ 300 mOsm was used as a zero-calcium control. Na⁺ Ringer was used as an external solution and contained the following: 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, with a pH of 7.4 and an osmolarity of ~ 300 mOsm. For internal solutions used, free Ca²⁺ concentrations were calculated with MaxChelator (<https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/>), assuming a temperature of 25°C and a pH of 7.2.

Inside-out patch clamp experiments were conducted with an Axon200B amplifier (Molecular Devices, San Jose, CA), using a holding potential of 0 mV between ramp protocols. Pipette resistances ranged from 2.5 to 5 M Ω , with an internal solution containing 140 mM KCL, 10 mM HEPES, and 1 mM MgSO₄. The external bath solution contained 140 mM KCl, 10 mM HEPES 1 mM EGTA, 0.1 Dibromo-BAPTA, and 1 mM HEDTA, with varying free calcium concentrations also calculated via MaxChelator. For inside-out experiments, calcium concentrations were verified using a Ca²⁺ Calibration buffer kit (ThermoFisher Scientific, Waltham MA).

All mutant channels were patch-clamped sequentially with 250 nM free Ca²⁺ and 10 mM free Ca²⁺ internal KCl solutions, to ensure that calcium sensitivity was unaffected. Any mutant which exhibited either nA current at 250 nM free Ca²⁺ or sub-nA current at 10 mM free Ca²⁺ was deemed to have altered calcium sensitivity and excluded (in this case R287E, R287Q and N201D as well as two S181 mutations that introduced bulky, aromatic residues, S181Y and S181W). Currents were elicited by either a 200-ms voltage ramp from -120 to 40 mV applied every 10 s (for whole-cell recordings), or a 1-s voltage ramp from -100 to 100 mV applied every 10 s (for inside-out experiments). For whole-cell recordings, the fold increase of slope conductance at -80 mV following NS309 perfusion was taken as a measure of channel modulator activity. Concentration response curves were fitted using GraphPad Prism9 (GraphPad Software, La Jolla, CA). All data points represent at least three independent experiments ($n \geq 3$) presented as mean $\pm$ standard deviation (SD), and EC₅₀s are reported with 95% confidence intervals (CI).

Results

NS309 can produce maximal responses greater than the endogenous agonist calcium on intermediate- and small-conductance calcium-activated potassium channels

NS309 has previously been shown to leftward shift the calcium concentration response curve of K_{Ca}2.2 and K_{Ca}2.3 channels (Hougaard et al. 2007; Pedarzani et al. 2005) and therefore has been termed a positive allosteric gating modulator that enhances the sensitivity of K_{Ca} channels to their endogenous ligand,

calcium. However, since no experiments studying the effect of NS309 on the calcium sensitivity of $K_{Ca3.1}$ are available in literature, we performed inside-out experiments on HEK cells transfected with $K_{Ca3.1}$, where we varied the intracellular calcium concentration and observed the response in the presence and absence of NS309 (Fig. 1A). In the presence of 10 mM of NS309, the calcium EC_{50} for $K_{Ca3.1}$ activation shifted from 430 nM to 31 nM. However, in addition to a quite dramatic left-ward shift there also was an increase in maximum effect even at saturating free calcium concentrations (Fig. 1A). Intriguingly, in whole-cell experiments, where we kept the intracellular calcium concentration constant but varied the concentration of NS309 in the bath, the EC_{50} of NS309 shifted from 74 nM in the presence of 250 nM of free calcium to 8.6 nM in the presence of 3 mM of free calcium (Fig. 1B). Both the increase of calcium sensitivity by NS309 and the increase in NS309 potency at higher calcium concentrations confirm that NS309 modulates the calcium dependent gating of $K_{Ca3.1}$.

Since we were somewhat astonished to see that NS309 can induce responses that are greater than the response to saturating calcium (Fig. 1), we next investigated if this “superagonistic” effect was exclusive to $K_{Ca3.1}$ or would also occur with the K_{Ca2} channels. In whole-cell experiments, perfusion of 10 mM of NS309 produced robust, on average 2.3-3.5 fold current increases on all K_{Ca} channel subtypes when cells were dialyzed with 10 mM of free calcium. The three K_{Ca2} channels, $K_{Ca2.1}$, $K_{Ca2.2}$ and $K_{Ca2.3}$, are slightly less sensitive to calcium than $K_{Ca3.1}$, with reported calcium EC_{50} values ranging from roughly 400 to 900 nM but are fully saturated with 10 mM of free calcium (Kaczmarek et al. 2017). No significant difference was observed between the current increases on $K_{Ca3.1}$ and the three K_{Ca2} channels demonstrating that NS309 acts as a superagonist on all four of these calmodulin gated channels (Fig. 1C and 1D).

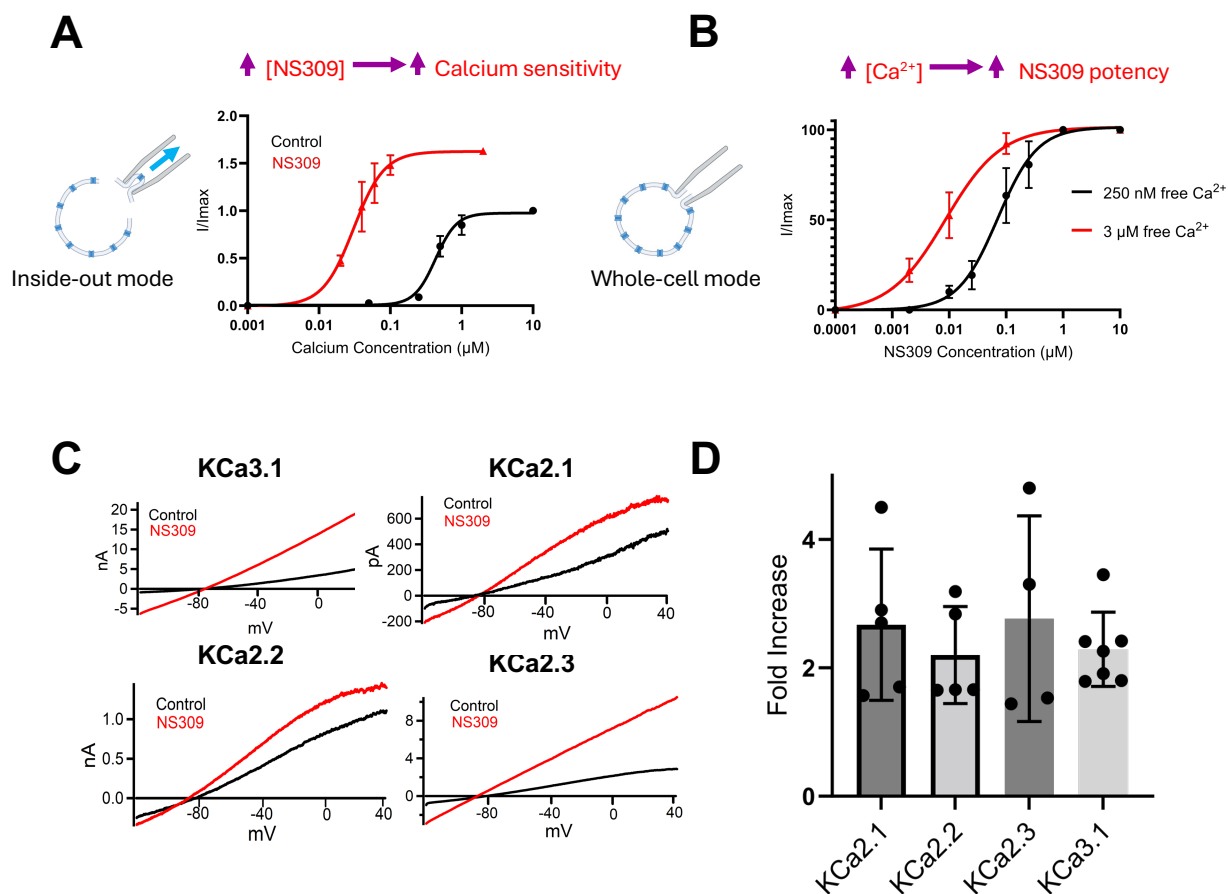


Figure 1. NS309 exhibits superagonistic properties on KCa channels. A. Ca^{2+} -concentration response curve for KCa3.1 activation measured from inside out patches in absence or presence of 1 μ M NS309, displaying a calcium EC_{50} of 430 nM for the control curve (95% CI: 365-502 nM, n_H 2.9; $n=3$ for all data points) and 31 nM in the presence of NS309 (95% CI: 22-41 nM, n_H 2.0, $P < 0.0001$; $n=2$ for 20 nM and 40 nM data points, and $n=3$ for remaining). B. NS309 concentration response curves obtained via whole-cell patches in 250 nM free calcium (black) and 3 μ M free calcium (red), with measured NS309 EC_{50} values of 74 nM (95% CI: 55-95 nM, n_H 1.33) and 8.6 nM (95% CI: 2.6-20 nM, n_H 0.96, $P < 0.0001$) respectively. C. Whole-cell recordings of KCa2.1, 2.2, 2.3 and 3.1 in saturating calcium conditions (10 μ M intracellular calcium), with the effect of a 10 μ M NS309 perfusion shown in red. D. Scatter plot displaying NS309's degree of superagonism on KCa2.1, KCa2.2, KCa2.3 and KCa3.1 in saturating calcium conditions (error bars represent SD from the mean). P values were calculated using an extra sum-of-squares F test (GraphPad Prism 10; GraphPad Software, La Jolla, CA). Each data point represents mean $\pm$ SD.

Virtual exploration of KCa3.1 for potential NS309 binding sites using Schrodinger's GLIDE

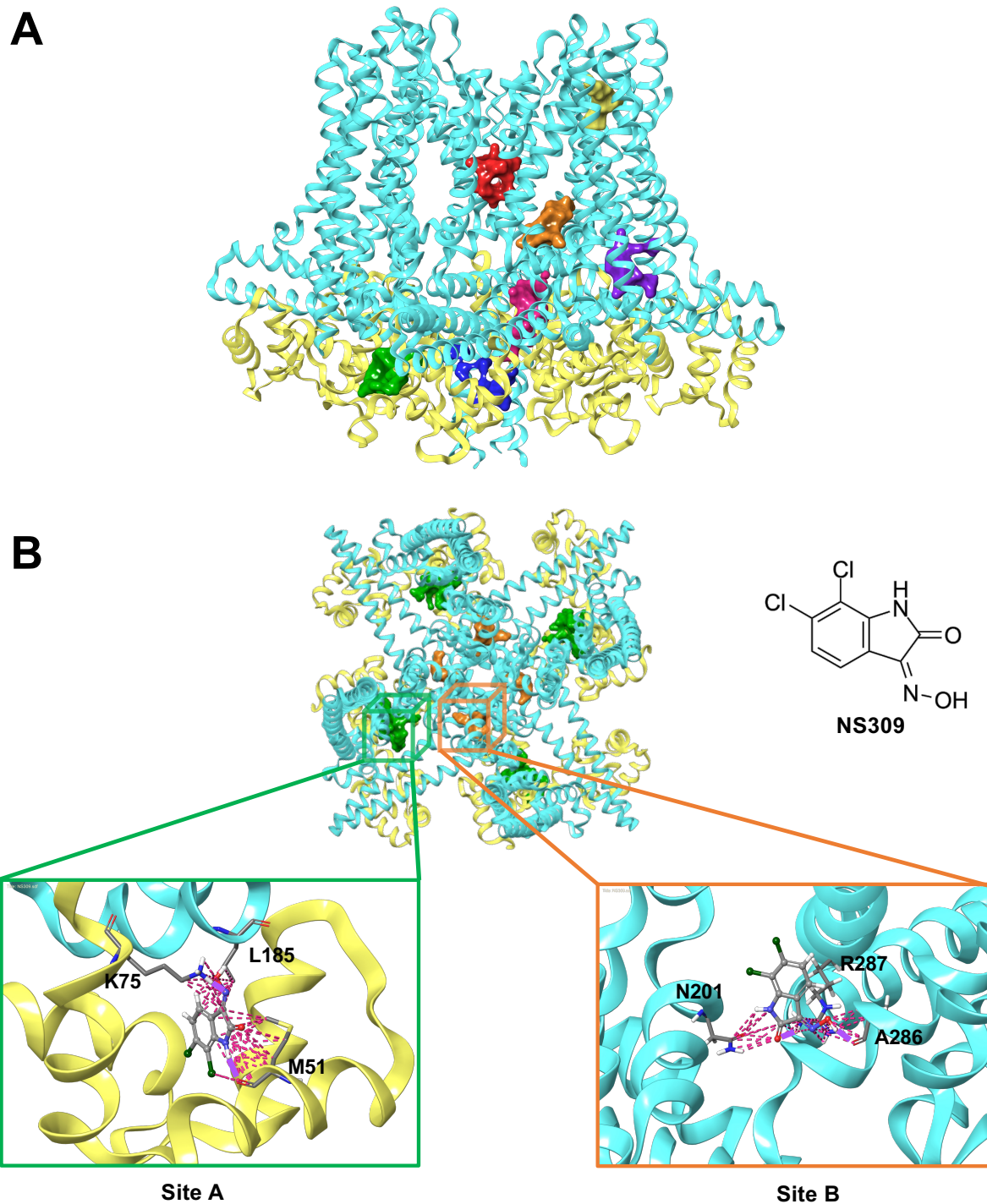


Figure 2. Exploration of potential KCa3.1 binding sites using Schrödinger's GLIDE. A. 7 distinct possible binding sites for KCa3.1 modulation, as highlighted via the SiteMap function of Schrödinger's GLIDE. B. Standard precision GLIDE Docking of NS309 in Sites A (green) & B (orange), with binding free energy scores reported at -6.9 and -7.05 kcal/mol respectively. Van der Waal's interactions are shown as dashed pink lines and H-bonds are shown in solid purple.

As a large tetrameric protein, K_{Ca}3.1 contains many potential binding sites for small molecule modulators. We used Schrödinger's molecular modeling software platform to identify pockets potentially capable of accommodating NS309. The full length cryo-EM structure of K_{Ca}3.1 in open state 1 (PDB ID: 6CNN) was first screened using Schrödinger's SiteMap feature. This initial exploration identified seven distinct possible binding sites, each represented by a colored object in the K_{Ca}3.1 structure, with calmodulin depicted as pale-yellow ribbons and the channel itself in light blue (Fig. 2A). Some of these highlighted pockets were previously known, including the triaryl methane binding site (Nguyen et al. 2017) located underneath the selectivity filter (shown in red) and the SKA-type activator binding pocket (Shim et al. 2019) located within the interface of calmodulin's N-lobe and the S₄₅A helix in the S4-S5 linker (shown in green in Fig. 2A). Another site located between the calmodulin's constitutively bound C-lobe and the HA and HB helices of K_{Ca}3.1 (shown in navy blue on the site map in Fig. 2A) is strikingly similar to the binding site of CyPPA, a low micromolar activator of K_{Ca}2.2 and K_{Ca}2.3 channels (Nam et al. 2022). The four remaining pockets identified by SiteMap were located between the lower half of the S2 transmembrane helix and the CaM-BD of the HB segment (purple), in the enclosed space between the S1-S4 transmembrane helices (yellow), between the tail ends of the HB helices of adjacent subunits (magenta), and finally a pocket sitting just above the S4-S5 linker S₄₅B helix between the S5 and S6 transmembrane segments (orange). To assess which of these potential binding pockets best fits NS309, the molecule was docked into each of these sites using the standard precision mode of Schrödinger's small molecule ligand docking platform, Glide. Of the seven binding pockets, the yellow pocket within the S1-S4 cage "kicked out" the compound, not having enough space for the molecule. The red, purple, magenta, and navy-blue pockets were able to accommodate NS309, albeit with relatively low binding free energies of -4.64 (red), -4.11 (purple), -4.88 (magenta), and -4.82 kcal/mol (navy-blue). The green and orange pockets (designated as Site A and Site B; shown in a top-down view in Fig. 2B) produced much more favorable binding energy scores of -6.9 and -7.05 kcal/mol, respectively. Within both pockets, NS309 was predicted to form two hydrogen bonds with receptor side chains, along with a plethora of van der Waals interactions. In Site A (green pocket), NS309 was predicted to form hydrogen bonds with K75 and M51 of the CaM N-lobe, in addition to forming multiple van der

Waals interactions with L185 on K_{Ca}3.1's S₄₅A segment in the S4-S5 linker. In Site B (orange pocket), NS309 was instead predicted to form hydrogen bonds with R287 of one subunit's S6 transmembrane segment and A286 from the adjacent subunit's S6 segment, again alongside multiple van der Waals, specifically with residue N201 of S₄₅B. Both Site A and Site B therefore seemed attractive possible binding sites.

Ligand docking simulation using RosettaLigand

We next used RosettaLigand to re-dock NS309 in the two most promising binding pockets: Site A and Site B. Unlike our docking simulations with Glide's standard precision mode, which used a rigid receptor and produced a single docking pose, RosettaLigand allowed us to dock NS309 with much higher receptor sidechain flexibility, enabling a pseudo induced-fit and thus theoretically increasing the accuracy of the results. RosettaLigand also allowed us to generate more starting poses for NS309 within each pocket, enabling us to use convergence of the top poses as an indicator of how favorable a particular pose might be. The more aligned the top poses are, the stronger the computational reproducibility of the results.

NS309 was manually placed into Site A and Site B and 10,000 poses in each pocket generated with RosettaLigand. Of those 10,000, the top 1,000 were sorted based on total energy scores of the NS309-K_{Ca}3.1 complex. These top 1,000 poses were then further filtered, this time by interface energy scores, down to the top ten energetically most favorable poses, which can be seen overlaid in Figure 3. Visually, there was an immediately obvious difference in convergence in Site A versus Site B. In Site A, all top ten poses were nearly identical (Fig. 3A). In Site B in contrast, the top ten poses were far more diverse, suggesting that this site is less favorable for NS309 (Fig. 3C). In Site A, NS309 was predicted to form a single hydrogen bond between its oxime hydrogen and residue E54 of calmodulin's N-lobe in all top ten poses. The remaining interactions between NS309 and the protein complex were van der Waals interactions, mainly involving calmodulin residues K75, M51, M71 and V55, and residue L185 in K_{Ca}3.1's S4-S5 linker (Fig. 3B). For Site B, although the top ten Rosetta-generated poses visibly differed, 5 of the ten poses

showed the same predicted interactions with NS309's oxime oxygen and hydrogen making hydrogen bonds with residues R287 and A286 of K_{Ca}3.1's S6 transmembrane segment, very similar to the interactions predicted by Schrödinger's Glide. However, RosettaLigand also predicted an additional hydrogen bond forming between NS309's amine hydrogen and residue N201 at the tail end of K_{Ca}3.1's S45B segment. All remaining interactions were van der Waals interactions involving those same three Site B residues.

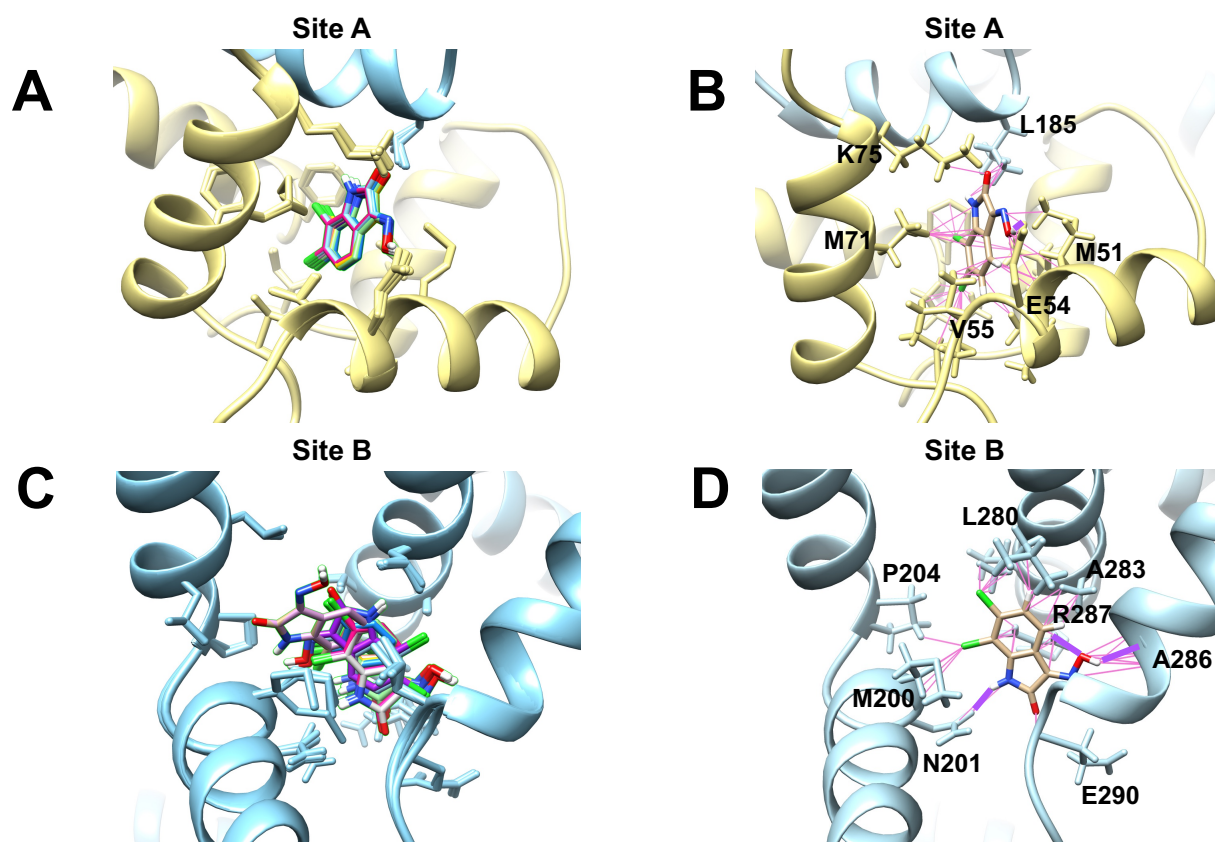


Figure 3. Rosetta Docking of NS309 in Sites A & B. A, Convergence of top 10 poses within Site A. B, top ranked pose in Site A, with VDW forces in pink and H-bonds in purple. C, Convergence of top 10 poses within Site B. D, top ranked pose in Site B, with VDW interactions in pink and H-bonds in purple.

NS309 binds in the interface between calmodulin's N-lobe and the S4-S5 linker of both K_{Ca}3.1 and K_{Ca}2.2

Based on the molecular modeling results we decided to probe both Site A and Site B by mutagenesis. Given that Site A had been previously explored when mapping the binding site of SKA-111 (Shim et al. 2019),

the two mutations which had induced the largest shifts in SKA-111 potency were reused for our purposes here. These mutations were the single mutant L185A, a residue featured heavily in both of our *in silico* docking experiments, and the double mutant S181A-L185A. Whole-cell patch-clamp experiments revealed that both mutants produced a significant rightward shift of the NS309 concentration response curve, pushing NS309's EC_{50} from 74 nM (wild-type, shown in black in Fig. 4A) to 1.27 μ M for the L185A mutant and 1.10 μ M for the S181A-L185A double mutant (shown in yellow and purple in Fig. 4A, respectively). This difference in NS309 potency is further highlighted in two representative whole-cell recordings shown in Fig. 4B and Fig. 4C. While 250 nM of NS309 is sufficient to increase wild-type currents by 36-fold (Fig. 4B), more than 10 mM of NS309 is required to produce a similar effect on the S181A-L185A double mutant (Fig. 4C). Given the robust effect of the L185A and the S181A-L185A mutant and the fact that all other Site A residues predicted to interact with NS309 are on calmodulin's N-lobe, we made no further mutations to test Site A.

For Site B we decided to focus our mutagenesis on residues N201 and R287 because all predicted interactions between NS309 and A286 were with the residue's backbone rather than the sidechains. Varying concentrations of NS309 were tested on mutant channels N201A, N201F, and R287, and EC_{50} values were calculated for each. Unlike the Site A mutations, most of these Site B mutants failed to exert a significant effect on NS309 potency. Shown in Fig. 4A in red and blue, mutations N201A and R287A responded very comparably to the wild-type channel, with EC_{50} values of 85 and 98 nM respectively. N201F resulted in a slightly larger rightward shift, with an observed EC_{50} value of 195 nM compared to the 74 nM EC_{50} of the wild-type channel. Although, statistically significant, this shift was much smaller than the shifts induced by the Site A mutations and presumably due to the introduction of the bulky phenylalanine residue. A smaller alanine residue in the same position did not affect change the NS309 EC_{50} (Fig. 4A).

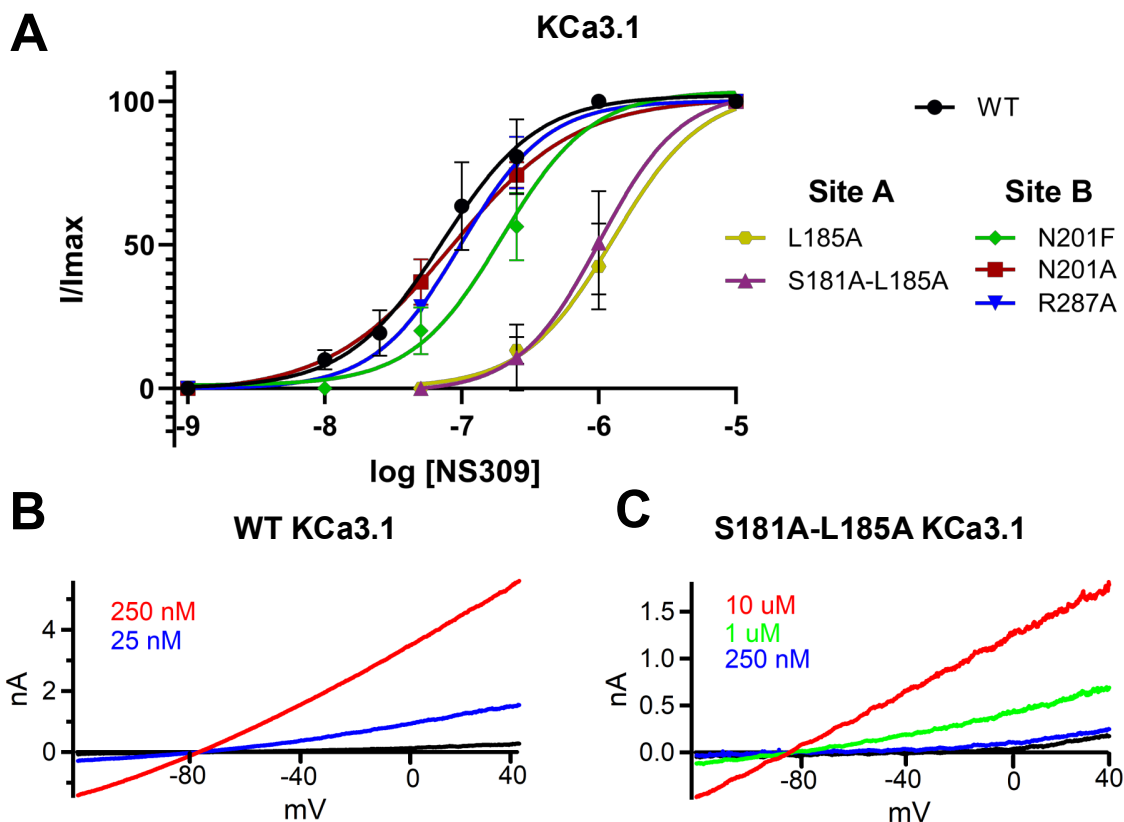


Figure 4. Effect of NS309 on various KCa3.1 mutations within Site A and B. A. Concentration response curves of NS309, on WT KCa3.1 in black ($EC_{50} = 74$ nM, 95% CI: 55-95 nM, n_H 1.33), L185A in yellow ($EC_{50} = 1.27$ μM, 95% CI: 945 nM-1.83 μM, n_H 1.350, $P < 0.0001$), S181A-L185A double mutant in purple ($EC_{50} = 1.1$ μM, 95% CI: 730 nM-1.61 μM, n_H 1.527, $P < 0.0001$), N201F in green ($EC_{50} = 195$ nM, 95% CI: 138-264 nM, n_H 1.34, $P < 0.0001$), N201A in red ($EC_{50} = 85$ nM, 95% CI: 64-112 nM, n_H 1.2, $P = 0.4679$) and R287A in blue ($EC_{50} = 98$ nM, 95% CI: 77-125 nM, n_H 1.51, $P = 0.1309$). Each data point on curve represents an n of 3, displayed as mean $\pm$ SD. B, the effect of NS309 at 25 nM and 250 nM concentrations on WT KCa3.1 ($EC_{50} = 74$ nM). C, the effect of NS309 at 250 nM, 1 μM and 10 μM concentrations on the S181A-L185A double mutant ($EC_{50} = 1.1$ μM). For both recordings, a voltage ramp protocol was applied, going from -120 to +40 mV over 200 ms.

An analogous mutagenesis experiment was repeated on the related K_{Ca}2.2 channel, this time using just the equivalent Site A double mutant S288A-L292A (see Fig. 5). As expected, we observed a large rightward shift in the NS309 concentration response curve. Unfortunately, solubility issues prevented further testing at higher NS309 concentrations, but the difference in NS309 efficacy on the WT versus Site A double mutant was still patently clear, increasing the EC_{50} from 1.7 μM to more than 83 μM (Fig. 5A). Whole-cell recordings shown in Figure 5B and 5C highlight these differences in WT and mutant response, with NS309 increasing WT K_{Ca}2.2 currents at 10 μM by 57-fold (Fig. 5B), while the double mutant required a more than ten-fold higher concentration to achieve the same magnitude of current increase (Fig. 5C).

Taken together, the results of these mutagenesis experiments demonstrate that, like SKA-111, NS309 is binding in the interface between calmodulin's N-lobe and the S4-S5 linker in both $K_{Ca3.1}$ and $K_{Ca2.2}$ channels.

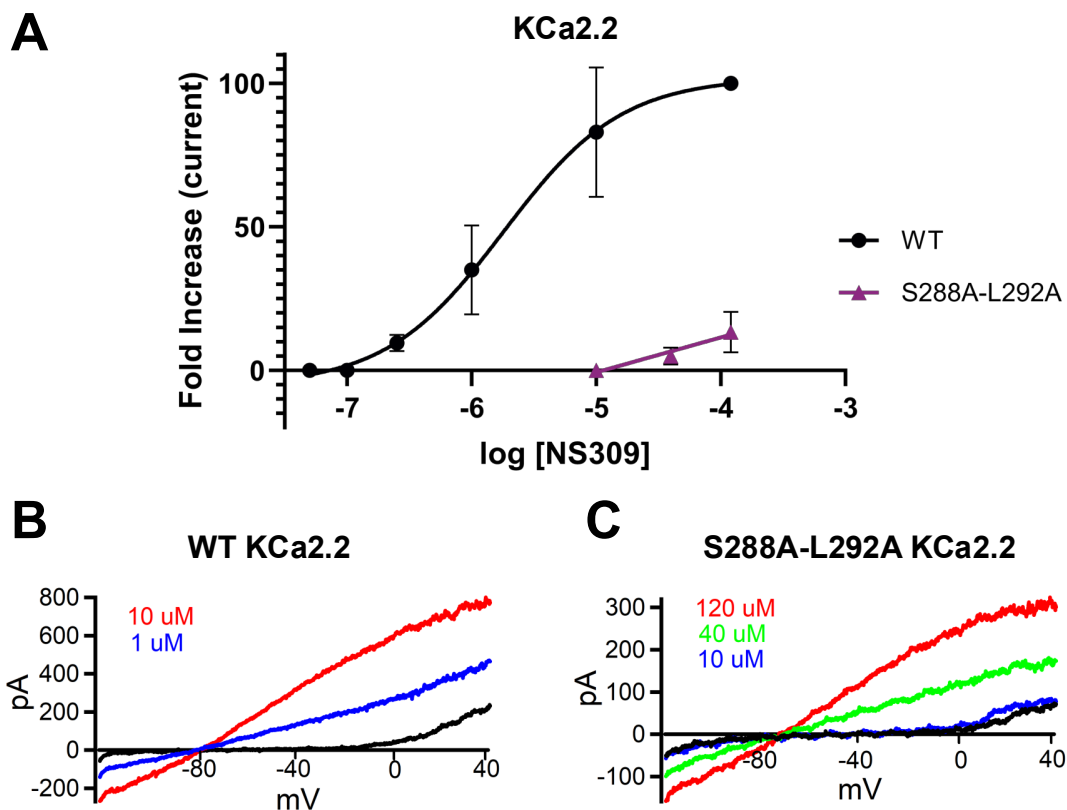


Figure 5. Effect of NS309 on the KCa2.2 Site A double mutant S288A-L292A. A. Concentration response curves of NS309 on WT KCa2.2 in black ($EC_{50} = 1.7 \mu M$, 95% CI: 912 nM- 4.2 μM) and S288A—L292A double mutant in purple ($EC_{50} > 83 \mu M$). Each data point represents an n of 3, displayed as mean $\pm$ SD. B, the effect of NS309 at 1 μM and 10 μM concentrations on WT KCa2.2. C, the effect of NS309 at 10 μM , 40 μM and 120 μM concentrations on the S288A-L292A double mutant. For both recordings, a voltage ramp protocol was applied, going from -120 to +40 mV over 200 ms.

NS309 promotes longer sustained widening of the inner gate for $K_{Ca3.1}$ in MD simulations

In order to gain mechanistic insight into how the interactions between NS309 and the S4-S5 linker can result in $K_{Ca3.1}$ activation we performed all-atom molecular dynamics simulations with either two or four NS309 molecules placed into the interfaces between the calmodulin N-lobe and the S4-S5 linker in the

position determined by the RosettaLigand docking (Figure 3A and 3B). As a control we also performed MD simulations with no NS309 bound. Over the course of 500 ns long MD simulations, all NS309 molecules remained stably bound within the binding pockets and no dissociation events were observed. NS309 made hydrogen bonds with S181 and hydrophobic interactions with L185 (Figure 6), consistent with the mutagenesis studies that suggested these K_{Ca}3.1 S4-S5 linker residues are important for the binding of NS309. The hydrogen bond between NS309 and S181 was intermittently present for around 41% of the simulation time, sometimes being formed with the backbone carbonyl oxygen and sometimes with the side chain hydroxyl group. The hydrophobic contact with L185 is relatively more stable, being present for 68% of the simulation. The most stable contact (present for over 95% of the simulation time) was a hydrogen bond between NS309 and E54 in the calmodulin N-lobe (Figure 6D), confirming that NS309 makes stable contacts at the channel-calmodulin interface. The aromatic ring and the chlorine atoms of NS309 additionally make multiple van der Waals contacts with five CaM residues, namely V55, F69, M71, K75, and F93 as well as I182 of K_{Ca}3.1.

Another interesting feature revealed by the MD simulations was the presence of water molecules around NS309. Over the course of the simulation, the number of water molecules making hydrogen bonds with NS309 decreased from six to two (Figure 7A) as NS309 established hydrogen bonds with channel residues and “settled down”. Early in the MD runs a water molecule was observed making a bridge between the indole group of NS309 and the sidechain hydroxyl as well as the backbone carbonyl group of S181 (Figure 7B). Later the water had been “pushed out”, and the indole group of NS309 was directly hydrogen bonding to S181, first to the sidechain hydroxyl group and then to the backbone carbonyl group (Figure 7B). We also observed one or two water molecules hydrogen bonding with the oxime nitrogen of NS309 while the hydroxyl group of the oxime was hydrogen bonding with the backbone carbonyl group of E54 of calmodulin (Figure 7A).

A closer inspection of the hydrophobic gate defined by V282 in the S6 segment (Figure 8A) revealed that the channel gate remains significantly wider when two or four NS309 molecules are bound to

K_{Ca}3.1 as compared to the simulations of the channel with no NS309 present. Over the course of the simulation trajectories, the gate of the channel tended to be significantly narrower (Figure 8B) without NS309. We had purposely chosen open state 2 (6CNO) for the MD simulations since this state is wider at the level of V282 (Lee and MacKinnon 2018). With two or four NS309 bound, the hydrophobic gate remains relatively wide (Figure 8C and 8D). The geometric mean in the no ligand case was 6.00 ± 0.30 Å, significantly smaller than the two-ligand case (8.02 ± 0.14 Å) and smaller than the four-ligand case with a mean of 7.46 ± 0.38 Å (values reported as mean $\pm$ standard error of mean, SE; Supplementary Table 1). The minimum gate diameter of 7 Å likely to conduct a partially hydrated potassium ion is indicated as a black dashed line in Figure 8B, 8C, and 8D. There was no statistical difference between the distances of the hydrophobic gate with two or four NS309 bound (p-value = 0.38) suggesting that two NS309 molecules may be sufficient for the agonist effect.

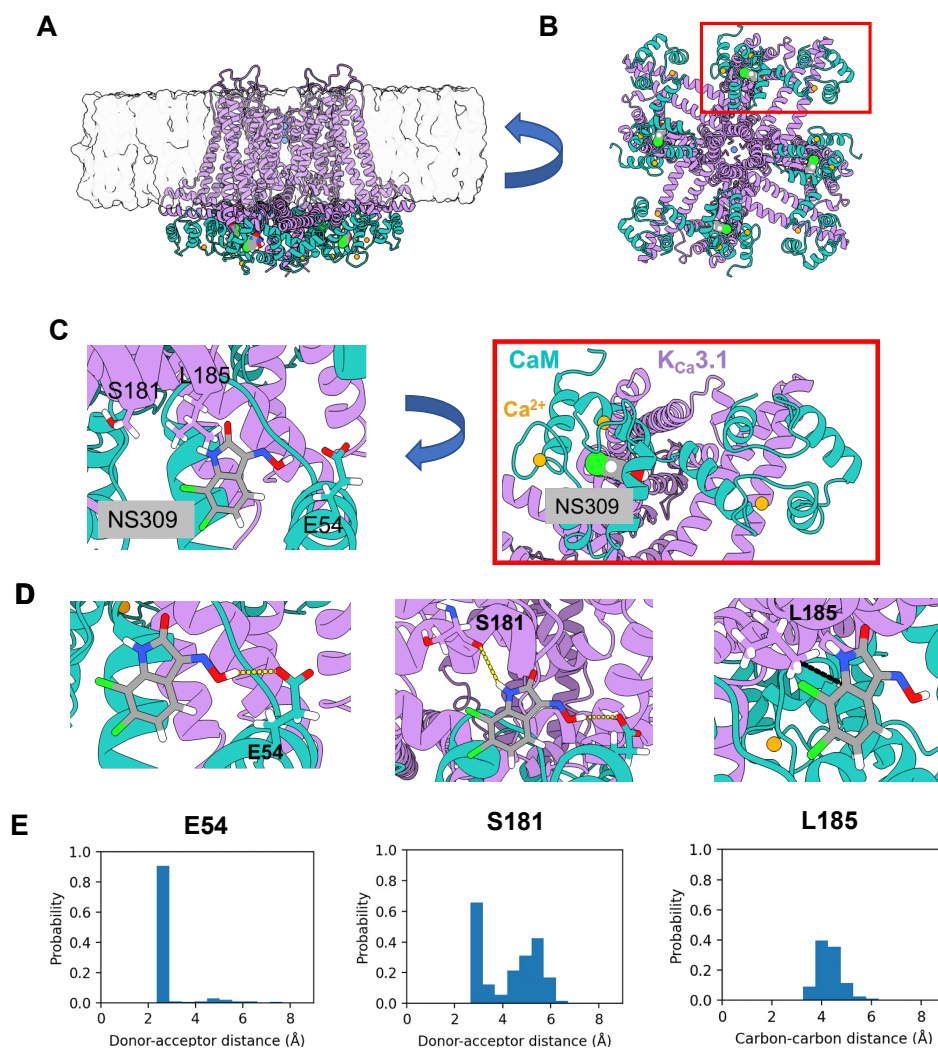


Figure 6. KCa3.1 and NS309 Molecular Dynamics Simulations setup. (A) Side view of KCa3.1 in the lipid membrane. (B) Channel (purple) with four calmodulin (teal) and four bound NS309 molecules viewed from the intracellular side. (C) View of KCa3.1 within the lipid membrane. (D) Inset from panel A showing one of the NS309 binding sites viewed from the side and from below showing the proximity to S181 and L185 in the S4-S5 linker of the channel and E54 in calmodulin. (E) Representative frames of the course of the MD simulations showing hydrogen bonds (dotted yellow line) between NS309 and E54 and S181 and hydrophobic contacts (dotted black line) with L185. (F) Histograms of donor to acceptor distances between S181 and E54 NS309 as well as carbon-carbon distance for hydrophobic contact between L185 and NS309.

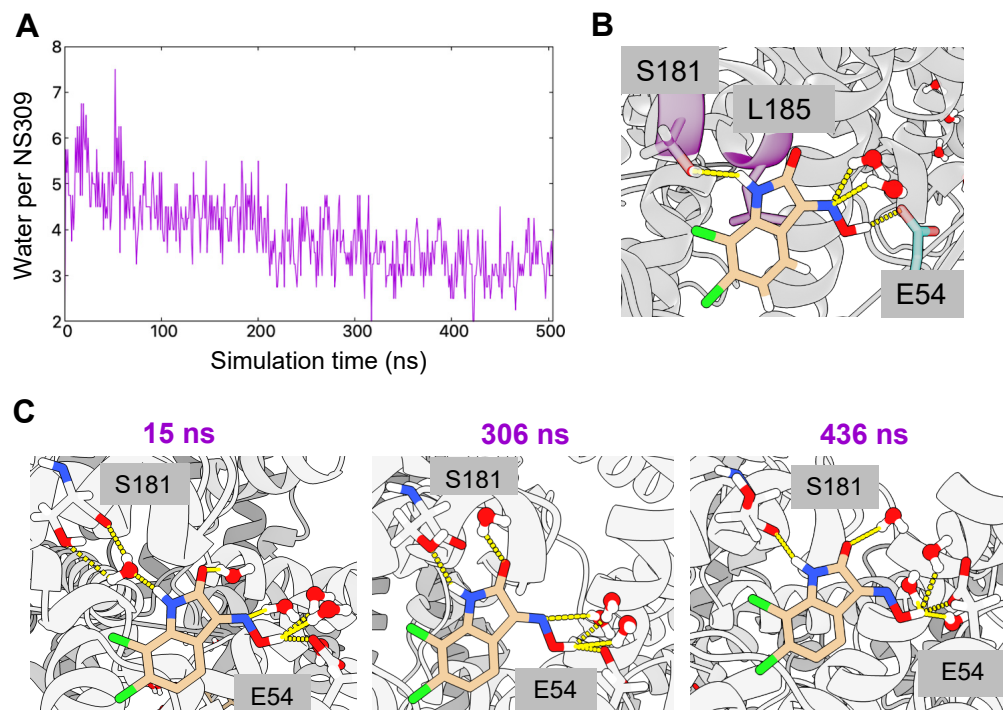


Figure 7. Water molecules interacting with NS309. (A) Number of water molecules per ligand over the time course of the simulation. Two to six water molecules typically make hydrogen bonds with each NS309 molecule. (B) A representative frame of NS309 making hydrogen bonds with S181, E54 in calmodulin, and two water molecules is shown next to the time course plot. (C) Evolution of hydrogen bonding with the indole group. Frame 15 of the simulation shows a water molecule mediating interactions with S181. Frame 306 shows a hydrogen bond with the hydroxyl group of S181, displacing the water molecule. Frame 432 shows a hydrogen between NS309 and the carbonyl oxygen of S181 which remains stable for the rest of the simulation.

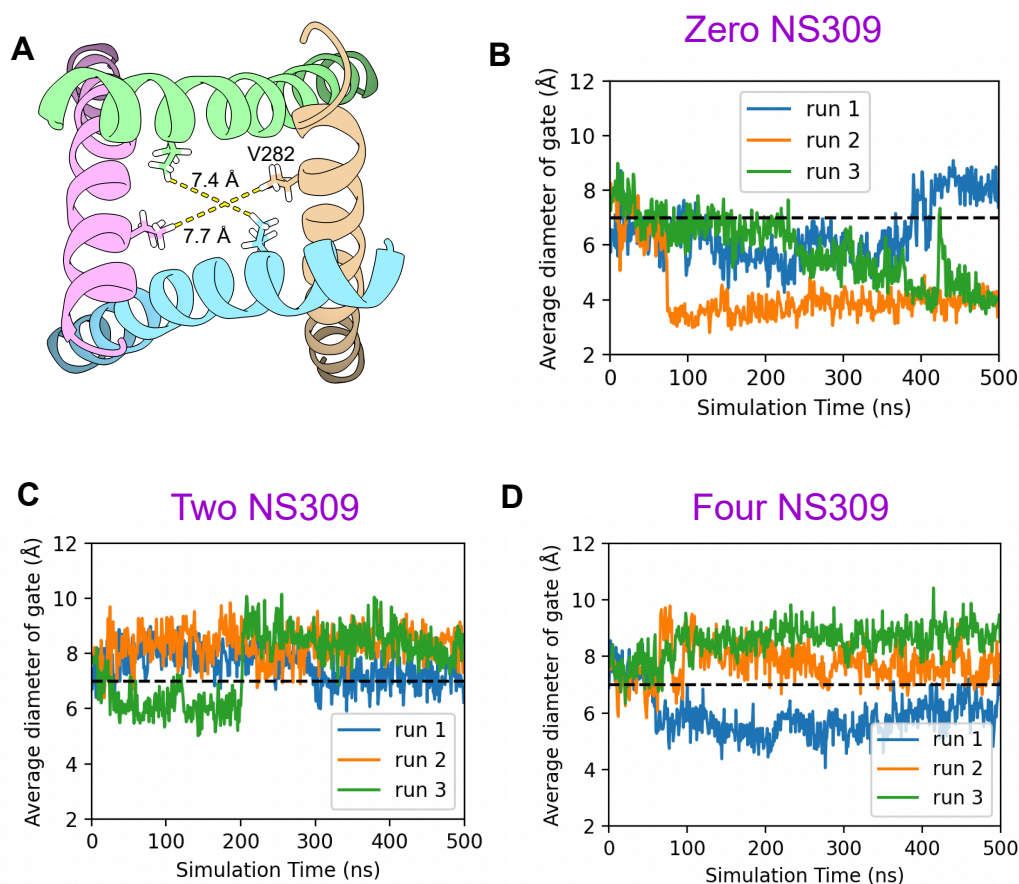


Figure 8. Diameter of hydrophobic gate over molecular dynamics simulation. (A) Representative diameter of hydrophobic gate following system equilibration. V282 is shown in a stick representation on all four chains. Average diameter of the hydrophobic gate over the course of the trajectory for each of the three simulations run for (B) zero NS309, (C) two NS309, and (D) four NS309 molecules bound to the KCa3.1 channel. A diameter of 7 Å is shown as a black dashed line indicating the minimum gate diameter likely to conduct K⁺ ions.

NS309 has no effect on constitutively open KCa3.1 mutants

Gain of function mutations causing constitutively open KCa3.1 channels have been described in a subset of patients with hereditary xerocytosis, a disease characterized by hemolytic anemia associated with erythrocyte dehydration (Andolfo et al. 2015; Glogowska et al. 2015). These gain of function mutations frequently involve residues situated at or near the channel's cytoplasmic gate, such as V282 and A279 (Fig. 9B), and typically contain substitutions that are more hydrophilic in nature (Garneau et al. 2009). Our molecular dynamics simulation showing that NS309 prevents the hydrophobic gate of KCa3.1 from narrowing suggest that NS309 exerts its agonistic effects by increasing open probability (P_O). We therefore

hypothesized that NS309 would have no effect on $K_{Ca3.1}$ gain of function mutations. Whole-cell recordings of two constitutively open mutants, V282G and A279G, confirmed that in contrast to wild-type channels, these mutants exhibited large currents with amplitudes in the nano ampere range even at low intracellular calcium concentrations or in the complete absence of calcium in a potassium fluoride-based pipette solution (Fig. 9A). Interestingly, 10 mM of NS309 had no agonistic effect on the V282G and A279G mutants (Fig. 9C), suggesting that NS309 cannot further increase the P_o of these already fully open $K_{Ca3.1}$ channels.

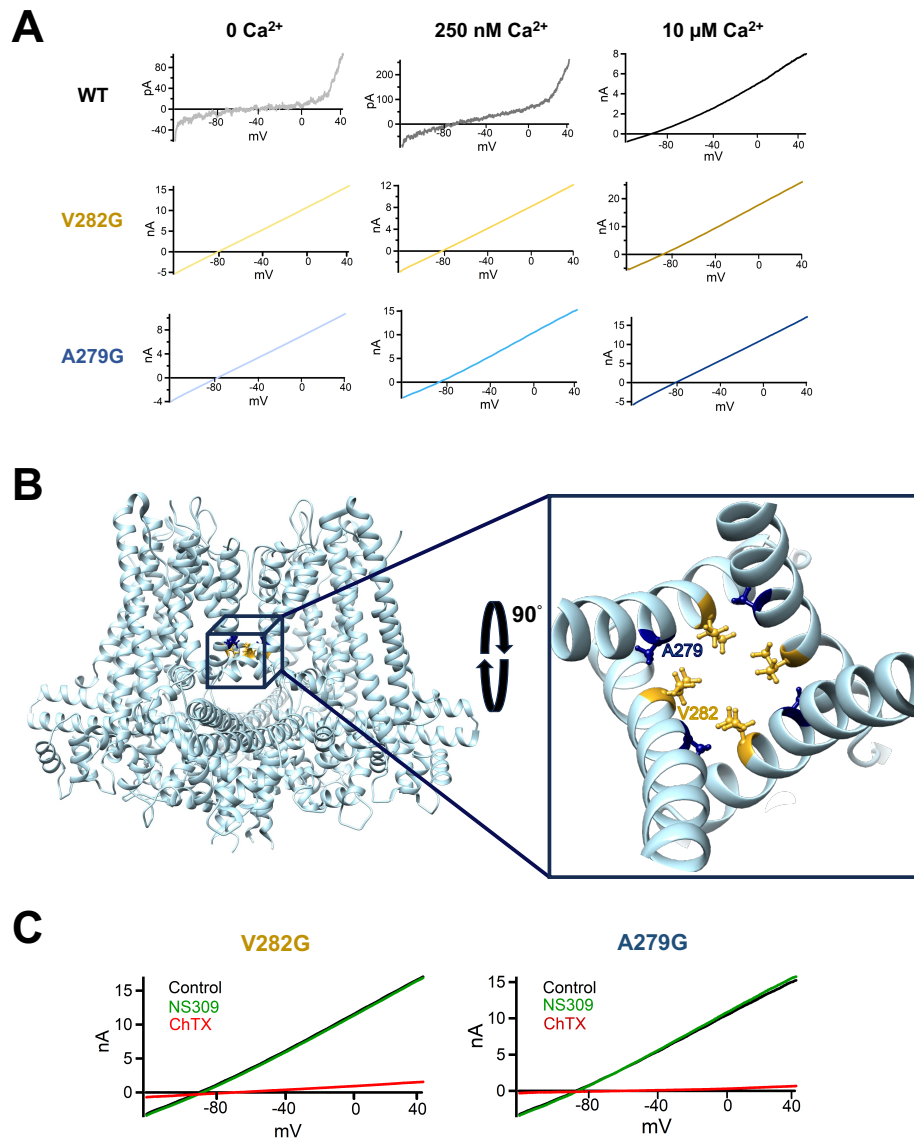


Figure 9. Characterization of two gain-of-function $K_{Ca3.1}$ mutations. A, Whole-cell recordings of wild-type and constitutively

open V282G and A279G mutant channels in response to various levels of intracellular calcium. B. Magnified view of K_{Ca}3.1's cytoplasmic gate, with pivotal residues A279 and V282 displayed in dark blue and gold, respectively. C. Whole-cell recordings of V282G and A279G using 250 nM intracellular calcium, with a 10 μ M NS309 perfusion shown in green and a 300 nM Charybdotoxin perfusion in red. Lack of effect on both mutations implies that NS309 operates at least partially by changing P_o. All Whole-cell recordings represent an n of at least 3.

Discussion

Similar to other ligand gated ion channels like nicotinic acetylcholine (nACh) receptors or γ -aminobutyric acid type A (GABA_A) receptors, small- and intermediate-conductance calcium-activated K⁺ channels have positive allosteric modulators (PAMs), that increase sensitivity to their endogenous ligand, which in this case is intracellular calcium. In keeping with previous reports (Hougaard et al. 2007; Pedarzani et al. 2005), we here observed that NS309, the most potent and widely used K_{Ca} activator, shifted the calcium-concentration response curve of K_{Ca}3.1 to the left, making the channel apparently more sensitive to calcium. However, NS309 was also able to elicit responses that are greater than the responses to saturating calcium. In analogy to GABA_A or G-protein coupled receptor pharmacology, we suggest calling this phenomenon “superagonism”, a term that is employed for agonists like neurosteroids that can produce a maximal response greater than the endogenous ligand and thus have an efficacy of more than 100%.

Using a combination of molecular modeling and mutagenesis we identified the binding site of NS309 in the interface between the N-lobe of the channel associated calmodulin and the S₄₅A helix in the S4-S5 linker of K_{Ca}3.1. This site, which we here termed Site A, is not a novel binding site. It was first hypothesized to be the functional binding pocket for positive gating modulators when the MacKinnon laboratory published the cryo-EM structure of K_{Ca}3.1 in the closed and open state in 2018 (Lee and MacKinnon 2018). Our own laboratory later confirmed this hypothesis when we showed that the benzothiazole SKA-111 is binding to S181 and L185 in the S₄₅A helix of K_{Ca}3.1 using site-directed mutagenesis (Shim et al. 2019). However, it remained unknown whether this site was unique to the benzothiazole compounds, or if it was also capable of accommodating other pharmacophores, such as that of NS309. Molecular dynamics simulations corroborated stable binding of NS309 to S181 and L185 in the

S₄₅A helix of K_{Ca}3.1 and to E54 in the calmodulin N-lobe and over the course of 500 ns simulations showed the molecule “settling down” into its binding site by displacing three water molecules out of the interface pocket. This loss of water leads to the establishment of a hydrophobic interface between NS309 and the binding pocket, allowing the lipophilic side of NS309 to make numerous van der Waals contacts with CaM and K_{Ca}3.1. The extensive hydrophobic contacts potentially explain the high potency of NS309 and the importance of the chlorine substituents on the indole. The role of individual water molecules is uniquely captured by the MD simulations and remains a shortcoming of docking software like RosettaLigand that do not explicitly model solvent.

The MD simulations also revealed that NS309 most likely exerts its agonistic effects by promoting longer sustained widening of the inner gate of K_{Ca}3.1 located at V282 in the S6 segment (Klein et al. 2007; Lee and MacKinnon 2018). These *in silico* findings are in line with previous experimental data showing that NS309 increases the open probability (P_o) of K_{Ca}2.2 channels (Li et al. 2009; Pedarzani et al. 2005; Zhang, Pascal and Zhang 2013) and suggest that NS309 similarly increases the open probability of K_{Ca}3.1. We here used the constitutively open K_{Ca}3.1 mutant, V282G, which had previously been shown to have a P_o of 0.98 in both the presence and the absence of saturating calcium (Garneau et al. 2009) and reasoned that NS309 would not be able to further enhance K⁺ flux through this mutant if its mechanism of action is to increase channel open probability. This hypothesis was confirmed by the observation that NS309 had no effect on the V282G mutant and another constitutively open K_{Ca}3.1 mutant, A279G, which also disrupts the hydrophobic inner gate (Garneau et al. 2009).

But how exactly can NS309 increase the open probability of the inner gate of K_{Ca}3.1? Given its binding site in the interface of calmodulin’s N-lobe and the S4-S5 intracellular linker, the most intuitive explanation is that the molecule is “acting as glue” between the two proteins. By strengthening the interaction between calmodulin and the S₄₅A helix in S4-S5 linker of K_{Ca}3.1, the N-lobe of calmodulin could theoretically spend more time “holding” the channel in its’ conductive state, thus increasing the channel’s open probability beyond what is possible with the endogenous ligand, calcium. Without this

NS309-driven stabilization, the channel's hydrophobic gating residues, V282 in particular, are more likely to force the channel back into an energetically more favorable, non-conducting state. Both our MD simulations and the experiments with the constitutively open mutant channels, seem to support this hypothesis. This hypothesis is further in line with a previous study from Li and Aldrich suggesting that NS309 stabilizes the interaction between calmodulin and K_{Ca}2.2 so that less calcium is required for channel opening, and who observed that a calmodulin mutant with one EF-hand motif mutation in the N-lobe could only gate K_{Ca}2.2 channels in the presence of NS309 (Li et al. 2009). Otherwise, two functional N-lobe EF-hands were required for a stable interaction between calmodulin and productive gating inducing a high open probability (Li et al. 2009). We here confirmed this classic hypothesis that NS309 stabilizes the interaction between the calmodulin N-lobe and the K_{Ca} channel α -subunit during gating for K_{Ca}3.1 and provide a re-interpretation of the mechanism of action of NS309 in a structural context. NS309 acts as a stabilizing force within the gating interface of K_{Ca}3.1 and reduces the probability of the hydrophobic inner gate located around V282 in the S6 segment to close.

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the paper and its Supplemental Data. Models in pdb format are available on request from the authors.

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Wrote or contributed to the writing of the manuscript: Joshua A. Nasburg, Kyle C. Rouen, Heesung Shim, Miao Zhang, Igor Vorobyov, Heike Wulff

Footnotes

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Figure Legends

Figure 1. NS309 exhibits superagonistic effects on K_{Ca} channels. (A) Ca²⁺-concentration response curve for K_{Ca}3.1 activation measured from inside out patches in absence or presence of 1 μ M NS309, displaying a calcium EC₅₀ of 430 nM for the control curve (95% CI: 365-502 nM; n_H 2.9; n = 3 for all data points) and 31 nM in the presence of NS309 (95% CI: 22-41 nM, P < 0.0001; n_H 2.0; n = 2 for 20 nM and 40 nM data points, and n = 3 for remaining concentrations). (B) NS309 concentration response curves obtained via whole-cell patches in 250 nM free calcium (black) and 3 mM free calcium (red), with measured NS309 EC₅₀ values of 74 nM (95% CI: 53-96 nM; n_H 1.3; n = 3) and 8.6 nM (95% CI: 2.6-20 nM; n_H 1.0; n = 3; P < 0.0001) respectively. P values for curve comparison were calculated using an extra sum-of-squares F test (GraphPad Prism 10; GraphPad Software, La Jolla, CA). C, Whole-cell recordings of K_{Ca}2.1, K_{Ca}2.2, K_{Ca}2.3 and K_{Ca}3.1 in saturating calcium conditions (10 μ M intracellular calcium), with the effect of a 10 μ M NS309 perfusion shown in red. D. Scatter plot showing current increases for K_{Ca}2.1, K_{Ca}2.2, K_{Ca}2.3 and K_{Ca}3.1 induced by 10 mM of NS309 at 10 μ M intracellular calcium (n = 5). All data are mean $\pm$ SD.

Figure 2. Search for small molecule binding sites in the K_{Ca}3.1 channel using Schrödinger's SiteMap.

(A) Seven distinct possible binding sites for K_{Ca}3.1 modulation were identified by the SiteMap function of Schrödinger suite. (B) Standard precision GLIDE Docking of NS309 in Sites A (green) & B (orange), with binding free energy scores reported at -6.9 and -7.05 kcal/mol respectively. Van der Waal's interactions are shown as dashed pink lines and H-bonds are shown in solid purple.

Figure 3. Rosetta Docking of NS309 in Sites A & B. (A) Convergence of top 10 poses within Site A. (B) Top ranked pose in Site A, with VDW forces in pink and H-bonds in purple. (C) Convergence of top 10 poses within Site B. (D) Top ranked pose in Site B, with VDW interactions in pink and H-bonds in purple.

Figure 4. Effect of NS309 on K_{Ca}3.1 mutations within Site A and B. (A) Concentration response curves of NS309 on WT K_{Ca}3.1 in black ($EC_{50} = 74$ nM, 95% CI: 55-95 nM, n_H 1.3), L185A in yellow ($EC_{50} = 1.27$ μ M, 95% CI: 945 nM-1.83 μ M, n_H 1.4, $P < 0.0001$), S181A-L185A double mutant in purple ($EC_{50} = 1.1$ μ M, 95% CI: 730 nM-1.61 μ M, n_H 1.6, $P < 0.0001$), N201F in green ($EC_{50} = 195$ nM, 95% CI: 138-264 nM, n_H 1.3, $P < 0.0001$), N201A in red ($EC_{50} = 85$ nM, 95% CI: 64-112 nM, n_H 1.2, $P = 0.4679$) and R287A in blue ($EC_{50} = 98$ nM, 95% CI: 77-125 nM, n_H 1.5, $P = 0.1309$). Each data point on curves represents an n of 3, displayed as mean $\pm$ SD. (B) Effect of NS309 at 25 nM and 250 nM concentrations on WT K_{Ca}3.1. (C) Effect of NS309 at 250 nM, 1 μ M and 10 μ M concentrations on the S181A-L185A double mutant. For both recordings, a 200 ms voltage-ramp protocol from -120 to +40 mV was applied every 10 s.

Figure 5. Effect of NS309 on the K_{Ca}2.2 Site A double mutant S288A-L292A. (A) Concentration response curves of NS309 on WT K_{Ca}2.2 in black ($EC_{50} = 1.7$ μ M, 95% CI: 912 nM- 4.2 μ M) and S288A-292A double mutant in purple ($EC_{50} > 83$ μ M). Each data point on curve represents an n of 3, displayed as mean $\pm$ SD. (B) Effect of NS309 at 1 μ M and 10 μ M concentrations on WT K_{Ca}2.2. (C) Effect of NS309 at

10 μ M, 40 μ M and 120 μ M concentrations on the S288A-L292A double mutant. For both recordings, a voltage ramp protocol was applied, going from -120 to +40 mV over 200 ms.

Figure 6. K_{Ca}3.1 and NS309 Molecular Dynamics Simulations setup. (A) Side view of K_{Ca}3.1 in the lipid membrane. B. Channel (purple) with four calmodulin (teal) and four bound NS309 molecules viewed from the intracellular side. (B) View of K_{Ca}3.1 within the lipid membrane. (C) Inset from panel A showing one of the NS309 binding sites viewed from the side and from below showing the proximity to S181 and L185 in the S4-S5 linker of the channel and E54 in calmodulin. (D) Representative frames of the course of the MD simulations showing hydrogen bonds (dotted yellow line) between NS309 and E54 and S181 and hydrophobic contacts (dotted black line) with L185. E. Histograms of donor to acceptor distances between S181 and E54 NS309 as well as carbon-carbon distance for hydrophobic contact between L185 and NS309.

Figure 7. Water molecules interacting with NS309. (A) Number of water molecules per ligand over the time course of the simulation. Two to six water molecules typically make hydrogen bonds with each NS309 molecule. (B) A representative frame of NS309 making hydrogen bonds with S181, E54 in calmodulin, and two water molecules is shown next to the time course plot. (C) Evolution of hydrogen bonding with the indole group. Frame 15 of the simulation shows a water molecule mediating interactions with S181. Frame 306 shows a hydrogen bond with the hydroxyl group of S181, displacing the water molecule. Frame 432 shows a hydrogen between NS309 and the carbonyl oxygen of S181 which remains stable for the rest of the simulation.

Figure 8. Diameter of hydrophobic gate over molecular dynamics simulation. (A) Representative diameter of hydrophobic gate following system equilibration. V282 is shown in a stick representation on all four chains. Average diameter of the hydrophobic gate over the course of the trajectory for each of the

three simulations run for (B) zero NS309, (C) two NS309, and (D) four NS309 molecules bound to the K_{Ca}3.1 channel. A diameter of 7 Å is shown as a black dashed line indicating the minimum gate diameter likely to conduct K⁺ ions.

Figure 9. Characterization of two gain-of-function K_{Ca}3.1 mutations. (A) Whole-cell recordings of wild-type and constitutively open V282G and A279G mutant channels in response to various concentrations of intracellular calcium. Zero calcium was achieved by using a fluoride containing pipette solution. (B) Location of residues A279 (dark blue) and V282 (gold) in the lower pore of K_{Ca}3.1. (C) Whole-cell recordings of V282G and A279G mutants using 250 nM intracellular calcium. NS309 at 10 mM (green) does not increase currents. The K_{Ca}3.1 blocking peptide toxin charybdotoxin at 300 nM (red) inhibits currents in both mutants. All shown traces are one representative recording of at least 3 independent experiments.

CHAPTER 3: Apamin Structure and Pharmacology Revisited

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Abstract

Apamin is often cited as one of the few substances selectively acting on small-conductance Ca^{2+} -activated potassium channels ($\text{K}_{\text{Ca}2}$). However, published pharmacological and structural data remain controversial. Here, the molecular pharmacology of apamin was investigated by two-electrode voltage-clamp in *Xenopus laevis* oocytes and patch-clamp in HEK293, COS7, and CHO cells expressing the studied ion channels, as well as in isolated rat brain neurons. The microtitre broth dilution method was used for antimicrobial activity screening. The spatial structure of apamin in aqueous solution was determined by NMR spectroscopy. Apamin was tested against 42 ion channels (K_{Ca} , K_{V} , Na_{V} , nAChR, ASIC, and others) and confirmed its unique selectivity to $\text{K}_{\text{Ca}2}$ channels. No antimicrobial activity was detected for apamin against Gram-positive or Gram-negative bacteria. The NMR solution structure of apamin was deposited in the Protein Data Bank. The results presented here demonstrate that apamin is a selective nanomolar or even subnanomolar-affinity $\text{K}_{\text{Ca}2}$ inhibitor with no significant effects on other molecular targets. The spatial structure as well as ample functional data provided here support the use of apamin as a $\text{K}_{\text{Ca}2}$ -selective pharmacological tool and as a template for drug design.

Introduction

Highly selective molecules that can interact with specific ion channel isoforms serve as invaluable molecular tools for fundamental and applied pharmacology (Hille, 2001; Wulff et al., 2019). The history of using such substances goes hand-in-hand with the discovery and investigation of their molecular targets (Hille, 2001). Indeed, selective molecular probes helped to identify and characterize a number of physiologically critical ion channels (Kuzmenkov and Vassilevski, 2018). For example, marine guanidinium and scorpion polypeptide toxins were used in pioneering studies for the purification of voltage-gated Na^+ channels (Na_v) (Hartshorne and Catterall, 1981; Barhanin et al., 1983; Hanke et al., 1984). Moreover, Na_v are traditionally classified based on isoform sensitivity to tetrodotoxin (Narahashi, 2008). A plethora of other ion channel-selective compounds has been extracted from different animal venoms (Herzig et al., 2020).

In the 1960s, Habermann examined the venom of the honeybee *Apis mellifera* and purified a minor peptide component that he named “apamin” (Habermann and Reiz, 1965; Habermann, 1972); the toxin was shown to induce muscle spasms, jerks, and convulsions when injected into mice ($\text{LD}_{50} \approx 4 \text{ mg kg}^{-1}$, i.v.). The symptoms are apparently of central origin because the toxicity increases by a factor of 1,000–10,000 and the progression of poisoning is faster in case of i.c.v. injection, but the clinical picture stays the same (Habermann, 1977, Habermann, 1984). Primary structure determination showed that apamin contains 18 amino acid residues (Haux et al., 1967; Shipolini et al., 1967), with two intramolecular disulfide bonds (Cys1–Cys11 and Cys3–Cys15) (Callewaert et al., 1968). Moreover, the C-terminal residue is amidated; this post-translational modification is common for peptide toxins from animal venoms (Kuzmenkov et al., 2015).

Studies of the spatial structure of apamin began as early as the late 1970s, but no atomic coordinates have been deposited in the Protein Data Bank. The first NMR study provided an erroneous assignment of the secondary structure elements (Bystrov et al., 1980), which was corrected in further

works (Wemmer and Kallenbach, 1983; Pease and Wemmer, 1988). The most recent work (Le-Nguyen et al., 2007) presented both a solution NMR structure of apamin and an X-ray structure of its N-terminally acetylated analog, which differ in the packing of the N-terminal region, the length of the α -helix, and the χ^3 angles of the disulfide bridges. In addition, whereas apamin is monomeric in solution, the X-ray structure is a dimer, and that dimeric structure was deposited in the Cambridge Crystallographic Data Centre (<https://www.ccdc.cam.ac.uk>; database identifier: NIWFEF).

Apamin played a key role in the identification and characterization of a novel subset of Ca^{2+} -activated K^+ ion channels (K_{Ca}) (Banks et al., 1979; Lazdunski, 1983; Garcia et al., 1991). Since the early 1980s, this toxin has been widely utilized to differentiate K_{Ca} channels into “apamin-sensitive” and “apamin-insensitive” because it selectively affected only the small-conductance K_{Ca} channels ($\text{K}_{\text{Ca}2}$, SK2, or SK_{Ca}) and not intermediate-conductance ($\text{K}_{\text{Ca}3.1}$) or large-conductance ($\text{K}_{\text{Ca}1.1}$) K_{Ca} channels. (Burgess et al., 1981; Romey and Lazdunski, 1984; Pennefather et al., 1985). After the cloning of SK channels from rat and human brain and their expression in *Xenopus laevis* oocytes, the direct activity of apamin was shown on these molecular targets (Köhler et al., 1996; Grunnet et al., 2001a). A number of pharmacological studies using *X. laevis* oocytes and mammalian cells indicated that among the three SK isoforms apamin was more potent on $\text{K}_{\text{Ca}2.2}$ (SK2 or $\text{SK}_{\text{Ca}2}$; $\text{IC}_{50} = 0.03\text{--}0.14$ nM) and less potent on $\text{K}_{\text{Ca}2.1}$ (SK1 or $\text{SK}_{\text{Ca}1}$; $\text{IC}_{50} = 0.7\text{--}12$ nM) (Köhler et al., 1996; Shah and Haylett, 2000; Strøbaek et al., 2000; Grunnet et al., 2001a), whereas $\text{K}_{\text{Ca}2.3}$ (SK3 or $\text{SK}_{\text{Ca}3}$) channels showed an intermediate sensitivity ($\text{IC}_{50} = 0.6\text{--}4.0$ nM) (Ishii et al., 1997; Grunnet et al., 2001b; Grunnet et al., 2001a).

Those investigations led to a prevailing view in the literature that apamin is a selective probe for just SK channels. Quite surprisingly, data to support this claim by showing absence of activity on other targets are missing. Moreover, some published studies actually claim various off-target activities of apamin. For example, it was assumed that apamin affects Ca^{2+} and/or Na^+ channels in the embryonic chicken heart (Bkaily et al., 1985; Bkaily et al., 1991; Bkaily et al., 1992). On the contrary, in another

study human cardiac Ca^{2+} , Na^{+} , or K^{+} channels were not affected, with the exception of SK channels (Yu et al., 2014). Apamin was also reported to affect inward-rectifier K^{+} channels $\text{K}_{\text{ir}}3.1/3.4$ and the voltage-gated K^{+} channel $\text{K}_{\text{v}}1.3$ (Jin and Lu, 1998; Voos et al., 2017); however, the activity of the toxin on those channel isoforms expressed individually is yet to be shown.

To verify the molecular pharmacology of such an important compound as apamin and to resolve the contradictory claims, we performed a large-scale electrophysiological profiling of this toxin against various molecular targets. We conclude that apamin is indeed a selective inhibitor of small-conductance K_{Ca} channels.

Methods

Materials

For consistency, we used apamin purified from the honeybee *Apis mellifera* venom (product number A1289) and synthetic melittin (M4171) purchased from Sigma-Aldrich. TRAM-34 was synthesized as described (Wulff et al., 2000). Other low-molecular-weight compounds were purchased from Sigma-Aldrich: acetylcholine chloride (ACh; A6625), capsaicin (M2028), capsazepine (C191), glycine (G7126), kainic acid monohydrate (K0250), and N-methyl-D-aspartic acid (NMDA; M3262).

Nomenclature of targets and ligands

Ion channel targets and their ligands are presented according to IUPHAR/BPS Guide to PHARMACOLOGY (<http://www.guidetopharmacology.org>) and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021). For potassium channel ligands of protein and peptide nature, readers are advised to consult the Kalium database (<https://kaliumdb.org>) (Kuzmenkov et al., 2016; Tabakmakher et al., 2019).

Analytical chromatography

Apamin purity was confirmed using reversed-phase (RP) HPLC on a Vydac 218TP54 C₁₈ column (4.6 × 250 mm; Separations Group) in a linear gradient of acetonitrile concentration (0%–60% in 60 min) in the presence of 0.1% trifluoroacetic acid (TFA). 1525 Binary HPLC pump and 2489 UV/Visible detector under the control of Breeze 2 software (all from Waters) were used for chromatography.

Mass spectrometry

Molecular mass measurements were performed using MALDI on an Ultraflex III TOF-TOF instrument (Bruker). 2,5-Dihydroxybenzoic acid (Sigma-Aldrich) was used as a matrix. Measurements were carried out in the reflector mode, which enabled isotopic resolution. Calibration was performed using the ProteoMass Peptide MALDI-MS Calibration Kit (Sigma-Aldrich). Mass spectra were analyzed with the Data Analysis 4.3 and Data Analysis Viewer 4.3 software (Bruker).

Peptide concentration measurements

Apamin and melittin concentrations were determined by UV spectrophotometry. To obtain absorption spectra of those substances in the UV range, lyophilized peptides were dissolved in 0.5 ml of water (Milli-Q produced on a Merck Millipore Water Purification System). We used a UV-1800 spectrophotometer (Shimadzu) and quartz cuvettes with an optical path length of 1.0 cm; water served as a reference solution. Apamin does not contain aromatic amino acid residues; therefore, absorbance at 205 nm was used to measure the concentration (Anthis and Clore, 2013). Melittin contains tryptophan, and so its absorbance was measured at 280 nm and the calculations were performed accordingly (Beaven and Holiday, 1952).

Antimicrobial assay

Apamin was tested against Gram-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* subsp. *aureus* ATCC 29213) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) following the previously described modification of the microtitre broth dilution method (Vassilevski et al., 2010). Those bacterial strains were also subjected to the treatment by melittin in the same concentration range to serve as a positive control.

Briefly, bacteria were cultured in a low-salt LB medium. The two-fold microtitre broth dilution assay was carried out in 96-well sterile plates in a final volume of 100 μL . Mid-exponential-phase cultures were diluted to a final concentration of 10^5 colony-forming units $\cdot\text{mL}^{-1}$. Pure peptides were dissolved in 10 μL of water and added to 90 μL of the bacterial dilution. The samples, a non-treated control and a sterility control were tested in five independent experiments ($n = 5$). The microtitre plates were incubated for 24 h at 37°C , and growth inhibition was determined by measuring the absorbance at 620 nm. Minimum inhibitory concentration (MIC) is expressed as the lowest concentration of peptide that caused 100% bacterial growth inhibition.

NMR spectroscopy

For NMR studies 1 mg of apamin was dissolved in 320 μL of $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture (95:5) and placed into a 5-mm Shigemi NMR tube. The sample pH was adjusted to 3.2. NMR spectra of apamin were recorded using a 600-MHz Bruker Avance III NMR spectrometer, equipped with a triple-resonance cryogenic probe, at 25°C . We recorded the following spectra: DQF-COSY, NOESY (120 ms), ROESY (200 ms), TOCSY (80 ms), ^{13}C and ^{15}N -HSQC at natural abundance. $^3J_{\text{HNHA}}$ were measured by the line shape analysis of cross-peaks in NOESY spectra, whereas $^3J_{\text{HAHB}}$ couplings of AMX spin systems were determined by the line shape analysis of cross-peaks in 2D TOCSY spectra.

3D structure calculation was performed using the simulated annealing/molecular dynamics protocol as implemented in the CYANA software package version 3.98 (Güntert et al., 1997). The disulfide linkages were introduced based on previously published data (Callewaert et al., 1968). 100

structures were obtained starting from random conformations and the 10 best were then selected for further analysis. Visual inspection of the calculated structures and figure drawings were performed using PyMOL (Schrödinger) and MOLMOL (Koradi et al., 1996) software.

Expression of ion channels in X. laevis oocytes

The following genes encoding ion channel subunits were expressed in *Xenopus* oocytes: for voltage-gated potassium channels, K_v [rK_v1.1 (GenBank accession number: NM_173095), rK_v1.2 (NM_012970), hK_v1.3 (NM_002232), rK_v1.4 (NM_012971), rK_v1.5 (NM_012972), rK_v1.6 (NM_023954), hK_v2.1 (NM_004975), hK_v3.1 (NM_004976), rK_v4.3 (NM_031739), hK_v7.1 (NM_000218), hK_v7.2/7.3 (NM_004518/NM_004519), hK_v10.1 (EAG1; NM_172362), hK_v11.1 (hERG; NM_000238), *Shaker-IR* from *Drosophila melanogaster* (NM_167595; amino acids 6–46 deleted), and KQT-1 from *Caenorhabditis elegans* (NM_171710)], inward-rectifier potassium channels, K_{ir} [mK_{ir}3.1/3.2 or GIRK1/2 (NM_008426/XM_011246104)], voltage-gated sodium channels, Na_v [rNa_v1.1 (NM_030875), rNa_v1.2 (NM_012647), rNa_v1.3 (NM_013119), rNa_v1.4 (NM_013178), hNa_v1.5 (NM_198056), mNa_v1.6 (NM_001077499), hNa_v1.7 (NM_002977), rNa_v1.8 (NM_017247), rβ1 (NM_001271045), hβ1 (NM_001037), and the arthropod channels BgNa_v1 from *Blattella germanica* (U73583) and VdNa_v1 from *Varroa destructor* (AY259834), and TipE from *D. melanogaster* (NM_079196)], transient receptor potential channels, TRP (human TRPV1, NM_080704), nicotinic acetylcholine receptors, nAChR [human α1β1γδ (NM_001039523, NM_000747, NM_005199, NM_000751), α4β2 (NM_000744, NM_000748), and α7 (NM_000746)].

Linearized plasmids bearing the ion channel genes were transcribed using the mMESSAGE mMACHINE SP6 or T7 transcription kits (Ambion) to prepare the respective cRNA. The harvesting of stage V–VI oocytes from anaesthetized female *X. laevis* frogs was described previously (Liman et al., 1992; Peigneur et al., 2021). Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng

nI⁻¹ using a microinjector (Drummond Scientific). The oocytes were incubated at 16°C in ND96 solution containing (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2; and HEPES, 5 (pH 7.4), supplemented with 50 mg I⁻¹ gentamicin sulfate.

Expression of ion channels in eukaryotic cells

HEK293 cells stably expressing hK_{Ca}1.1 (BK, GenBank accession number: NM_002247), hK_{Ca}2.1 (SK1, NM_002248), and hK_{Ca}3.1 (IK, AH009923) or COS7 cells stably expressing hK_{Ca}2.3 (SK3, AJ251016) were generated and cultured as previously described (Sankaranarayanan et al., 2009). HEK293 cells stably expressing rK_{Ca}2.2 (SK2, NM_019314) were a gift from Dr. Mio Zhang (Chapman University, Irvine).

CHO cells were used for transient expression of acid-sensing ion channels (ASIC). These cells were cultured in a CB-150 CO₂ incubator (Binder) at 37°C in a humidified atmosphere of 5% CO₂. Cells were maintained under standard culture conditions (DMEM/F12, 10% fetal bovine serum, and 50 mg I⁻¹ gentamicin) in 35 mm² Petri dishes. Transfection was performed using 0.5 µg of plasmids encoding rASIC1a (NM_024154), rASIC2a (NM_001034014), or rASIC3 (NM_173135) gifted by Dr. Alexander Staruschenko (University of South Florida, Tampa) with 0.5 µg of a GFP-encoding plasmid and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Patch-clamp experiments were performed 48 h after transfection.

Isolation of rat neurons

Wistar rats (12–18 days old, both sexes) were deeply anaesthetized with isoflurane and sacrificed by cervical dislocation followed by decapitation. The brains were quickly removed and immersed in ice-cold (2°C–4°C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 124; KCl, 5; CaCl₂, 1.3; MgCl₂, 2; NaHCO₃, 26; NaH₂PO₄, 1.24; and D-glucose, 10; aerated with carbogen (95% O₂, 5% CO₂). Transverse slices, comprising hippocampus and striatum, were cut with a 7000 SMZ-

2 vibratome (Campden Instruments) and stored at room temperature (22°C–24°C) in ACSF aerated with carbogen. Neurons were isolated from the slices by vibrodissociation (Vorobjev, 1991). The effects of apamin on ionotropic glutamate receptors (iGluR) were studied on hippocampal pyramidal neurons of the CA1 area expressing GluN2A/B NMDA receptors (Monyer et al., 1994; Foster et al., 2010) and Ca^{2+} -impermeable GluA2-containing AMPA receptors (Wenthold et al., 1996; Seifert et al., 2000) and on giant cholinergic interneurons of the striatum expressing Ca^{2+} -permeable GluA2-lacking AMPA receptors (Buldakova et al., 1999). The experiments on ASIC were carried out on hippocampal interneurons of the *lacunosum-moleculare* and *radiatum* layers of the CA1 region, which express ASIC1a/2 heteromers (Weng et al., 2010).

Two-electrode voltage-clamp

Recordings were performed at room temperature (18°C–22°C) using a Geneclamp 500 amplifier (Molecular Devices) controlled by a pClamp data acquisition system (Axon Instruments). Whole-cell currents from oocytes were recorded 1–4 days after cRNA injection. Bath solution composition was ND96, or HK containing (in mM): NaCl, 2; KCl, 96; CaCl_2 , 1.8; MgCl_2 , 2; and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept at 0.7–1.5 M Ω . Elicited currents were sampled at 1 kHz and filtered at 0.5 kHz (for potassium currents) or sampled at 20 kHz and filtered at 2 kHz (for sodium currents) using a four-pole low-pass Bessel filter. Leak subtraction was performed using a $-P/4$ protocol.

Currents were evoked by a 100 ms (Na_v) or 500 ms (K_v) depolarization to the voltage corresponding to the maximal activation of the channels in control conditions from a holding potential of -90 mV. For nAChR experiments, the oocytes were voltage-clamped at a holding potential of -70 mV and continuously superfused with ND96 via gravity-fed tubes at $0.1\text{--}0.2$ ml min^{-1} , with 5 min incubation times for the bath-applied peptides. ACh was applied via gravity-fed tubes until peak current amplitude was obtained (1–3 s), with 1–2 min washout periods between applications. The nAChR were gated by a

particular time duration pulse of ACh for the respective nAChR subtype (200 μ M for $\alpha 1\beta 1\gamma\delta$ and $\alpha 4\beta 2$; 100 μ M for $\alpha 7$) at 2 ml min⁻¹. Data were sampled at 500 Hz and filtered at 200 Hz. TRP currents were measured in ND96 at -90 mV during 400 s. Capsaicin (2 μ M) was used as an agonist and capsazepine (10 μ M) as an antagonist of TRPV1. Peak current amplitude was measured prior to and following the application of the peptide. All data were obtained in at least five independent experiments ($n \geq 5$).

Patch-clamp of HEK293 or COS7 cells

All experiments were conducted with an EPC-10 amplifier (HEKA) in the whole-cell configuration with a holding potential of -80 mV. Pipette resistances averaged around 2.5 M Ω . Solutions of apamin in Na⁺ Ringer were freshly prepared during the experiments from 100 μ M stock solutions in Roswell Park Memorial Institute medium (RPMI). For current measurements, we used an internal pipette solution containing (in mM): K⁺ aspartate, 160; MgCl₂, 2.08; HEPES, 10; EGTA, 10; and CaCl₂, 8.55 (1 μ M free Ca²⁺); pH 7.2; osmolarity 310 mOsm. Free Ca²⁺ concentrations were calculated with MaxChelator (developed by Chris Patton, Stanford University) assuming a temperature of 25°C, pH 7.2, and ionic strength of 160 mM. Na⁺ Ringer was used as an external solution containing (in mM): NaCl, 160; KCl, 4.5; CaCl₂, 2; MgCl₂, 1; and HEPES, 10; pH 7.4; osmolarity, 315 mOsm. Please note that the provided concentrations are what was weighed in to achieve an initially hyperosmolar solution that was diluted with water to 310 mOsm for the internal and 315 mOsm for the external solution as measured with a VAPRO Vapor pressure osmometer (Wescor). A slight 5 mOsm difference in osmolarity between internal and external solution improves the sealing rate.

K_{Ca2} and K_{Ca3.1} currents were elicited by 200-ms voltage ramps from -120 to 40 mV applied every 10 s, and the fold decrease of slope conductance at -80 mV was taken as a measure of apamin-induced channel inhibition. TRAM-34 was used to block K_{Ca3.1}. K_{Ca1.1} channel activity was recorded with a step protocol, in which cells were stepped to +40 mV for 100 ms at 30-s intervals. Concentration-

dependent current inhibition was fitted with the Hill equation using Prism 8 (GraphPad Software). All data were collected in at least five independent experiments ($n \geq 5$).

Patch-clamp of rat neurons or CHO cells

In this case, the holding potential was set at -70 mV. Signals were filtered at 10 kHz and sampled at 20 kHz. Drugs were applied using an RSC-200 (BioLogic) perfusion system. The extracellular solution contained (in mM): NaCl, 143; KCl, 5; CaCl_2 , 2.5; D-glucose, 10; and HEPES, 10; and the pH was adjusted to 7.4 with HCl. 10 mM MES was used in the extracellular solution in experiments on ASIC. The patch pipettes (2.5–3.5 M Ω) were made from borosilicate glass (WPI) using a P-97 puller (Sutter Instruments). The pipette solution contained (in mM): CsF, 100; CsCl, 40; NaCl, 5; CaCl_2 , 0.5; EGTA, 5; and HEPES, 10; and the pH was adjusted to 7.2 with CsOH. All experiments were performed at room temperature (22°C–24°C). NMDA receptors were activated by 100 μM NMDA and 10 μM glycine. AMPA receptors were activated by 100 μM kainate. ASIC of rat neurons were activated by pH drops from pH 7.4 to 6.5. ASIC1a, ASIC2a, and ASIC3 homomers were expressed in CHO cells and activated by pH 6.5, 5.0, and 6.8, respectively. Lyophilized apamin was freshly dissolved in extracellular solution prior to the experiment. After recording the control response, the patched cell was superfused for 40 s with extracellular solution containing apamin, and then the response to co-application of the agonist and apamin was recorded. Offline data analyses were performed using Origin 9.1 (OriginLab) software. All data were collected from at least five experiments ($n \geq 5$).

Data analysis and statistics

Data and statistical analysis comply with recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). All data points in the apamin concentration-response curves on K_{Ca} channels are means $\pm$ standard deviation (SD) from at least five independent experiments. IC_{50} s are reported with 95% confidence intervals (CI). The CI is based on the fit for the averaged currents. Student's two-tailed unpaired t -test was used to assess the significance of the effect of apamin in rat

neurons (current in the presence of apamin vs current in control). A value of $p < 0.05$ was considered statistically significant.

Results

Confirmation of apamin sample purity

Natural apamin isolated from bee venom was purchased from Sigma and subjected to analytical RP-HPLC. It presented a single symmetrical Gaussian peak (Figure 1A) corresponding to >95% purity. The fraction corresponding to the individual component was further inspected by MALDI mass spectrometry. The measured monoisotopic molecular mass ($[M + H]^+ = 2026.7$ Da; Figure 1A, inset) did not differ significantly from the calculated value for apamin (2026.9 Da; $\Delta = 0.2$ Da).

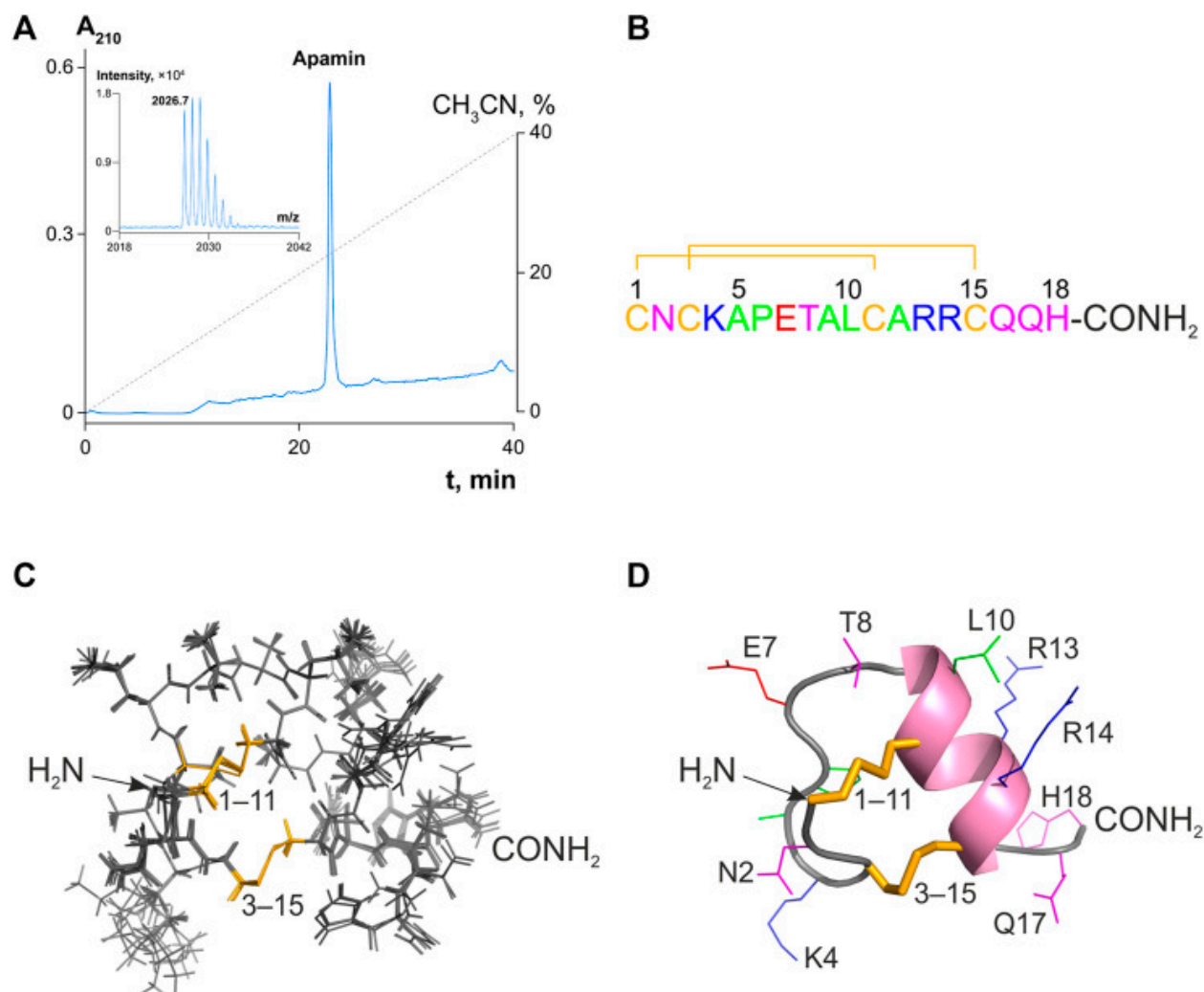


Figure 1. Analytical RP-HPLC of apamin and its structure in solution. **(A)** 10 nmol of peptide was injected onto a Vydac C₁₈ column (4.6 × 250 mm). Reflector-mode MALDI mass spectrum with an isotopic resolution of the purified peptide is shown in the inset. **(B)** Amino acid sequence of apamin. The numbering is shown above. Amino acid residues are colored with respect to their chemical properties: positively charged residues are shown in blue, negatively charged in red, hydrophobic in green, hydrophilic uncharged in magenta, and cysteines in yellow. Disulfide bridges are shown with lines. **(C)** A set of 10 NMR structures with the fewest restraint violations (PDB ID: 7OXF). Disulfide bridges are colored in yellow and labeled. **(D)** The spatial structure of apamin is shown in a ribbon representation. The α -helix is colored pink, and the rest of the main chain is gray. Side chains of the peptide are labeled and colored, the coloring is as in panel **(B)**. Disulfide bridges are shown as yellow sticks.

Apamin spatial structure in solution

As there is no apamin structure in the PDB, we solved it with NMR spectroscopy in solution based on the recorded J-couplings and NOE distances (Supplementary Figure S1). NMR chemical shifts, experimental restraints, and the spatial structure were deposited to the BMRB and PDB databases under the accession codes 34641 and 7OXF, respectively. The obtained structure fits all the experimental data

and is characterized by a low backbone RMSD value (0.24 Å), which confirms that it is well converged (Supplementary Table S1).

Apamin forms a short α -helix (Ala9–Gln16) and a β -turn (Asn2–Ala5; Figures 1B–D). Additionally, the amide group of Lys4 forms a hydrogen bond with the side chain carbonyl of Asn2, which is in agreement with the previously published decelerated hydrogen-deuterium exchange of Lys4 amide (Bystrov et al., 1980). Analysis of the structure reveals no electrostatic, π -cation, or stacking interactions that could stabilize the observed apamin conformation.

Antimicrobial activity

The antibacterial assay showed no activity of apamin against either Gram-positive or Gram-negative bacteria up to a concentration of 50 μ M. Melittin, on the other hand, displayed the expected antimicrobial effect. Its MIC values on *E. faecalis*, *S. aureus*, *E. coli*, and *P. aeruginosa* were 0.5, 3, 1.5, and 6 μ M, respectively.

Apamin pharmacology

We estimated the activity of apamin against five K_{Ca} , one K_{ir} , 15 K_V , 10 Na_V , three ASIC, and one TRP, as well as three nAChR (Table 1; Figures 2, ,3).3). The inhibitory effect was detected only for the three isoforms of K_{Ca2} (SK2 or SK $_{Ca}$) channels (Figures 2A–C). We constructed concentration-response curves for the susceptible channels (Figure 2D) and confirmed that apamin displays the expected nanomolar and subnanomolar affinity to these channels. The IC_{50} values were 4.1 nM, 87.7 pM, and 2.3 nM, respectively for $K_{Ca2.1}$, $K_{Ca2.2}$, and $K_{Ca2.3}$ (Table 1). Apamin had no effect on the intermediate-conductance $K_{Ca3.1}$ (IK) or large-conductance $K_{Ca1.1}$ (BK) currents at 5 μ M (Figures 2E,F, respectively).

IC_{50} with 95% Confidence Interval

Ca²⁺-activated K⁺ Channels (K_{Ca})						
K_{Ca}1.1	K _{Ca} 2.1	K _{Ca} 2.2	K _{Ca} 2.3	K _{Ca} 3.1		
N.E. [1.02 ± 0.07 (n = 5)]	4.1 nM	87.7 pM	2.3 nM	N.E. [0.99 ± 0.05 (n = 5)]		
	95% CI 3.3– 5.0 nM	95% CI 74.2–103.3 pM	95% CI 1.8– 2.9 nM			
Voltage-gated K⁺ channels (K_V)						
K_V1.1	K _V 1.2	K _V 1.3	K _V 1.4	K _V 1.5	K _V 1.6	K _V 2.1
N.E. [0.97 ± 0.02 (n = 5)]	N.E. [0.99 ± 0.03 (n = 5)]	N.E. [1.03 ± 0.04 (n = 5)]	N.E. [0.99 ± 0.02 (n = 5)]	N.E. [0.95 ± 0.04 (n = 5)]	N.E. [1.02 ± 0.01 (n = 5)]	N.E. [1.05 ± 0.03 (n = 6)]
K_V3.1	K _V 4.3	K _V 7.1	K _V 7.2/7.3	K _V 10.1	K _V 11.1	

N.E. [0.99 ± 0.02 ($n = 5$)]	N.E. [0.97 ± 0.03 ($n = 5$)]	N.E. [0.93 ± 0.05 ($n = 5$)]	N.E. [1.00 ± 0.01 ($n = 5$)]	N.E. [1.02 ± 0.04 ($n = 6$)]	N.E. [1.07 ± 0.06 ($n = 5$)]	
<i>Shaker-IR</i>	KQT-1					
N.E. [0.98 ± 0.04 ($n = 5$)]	N.E. [1.01 ± 0.02 ($n = 5$)]					
Inwardly rectifying K⁺ channels (K_{ir})						
K_{ir}3.1/3.2						
N.E. [1.02 ± 0.04 ($n = 6$)]						
Voltage-gated Na⁺ channels (Nav)						
Nav1.1	Nav1.2	Nav1.3	Nav1.4	Nav1.5	Nav1.6	Nav1.7
N.E. [0.93 ± 0.04 ($n = 5$)]	N.E. [0.97 ± 0.02 ($n = 6$)]	N.E. [0.98 ± 0.06 ($n = 5$)]	N.E. [1.06 ± 0.08 ($n = 5$)]	N.E. [1.00 ± 0.03 ($n = 5$)]	N.E. [1.09 ± 0.06 ($n = 6$)]	N.E. [0.93 ± 0.08 ($n = 5$)]

Nav1.8	BgNav1	VdNav1				
N.E. [0.95 ± 0.04 (n = 5)]	N.E. [0.96 ± 0.06 (n = 5)]	N.E. [0.97 ± 0.01 (n = 5)]				
Transient receptor potential channels (TRP)						
TRPV1						
N.E. [1.20 ± 0.09 (n = 5)]						
Nicotinic acetylcholine receptors (nAChR)						
α1β1γδ	α4β2	α7				
N.E. [1.06 ± 0.03 (n = 6)]	N.E. [0.95 ± 0.07 (n = 6)]	N.E. [1.10 ± 0.08 (n = 6)]				
Acid-sensing ion channels (ASIC)						
ASIC1a/2 native	ASIC1a	ASIC2a	ASIC3			

N.E. [0.99 ± 0.12 (<i>n</i> = 10)]	N.E. [0.99 ± 0.03 (<i>n</i> = 6)]	N.E. [1.01 ± 0.03 (<i>n</i> = 7)]	N.E. [0.99 ± 0.02 (<i>n</i> = 7)]			
Glutamate receptors (GluR)						
AMPA (Ca ²⁺ -impermeable)	AMPA (Ca ²⁺ -permeable)	NMDA				
N.E. [0.98 ± 0.03 (<i>n</i> = 7)]	N.E. [1.01 ± 0.02 (<i>n</i> = 6)]	N.E. [0.96 ± 0.07 (<i>n</i> = 14)]				

Table 1. Apamin potency against tested ion channels. K_{Ca} channels were expressed in HEK293 cells (K_{Ca}2.3, in COS7 cells); K_V, K_{ir}, Nav, TRP, and nAChR were expressed in *X. laevis* oocytes; homomeric ASIC1a, ASIC2a, and ASIC3 were expressed in CHO cells; native NMDA and Ca²⁺-impermeable AMPA receptors were investigated in hippocampal CA1 pyramidal cells; native Ca²⁺-permeable AMPA receptors were studied in giant cholinergic interneurons of the striatum; and native ASIC1a/2 heteromers were investigated in hippocampal interneurons of the *lacunosum-moleculare* and *radiatum* layers of the CA1 region. N.E., no effect at 5 μM concentration. The current ratio (*I*_{apamin}/*I*_{control}) with an indication of SD, and of the *n* is displayed in the square brackets.

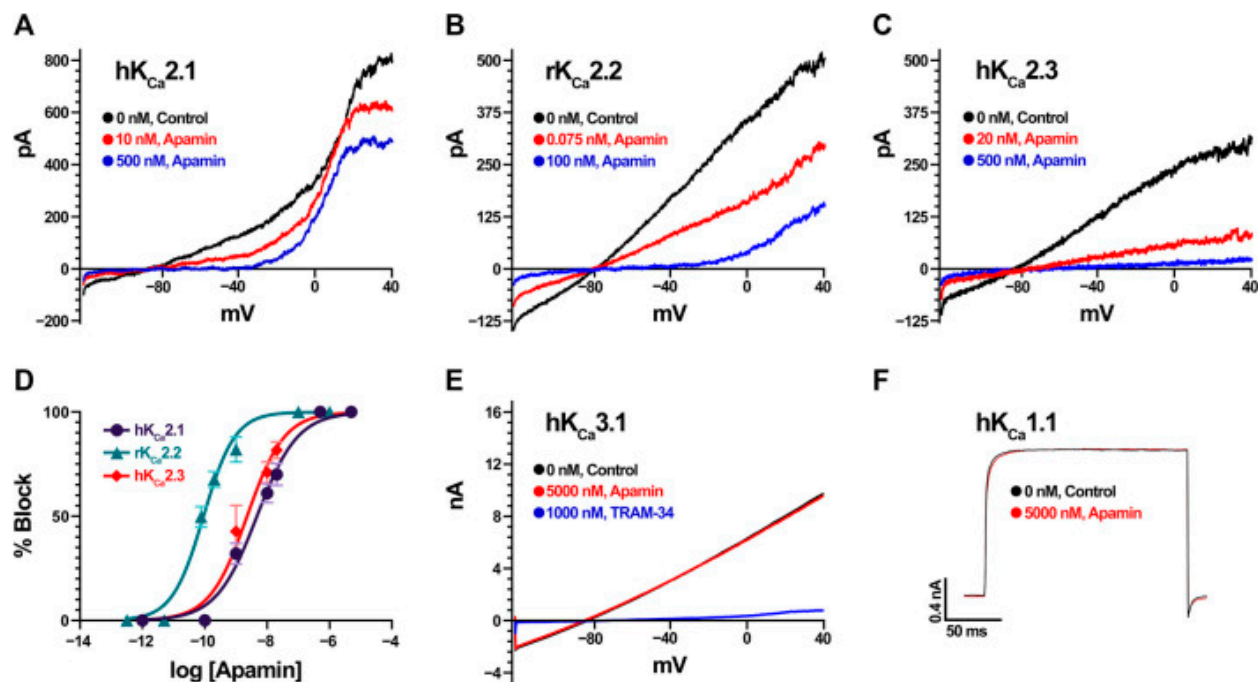


Figure 2. Apamin effects on K_{Ca} channels expressed in HEK293 or COS7 cells (in the case of $K_{Ca2.3}$). (A) Effect of increasing concentrations of apamin (10 nM and 500 nM in red and blue, respectively) on $K_{Ca2.1}$ currents. Note that the currents visible above 0 mV are carried by endogenous K_V channels in HEK cells. (B) Effect of increasing concentrations of apamin (75 pM and 100 nM in red and blue, respectively) on $K_{Ca2.2}$ currents. (C) Effect of increasing concentrations of apamin (20 nM and 500 nM in red and blue, respectively) on $K_{Ca2.3}$ currents. (D) Concentration-response curves for $K_{Ca2.1}$ (in dark purple), $K_{Ca2.2}$ (teal), and $K_{Ca2.3}$ (red). Data points are mean $\pm$ SD from five independent measurements per concentration. (E) $K_{Ca3.1}$ currents (in black) are insensitive to 5 μ M apamin (red) but are potently blocked by 1 μ M TRAM-34 (blue). (F) $K_{Ca1.1}$ currents evoked by depolarization steps to +80 mV (in black) are insensitive to 5 μ M of apamin (red).



Figure 3. Electrophysiological profiling of apamin. Shown are representative traces of currents through the corresponding ion channels in control (gray) and after application of 5 μ M toxin (blue). In the case of nAChR, blue bars indicate apamin application. In nAChR, arrows indicate agonist (ACh) application. In TRPV1, the open bar shows agonist (capsaicin, CAP) application, and gray bar, antagonist (capsazepine, CZP) application. In GIRK1/2, presentation of different bath solutions is shown as line segments. In ASIC traces, application of the activating pH is shown with gray bars. And in GluR traces, gray bars depict the application of agonists. Ca_{imp} , Ca^{2+} -impermeable; Ca_p , Ca^{2+} -permeable.

The effects of apamin on native iGluR (Ca^{2+} -permeable and Ca^{2+} -impermeable AMPA receptors as well as NMDA receptors) and ASIC were studied in isolated neurons of rat brain. Extracellular application of apamin alone did not produce noticeable effects on the holding currents. We compared the whole-cell currents through the receptors in control and in the presence of apamin. At a concentration of 5 μ M, it produced no effects on the three types of iGluR and ASIC. In the case of NMDA receptors we

noticed that the current in the presence of apamin tended to be slightly lower than in control (in some cells up to $\approx 15\%$), although this difference was not statistically significant ($I_{\text{apamin}}/I_{\text{control}} = 0.96 \pm 0.07$, $p > 0.05$, $n = 14$; Supplementary Figure S2).

Discussion

Apamin's 3D structure

Apamin was investigated several times, by different groups, using both crystallography and NMR spectroscopy. To our surprise, no atomic coordinates were deposited in the PDB, limiting the utility of those studies to further progress. We, therefore, performed a solution NMR study, solved our own structure (Figures 1C,D), and deposited it in the PDB (accession code: 7OXF).

At least four papers reported the spatial structure of apamin. The first study used NMR and was published by Bystrov and coworkers in 1980 (Bystrov et al., 1980). However, the reported structure appears erroneous due to the wrong positioning of the secondary structure elements (for instance, the α -helix was proposed to be formed in the region 6–13, which is incorrect). Two later NMR studies were published in 1983 and 1988 (Wemmer and Kallenbach, 1983; Pease and Wemmer, 1988) and reported a conformation that seems almost correct, with the α -helix in the region 9–17, and a β -turn formed by residues 2–5. Finally, the most recent work by Aumelas and coworkers (Le-Nguyen et al., 2007) reported an NMR structure of apamin and an X-ray structure of its analog. The NMR structure from that study is in perfect agreement with the apamin conformation observed in our work. The X-ray structure, on the other hand, presents a dimer and differs in the conformation of the N-terminus, the length of the α -helical segment (9–18 in the case of X-ray and 9–16 in NMR), and the dihedral angles of the disulfide bonds. These differences are apparently due to crystal packing constraints.

Apamin shows no antimicrobial effects

Honeybee *Apis mellifera* venom is well known to display antibacterial and antifungal effects, which are mostly due to its major component melittin, one of the best-studied cytolytic peptides (Habermann, 1972; Raghuraman and Chattopadhyay, 2007). Expectations of similar activity in apamin are reasonable because animals usually produce several similar toxins or even “libraries” of toxins, which is the case for both neurotoxic and cytolytic components (Vassilevski et al., 2009). However, apamin testing against Gram-positive and Gram-negative bacteria revealed no effect up to a very high concentration of 50 μ M. At the same time, melittin shows prominent antimicrobial effects at low micromolar concentrations, and since its content in bee venom is $\approx$ 20 times higher, the chances that apamin shares a similar mode of action are very low.

Apamin’s revisited pharmacology

Small-conductance Ca^{2+} -activated K^+ channels ($\text{K}_{\text{Ca}2}$, SK, or SK_{Ca}) are a group of three α -subunit isoforms ($\text{K}_{\text{Ca}2.1-2.3}$) that can form mature channels of either homo- or heterotetrameric structure (Köhler et al., 1996; Strassmaier et al., 2005). Similarly to many other K^+ channels, each SK α -subunit contains six transmembrane segments (S1–S6, with S5 and S6 contributing to the pore domain). Unlike K_{V} channels or large-conductance Ca^{2+} -activated K^+ channels (BK or $\text{K}_{\text{Ca}1.1}$), the gating of SK channels is insensitive to transmembrane voltage. $\text{K}_{\text{Ca}2}$ channels have no Ca^{2+} binding sites in their α -subunit, but they form a stable complex with calmodulin that acts as their Ca^{2+} sensor (Xia et al., 1998). All three isoforms of $\text{K}_{\text{Ca}2}$ are widely expressed in the central nervous system (Stocker and Pedarzani, 2000; Chen et al., 2004) and found in sensory neurons and the heart (Köhler et al., 1996; Xu et al., 2003; Tuteja et al., 2005; Skibsbbye et al., 2014); $\text{K}_{\text{Ca}2.2}$ is also important in the liver (Feranchak et al., 2004); and $\text{K}_{\text{Ca}2.3}$ is found in many tissues (Herrera et al., 2003; Tamarina et al., 2003; Chen et al., 2004). According to the Mouse Brain Atlas (<http://www.mousebrain.org>) (Zeisel et al., 2018), $\text{K}_{\text{Ca}2.1}$ is highly expressed in neurons of the cerebral cortex, midbrain red nucleus, hindbrain, and sensory neurons; $\text{K}_{\text{Ca}2.2}$ in the cerebral cortex, thalamus, hippocampus, spinal cord, midbrain, hindbrain, erector muscle, and sensory neurons, as well as glial cells;

and K_{Ca}2.3 in the diencephalon, nuclei of cranial nerves, medulla, thalamus, hypothalamus, ventral midbrain, hindbrain, cerebellum, enteric and erector muscle neurons, as well as enteric fibroblasts.

The number of known polypeptide or peptide toxins acting on K_{Ca}2 with high (nanomolar) affinity is limited. According to the Kalium database (<https://kaliumdb.org>) (Kuzmenkov et al., 2016; Tabakmakher et al., 2019), six of these substances were identified in scorpion venom (AmP05, maurotoxin, Pi-1, scyllatoxin, tamapin, and Ts9). Scyllatoxin (α -KTx 5.1) from *Leiurus quinquestriatus hebraeus* (Shakkottai et al., 2001) and tamapin (α -KTx 5.4) from *Mesobuthus tamulus* (Pedarzani et al., 2002), in particular, show high potency against K_{Ca}2.2 with IC₅₀ values in the subnanomolar range. Interestingly, apamin shares a similar sequence motif RXCQ with several scorpion toxins that inhibit K_{Ca} channels: X = R in apamin and AmP05, M in scyllatoxin, and P in Pi-1. In all these toxins the RXCQ motif is found in an α -helix. Replacement of “X” in the RXCQ motif of scyllatoxin with a positively charged residue (e.g., lysine or 2,4-diaminobutyric acid) resulted in derivatives with enhanced selectivity for K_{Ca}2.2 over K_{Ca}2.3 (Sabatier et al., 1993, 1994; Shakkottai et al., 2001). The importance and generality of this motif will be clarified in the future.

Structure-activity studies showed that one of the two adjacent arginine residues (Arg13 and Arg14) and Gln17 of apamin are key determinants of its activity (Vincent et al., 1975; Sandberg, 1979; Labbé-Jullié et al., 1991). Mutagenesis suggested that one of these Arg residues interacts with Asp341 (S5–P region) of K_{Ca}2.2 channel pore region, whereas Gln17 interacts with Asn368 (P–S6; numbering according to rK_{Ca}2.2, UniProt accession number: P70604) (Ishii et al., 1997). Two additional positions in the pore region were subsequently proposed to be involved in apamin sensitivity [His337 or His485 of K_{Ca}2.2 and K_{Ca}2.3 (Q9UGI6), respectively, and Asn345 of K_{Ca}2.2; all in S5–P] (Lamy et al., 2010). Those studies advocated in favor of apamin acting like a pore blocker. On the other hand, some investigations reported that the S3–S4 extracellular loop may be an essential molecular determinant of apamin sensitivity suggesting an allosteric mode of action and not direct pore blockage. Thus, a point mutation (Thr216Ser) significantly influences K_{Ca}2.1 (Q92952) sensitivity towards apamin (Nolting et al., 2007). In addition, the

three-amino-acid motif in the S3–S4 loop of K_{Ca}2 channels (²¹⁶TYA²¹⁸ in hK_{Ca}2.1, ²⁴⁴SYA²⁴⁶ in hK_{Ca}2.2, and ³⁹³SYT³⁹⁵ in hK_{Ca}2.3) was implicated in forming the binding interface for apamin (Weatherall et al., 2011). In the absence of a solved 3D structure of a K_{Ca}2 channel, it is difficult to predict the details of molecular interactions with apamin. One tempting possibility is that the long S3–S4 loop protrudes to the pore domain and together they form a common binding site for apamin.

It is apamin from the honeybee venom (Habermann and Reiz, 1965; Habermann, 1972) that is the most prominent peptide ligand of K_{Ca}2. Indeed, since the early 1980s apamin has been used as the main pharmacological agent to distinguish K_{Ca}2 channels from other K⁺ channels (Burgess et al., 1981; Romey and Lazdunski, 1984; Pennefather et al., 1985). Subsequent studies on cloned K_{Ca}2 confirmed that these three channel isoforms are the molecular targets of apamin (Köhler et al., 1996; Shah and Haylett, 2000; Strøbaek et al., 2000; Grunnet et al., 2001a). However, later studies also claimed some off-target activities of apamin. One series of publications reported inhibition of Ca²⁺ and Na⁺ channels in embryonic heart tissues (Bkaily et al., 1985, 1991, 1992). And another study pointed to K_V1.3 as a target (Voos et al., 2017). Our extensive electrophysiological measurements disagree with or directly disprove these claims. Apamin does not present any activity on neither of the expressed Na⁺ channels nor K_V1.3.

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Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author. NMR chemical shifts, experimental

restraints, and the spatial structure of apamin were deposited to the BMRB (accession code 34641) and PDB databases (7OXF).

Ethics statement

This study strictly complied with the International Guiding Principles for Biomedical Research Involving Animals and the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 18. III. 1986). Animal studies are reported in compliance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020). The use of the frogs was in accordance with the license number LA1210239 to Toxicology and Pharmacology, KU Leuven, and was approved by the Ethical Committee for animal experiments of KU Leuven (P186/2019). Wistar rats were bred and reared at a local animal facility of the Sechenov Institute of Evolutionary Physiology and Biochemistry. Animals were housed in ventilated plastic cages and had *ad libitum* access to food and water. 12/12 h light/dark cycle, temperature and humidity control in the rooms were secured. All experiments with rats were approved by the Local Bioethics Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences (Protocol no. 4/2021, 29 April 2021). Maximum efforts were made to minimize animal suffering and the number of animals used in the experiments.

Author contributions

AK and AV designed research; AK, SP, JN, ELP-J, KM, MN, HW, JT, and AV analysed data; AK, SP, ELP-J, KM, and MN performed research; AK performed the purification; KM established the NMR structure; SP, ELP-J, JN, and MN performed the pharmacological profiling of apamin; HW and JT supervised the pharmacology; AA supervised the NMR spectroscopy; AK, SP, JN, KM, MN, HW, and AV wrote the paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2022.977440/full#supplementary-material>

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CHAPTER 4: The erythroid K-Cl cotransport inhibitor [(dihydroindenyl)oxy]acetic acid blocks erythroid Ca^{2+} -activated K^+ channel KCNN4

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Abstract

Red cell volume is a major determinant of HbS concentration in sickle cell disease. Cellular deoxy-HbS concentration determines the delay time, the interval between HbS deoxygenation and deoxy-HbS polymerization. Major membrane transporter protein determinants of sickle red cell volume include the SLC12/KCC K-Cl cotransporters KCC3/SLC12A6 and KCC1/SLC12A4, and the KCNN4/KCa3.1 Ca^{2+} -activated K^+ channel (Gardos channel). Among standard inhibitors of KCC-mediated K-Cl cotransport, only [(dihydroindenyl)oxy]acetic acid (DIOA) has been reported to lack inhibitory activity against the related bumetanide-sensitive erythroid Na-K-2Cl cotransporter NKCC1/SLC12A2. DIOA has been often used to inhibit K-Cl cotransport when studying the expression and regulation of other K^+ transporters and K^+ channels. We report here that DIOA at concentrations routinely used to inhibit K-Cl cotransport can also abrogate activity of the KCNN4/KCa3.1 Gardos channel in human and mouse red cells and in human sickle red cells. DIOA inhibition of A23187-stimulated erythroid K^+ uptake (Gardos channel activity) was chloride-independent and persisted in mouse red cells genetically devoid of the principal K-Cl cotransporters KCC3 and KCC1. DIOA also inhibited YODA1-stimulated, chloride-independent erythroid K^+ uptake. In contrast, DIOA exhibited no inhibitory effect on K^+ influx into A23187-treated red cells of *Kcnn4*^{-/-} mice. DIOA inhibition of human KCa3.1 was validated (IC_{50} 42 μM) by whole cell patch clamp in HEK-293 cells. RosettaLigand docking experiments identified a potential binding site for DIOA in the fenestration region of human KCa3.1. We conclude that DIOA at concentrations routinely used to inhibit K-Cl cotransport can also block the KCNN4/KCa3.1 Gardos channel in normal and sickle red cells.

Introduction

The therapeutic regulation of erythrocyte volume has been acknowledged as a promising approach toward the development of adjunct treatments for sickle cell disease (1–3). The delay time for polymerization of deoxy-hemoglobin S (HbS) is an inverse function of the 30th–50th exponential power of deoxy-HbS concentration within the red blood cell (4). Red cell volume is controlled predominantly by K^+ transporters and channels. The most important of these transmembrane erythroid K^+ carriers are the K-Cl cotransporter KCC3 (with a lesser contribution from KCC1 and, in the human red cell, additional potential contribution from KCC4) (2, 5), and the Gardos channel—the intermediate conductance, Ca^{2+} -activated K^+ channel also known as KCNN4, KCa3.1, or IK1 (2, 6, 7). The Gardos channel is the principal (if not unique) K^+ conductance of the red cell (8). The Gardos channel inhibitor clotrimazole (9) and its higher affinity and higher specificity congeners TRAM-34 (10) and senicapoc (11) have all been shown to regulate red cell volume, leading to proposed clinical use as adjunct treatments for sickle cell disease. Inhibitors of the K-Cl cotransporters should, in theory, be helpful in this strategy to control red cell volume. However, unlike for the neuronally expressed KCC2, specific inhibitors of high-affinity targeting KCC3, KCC1, and KCC4 are less extensively developed (12).

Several KCC inhibitors of low specificity and affinity have been used for pharmacological identification of K-Cl cotransport activity and to inhibit erythroid K-Cl cotransport activity during studies of other red cell K^+ transport processes, including that mediated by the KCNN4 Gardos channel (12). The inexpensive loop diuretic bumetanide at the high concentration of 1 mM is commonly used to inhibit ~80% of K-Cl cotransport while also completely inhibiting red cell NKCC1-mediated Na-K-Cl cotransport. However, DIOA has been shown to inhibit K-Cl cotransport with IC_{50} of 10 μ M (13), reportedly without inhibition of NKCC1 at the 100 μ M concentration usually used for complete inhibition of K-Cl cotransport in red cells (14).

While assessing K-Cl cotransport in red cells from affected members of a family with hereditary xerocytosis (HX) secondary to KCNN4 heterozygous mutation V282M (15, 16), we noticed that the use of 100 μ M DIOA appeared to be associated with inhibition of A23187-stimulated KCNN4 activity in normal and HX red cells. Since the activity of K-Cl cotransport is often pharmacologically blocked during radioisotopic assessment of KCNN4 activity, this observation led us to characterize DIOA as a potentially confounding inhibitor of KCNN4.

Methods

Materials

Human blood obtained from research blood donors and from samples discarded after use for clinically indicated laboratory testing was used for these studies under protocols approved by the Institutional Review Boards of Boston Children's Hospital and Beth Israel Deaconess Medical Center. ^{83}Rb was obtained from Meta Isotopes, LLC (Lubbock, TX). R-(+)-[(2-*n*-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]acetic acid, also known as [(dihydroindenyl)oxy]acetic acid (DIOA) was from Sigma (St. Louis, MO). Senicapoc (SCP) was a gift from Pfizer, Inc. Salts and other drugs were obtained from Sigma.

Animals

Mouse blood was obtained by terminal exsanguination from mice bred, maintained, and used under Institutional Animal Care and Use Committee (IACUC) protocols approved by Beth Israel Deaconess Medical Center. C57Bl6/J wild-type (WT) mice were from our own colony. *Kcc1*^{-/-}; *Kcc3*^{-/-} mice (2, 5), *Kcnn4*^{-/-} mice (2, 17), and SAD mice (2) were maintained in our own colony, as previously described.

Solutions

Human chloride flux medium contained (in mM) 145 NaCl, 2 KCl, 1.5 CaCl₂, 0.15 MgCl₂, 10 glucose, 0.1 ouabain, 1.0 bumetanide, and 10 Tris-MOPS, pH 7.4 at 37°C. Human chloride-free sulfamate flux medium contained (in mM) 145 Na sulfamate, 2 KNO₃, 1.5 Ca(NO₃)₂, 0.15 Mg(NO₃)₂, 10 glucose, 0.1 ouabain, 1.0 bumetanide, and 10 Tris-MOPS, pH 7.4 at 37°C. All solutions for use with human cells exhibited osmolarities of 290–310 mosmol/kg H₂O by freezing point depression (Osmette A, Precision Instruments, Fisher Scientific). Mouse chloride flux medium contained 155 mM NaCl, and mouse chloride-free sulfamate flux medium contained 155 mM Na sulfamate. Other salt and drug concentrations were identical to their human counterparts except that ouabain was 1 mM in mouse solutions. All solutions for use with mouse cells exhibited osmolarities of 330–340 mosmol/kg H₂O by freezing point depression. 5 μM Ca²⁺ ionophore A23187 was added as specified. When in the presence of A23187, extracellular concentrations of CaCl₂ (in chloride media) and CaNO₃ (in chloride-free sulfamate media) were reduced to 100 μM. DIOA was used at 100 μM, Senicapoc was used at 200 nM, and YODA1 was used at 15 μM in both mouse and human solutions unless (in the case of DIOA) otherwise specified.

Unidirectional K⁺ Influx Studies

Whole blood was passed through cotton to remove the plasma and buffy coat. Red blood cells were washed 4× with isotonic choline chloride solution, and hematocrit was measured. Freshly isolated, washed red cells at 3% hematocrit were preincubated at 37°C in chloride-containing or chloride-free (sulfamate) transport media as described in *Solutions*. Influx was initiated by the addition of 30 μL packed, washed red cells to tubes containing transport media, drugs, and 5 μCi ⁸³RbCl (12) (total volume 1 mL) for 10 min (in the absence of A23187) or for 5 min (in the presence of A23187). Note that Rb⁺ salts have long been used to substitute for the higher energy, shorter-lived radioisotopes of K⁺ (12). For A23187 experiments, ionophore was added to a concentration of 5 μM at time zero, and aliquots of cell suspension removed after 5 min were immediately centrifuged through 0.8 mL cold transport medium containing 5 mM EGTA over a 0.3 mL cushion of n-butylphthalate. In the absence of A23187, aliquots were taken after 10-min incubation. Supernatants were aspirated, and the tube tip containing the cell pellet was cut off. Erythrocyte-

associated radioactivity was counted in a gamma counter (Isomedic 41600 HE, ICN Biomedicals, Costa Mesa, CA) and expressed as mmol/L cell $\times$ hour. Each condition for each experimental sample was tested in quadruplicate, and the mean of the quadruplicate samples was taken as one experimental value for that condition. Concentration-response curves presented in Fig. 8 represent fits of the data to the Hill equation:

$$Y = [y_{\min} + (y_{\max} - y_{\min}) / (1 + X / IC_{50})]$$

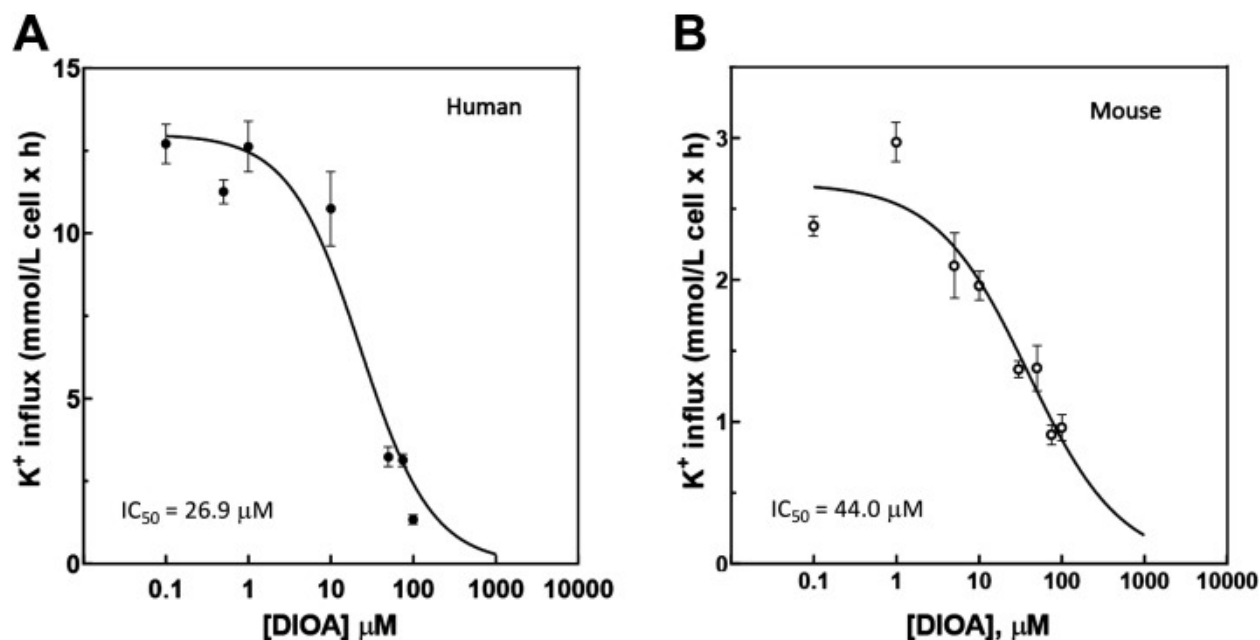


Figure 8. Concentration-response curves for DIOA inhibition of A23187-stimulated Gardos channel flux. *A*: DIOA concentration-response curve for inhibition of A23187-stimulated K⁺ influx into WT human red cells. *B*: DIOA concentration-response curve for inhibition of A23187-stimulated K⁺ influx into WT mouse red cells. DIOA, [(dihydroindenyl)oxy]acetic acid; WT, wild type.

Note that all influx values presented are measured in the presence of ouabain and bumetanide. Red cell exposure to A23187 in the presence of extracellular Ca²⁺ has been shown to hyperpolarize membrane potential by as much as ~60 mV (18).

Electrophysiology

Whole cell patch clamp experiments on human embryonic kidney (HEK) cells stably expressing hKCa3.1 were performed with a HEKA EPC-10 amplifier, as previously described (19). Briefly, cells were clamped to a holding potential of −80 mV and K_{Ca} currents were elicited by 200-ms voltage ramps from −120 to +40 mV applied every 10 s. The extracellular solution contained 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4, 300 mosmol/kg H₂O). The pipette (intracellular)

solution contained 154 mM KCl, 10 mM HEPES, 10 mM EGTA, 1.75 mM MgCl₂, and 8.5 mM CaCl₂ (pH 7.2, 290 mosmol/kg H₂O), yielding a free [Ca²⁺] of 1 μ M as calculated with MaxChelator. The concentration-response curve data were fit with the Hill equation (Fig. 10B) as described above for the flux data of Fig. 8 (see *Unidirectional K⁺ Influx Studies*).

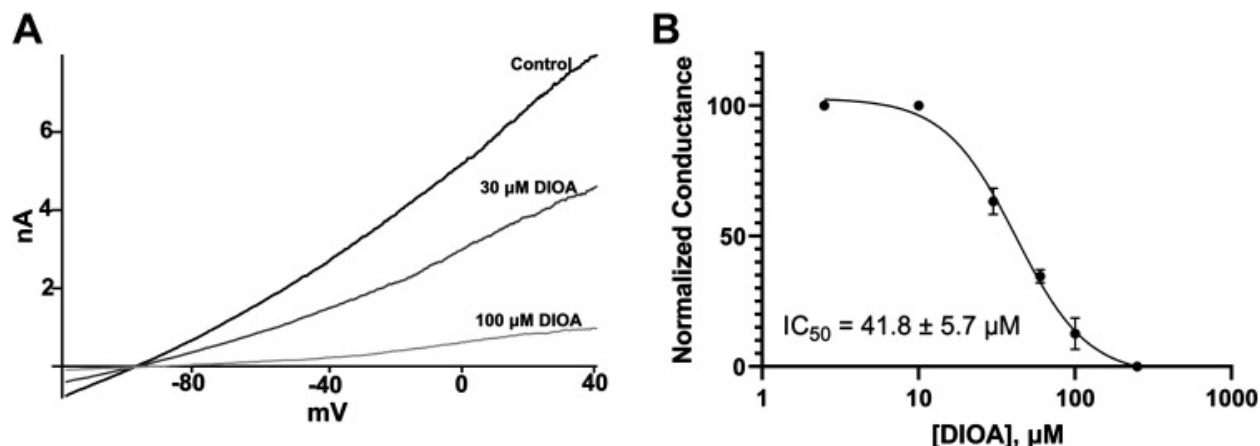


Figure 10. DIOA inhibits Ca²⁺-activated K⁺ currents in HEK-293 cells overexpressing recombinant human KCa3.1. *A*: current-voltage relationship of human KCa3.1 currents elicited by voltage ramps from -120 to $+40$ mV and recorded in whole cell patch mode in the absence (Control) and subsequent bath addition of DIOA first at 30μ M, and then at 100μ M. Cells were dialyzed with 1 mM free Ca²⁺ in the pipette solution *B*: normalized concentration-response curve showing DIOA inhibition of human KCa3.1-mediated K⁺ current. Values are means $\pm$ SE ($n = 3$). DIOA, [(dihydroindenyl)oxy]acetic acid.

Molecular Modeling

The cryo-EM structures of the full-length KCa3.1 channel (20) in the closed state (PDB: 6CNM) were refined using the Rosetta cryo-EM refinement protocol. Conformers of DIOA were generated using Open Eye OMEGA software v. 2.5.1.4, randomly placed within either the pore or the fenestration binding pocket of KCa3.1, and then taken through the three stages of RosettaLigand modeling, progressing from low-resolution conformational sampling and scoring to full atom optimization using Rosetta's all-atom energy function, as previously described in detail (19, 21). In total 10,000 models were generated for each site, from which the top 1,000 models of the lowest total energy score were selected. Among these 1,000 models, the top 10 models of lowest binding energy were identified and manually inspected for ligand/channel interactions and convergence. The lowest energy poses (typically also the most frequently sampled) are shown in Fig. 11.

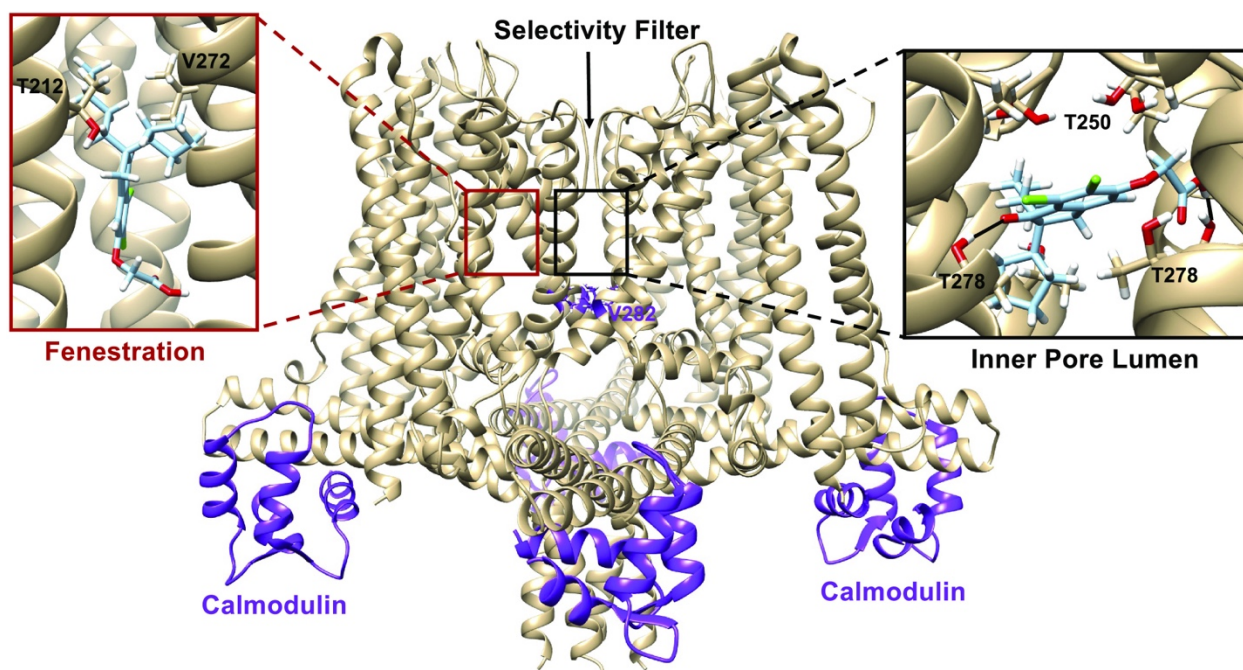


Figure 11. Molecular docking of DIOA to human KCa3.1. Transmembrane view of a Rosetta model of the cryo-EM structure of human KCa3.1 in the closed state (PDB:6CNM). The tetrameric channel is in light-brown ribbon presentation with the four associated calmodulins rendered in purple. The subunit in the back is shown semitransparent. The selectivity filter is indicated with a black arrow. The narrowest point of the inner pore is located at residue V282 (in purple at center of structure). DIOA is shown in stick presentation with carbon colored light blue, oxygen red, chlorine green, and hydrogen white. DIOA docks to the fenestration region with an energy of -15.8 REU (one of the four fenestrations is highlighted by a red box and shown magnified at *left*). DIOA can also dock within the inner pore (highlighted by black box, magnified at *right*) with an energy of -12.5 REU. At each docking site, the DIOA pose shown is that most frequently sampled and of lowest energy among the 10 poses of lowest energy. DIOA, [(dihydroindenyl)oxy]acetic acid; REU, Rosetta energy unit.

Statistical analysis

Two-way comparisons were conducted by Mann–Whitney test. Comparisons among multiple samples were conducted by Kruskal–Wallis ANOVA (GraphPad PRISM 8.4.3).

Results

DIOA inhibits KCNN4 in a Cl^- -independent manner in human red blood cells

In the absence of A23187, and in the absence or presence of the ion transport inhibitors senicapoc or DIOA, basal K^+ influx into normal (WT) human red cells was minimal in either chloride-containing or chloride-deficient (sulfamate-containing) medium (Fig. 1A). However, the calcium ionophore A23187 robustly stimulated K^+ influx in a chloride-independent manner typical of Gardos channel activity, and that activity was almost completely inhibited by 200 nM senicapoc. Moreover, DIOA at the 100 μM concentration routinely used to maximally inhibit erythroid K-Cl cotransport also inhibited Gardos channel activity as completely as did senicapoc, and in a manner independent of extracellular chloride (Fig. 1B). The same patterns of inhibition were evident in tests of red cells from patients with hereditary xerocytosis

(HX) secondary to the KCNN4 mutation V282M. (Fig. 1C and D). As we previously reported (15), KCNN4 V282M mutant HX red cells in this family exhibit reduced Gardos channel activity as measured by A23187-stimulated Gardos channel K^+ influx assay. Both 100 μ M DIOA and 200 nM senicapoc were equally effective as inhibitors of Gardos channel activity in both WT and HX human red cells. Levels of senicapoc-sensitive K^+ influx and DIOA-sensitive K^+ influx elicited by A23187 exposure were equivalent in WT human red cells (Fig. 2A and B) and (at the lower levels of activity noted above) in HX red cells with the heterozygous V282M mutation in KCNN4 (Fig. 2C and D).

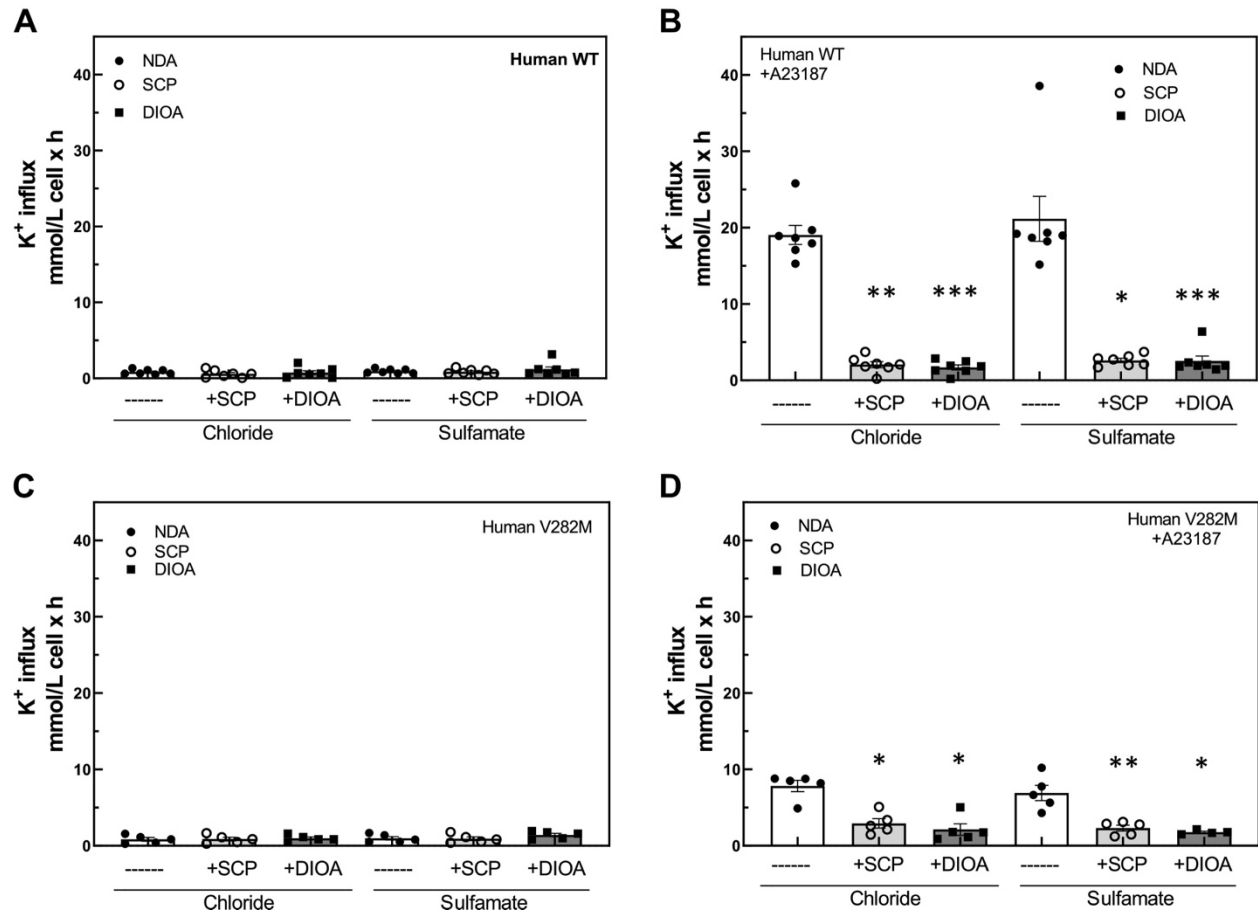


Figure 1. DIOA inhibits the Gardos channel in human red cells. *A*: basal K^+ influx into WT human red cells in chloride or sulfamate media in the absence of A23187. Cells were exposed to no drug (dashed line), to senicapoc (SCP, 200 nM) or to DIOA (100 μ M). *B*: A23187-stimulated K^+ influx into WT human red cells in chloride or sulfamate media, in the presence of no drug, senicapoc or DIOA. *C*: basal K^+ influx into human hereditary xerocytosis (HX) red cells carrying the KCNN4 mutation V282M, in chloride or sulfamate media in the absence of A23187. Cells were exposed to no drug, senicapoc, or DIOA. *D*: A23187-stimulated K^+ influx into human HX V282M red cells in chloride or sulfamate media, in the presence of no drug, SCP or DIOA. * P < 0.05; ** P < 0.01; *** P < 0.002 vs. no drug in chloride or in sulfamate, by Mann–Whitney test. DIOA, [(dihydroindenyl)oxy]acetic acid; NDA, no drug added; WT, wild type.

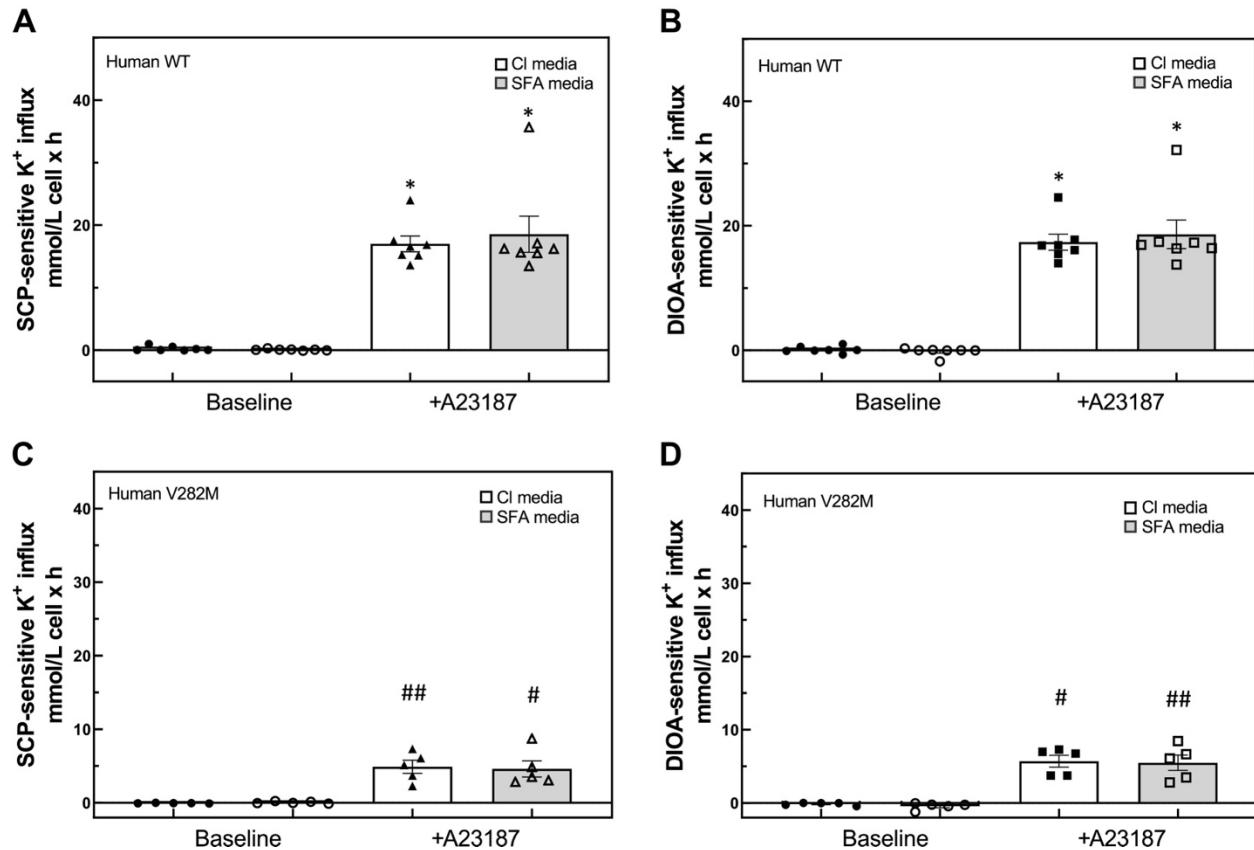


Figure 2. DIOA-sensitive K^+ influx in the absence or presence of A23187 is indistinguishable in magnitude from senicapoc-sensitive K^+ influx. *A*: basal and A23187-stimulated SCP-sensitive K^+ influx into human WT red cells. *B*: basal and A23187-stimulated DIOA-sensitive K^+ influx into human WT red cells. * $P < 0.001$ vs. baseline, by Mann–Whitney test. *C*: basal and A23187-stimulated SCP-sensitive K^+ influx into human HX V282M red cells. *D*: basal and A23187-stimulated DIOA-sensitive K^+ influx into human HX V282M red cells. These data are replotted from the assays portrayed in Fig. 1. # $P \leq 0.01$; ## $P \leq 0.0005$ vs. WT RBC in same condition (in *A* and *B*), by Kruskal–Wallis test with Dunn’s correction. DIOA, [(dihydroindenyl)oxy]acetic acid; HX, hereditary xerocytosis; SCP, senicapoc; WT, wild type.

DIOA inhibits KCNN4 in a Cl^- -independent manner in mouse red blood cells

In the absence of A23187, basal K^+ influx into normal (WT) mouse red cells was minimal in media containing chloride or sulfamate, whether in the absence or presence of senicapoc or DIOA (Fig. 3*A*). However, A23187 robustly stimulated K^+ influx in a chloride-independent manner typical of mouse red cell Gardos channel activity. That stimulated activity was inhibited to equivalent extents by 200 nM senicapoc and by 100 μ M DIOA. The lower A23187-stimulated Gardos channel activity in mouse red cells as compared with human red cells (Fig. 3*B*) and the elevated senicapoc-resistant background activity evident in mouse RBC versus human RBC (Fig. 3*B* and *C*) are consistent with our previous observations (22). A23187-stimulated K^+ influx sensitive to DIOA and that sensitive to senicapoc were indistinguishable in WT mouse red cells (Figs. 3*C* and *D*).

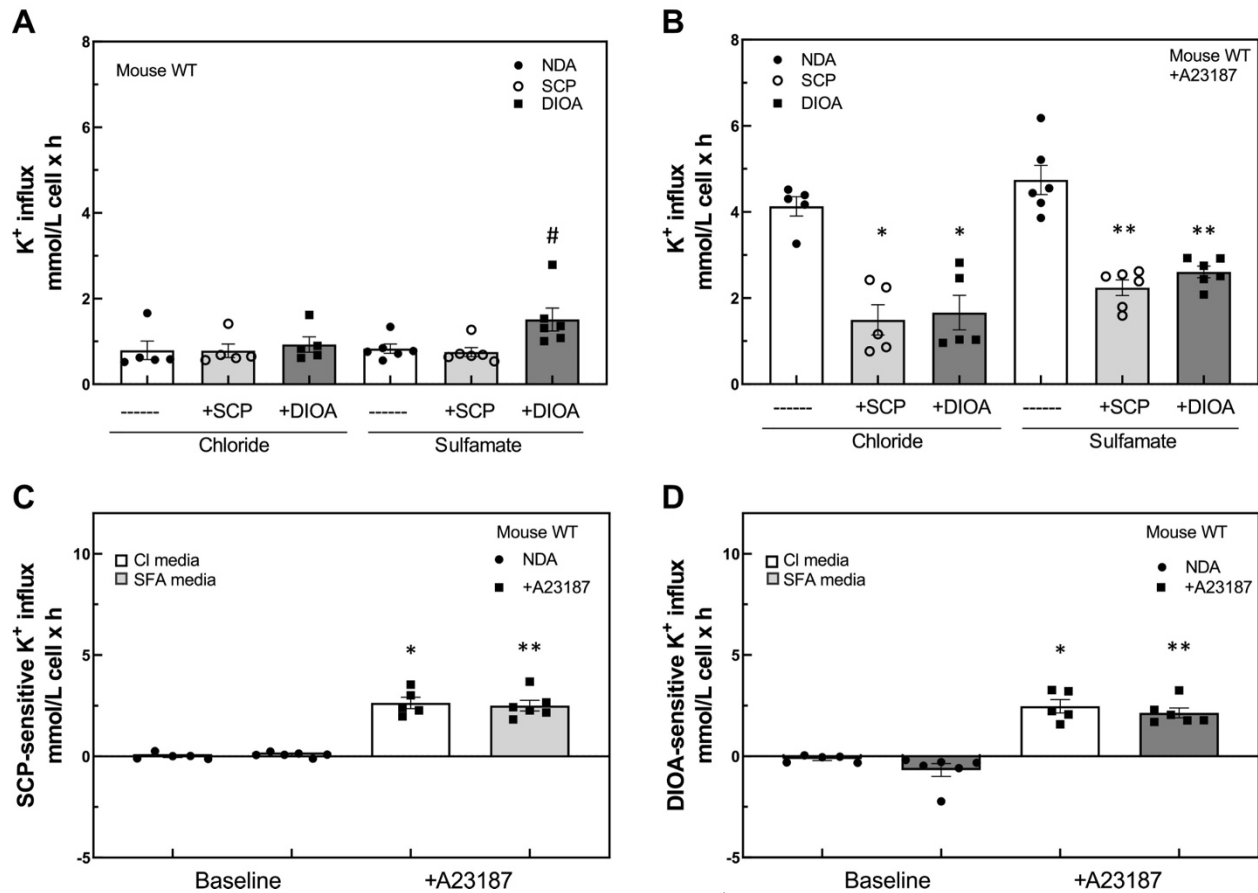


Figure 3. DIOA inhibits the Gardos channel in mouse red cells. *A*: basal K⁺ influx into WT mouse red cells in chloride or sulfamate media in the absence of A23187. Cells were exposed to no drug (dash), to SCP (200 nM), or to DIOA (100 μ M). #*P* < 0.02 vs. no drug in sulfamate (Mann–Whitney test). *B*: A23187-stimulated K⁺ influx into WT mouse red cells in chloride or sulfamate media, in the presence of no drug, SCP, or DIOA. *C*: basal and A23187-stimulated SCP-sensitive K⁺ influx into mouse WT red cells. *D*: basal and A23187-stimulated DIOA-sensitive K⁺ influx into mouse WT red cells. Data in *C* and *D* are derived from data in *A* and *B*. **P* < 0.008; ***P* < 0.003 vs. no drug added (NDA) in *B*; and vs. corresponding baseline in *C* and *D* (Mann–Whitney test). DIOA, [(dihydroindenyl)oxy]acetic acid; HX, hereditary xerocytosis; SCP, senicapoc; WT, wild type.

DIOA inhibition of KCNN4 gardos channel activity in mouse RBC persists in mouse RBC devoid of K-Cl cotransport and requires KCNN4 expression

Erythroid K-Cl cotransport in normal human RBC is nearly quiescent in the absence of activating stimuli that include hypotonic swelling, staurosporine, or urea (22, 23). However, mouse erythroid K-Cl cotransport exhibits a relatively higher basal activity in the absence of the same stimuli (22). To rule out a contribution from basal or atypically activated K-Cl cotransport to the DIOA-sensitive fraction of A23187-stimulated K⁺ influx, we examined K⁺ influx into *Kcc1*^{-/-}; *Kcc3*^{-/-} mouse red cells in the presence of extracellular chloride. These red cells lack nearly all K-Cl cotransport sensitive to hypotonic swelling, staurosporine, or urea and exhibit reduced levels of unstimulated K⁺ influx (2, 5). Figure 4*A* shows that *Kcc1*^{-/-}; *Kcc3*^{-/-} mouse red cells exhibit a 44.3% reduction in basal K⁺ influx insensitive to senicapoc or to DIOA. A23187 stimulated K⁺ influx 4.6-fold over baseline (Fig. 4*A*), roughly comparable to the 4.2-

fold stimulation exhibited by WT mouse red cells. Senicapoc inhibited A23187-stimulated K⁺ influx by 64.3%, whereas inhibition by DIOA was 47.5% (Fig. 4A). Thus, senicapoc-sensitive and DIOA-sensitive fractions of A23187-stimulated K⁺ influx into red cells of *Kcc1*^{-/-}; *Kcc3*^{-/-} mice did not differ significantly (Fig. 4B and C).

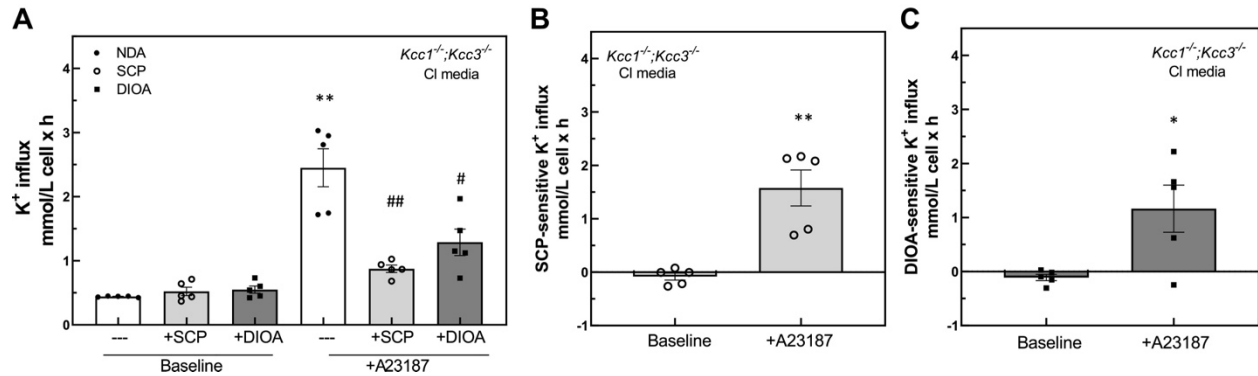


Figure 4. DIOA inhibits the Gardos channel in mouse red cells genetically devoid of K-Cl cotransport activity. *A*: basal and A23187-stimulated K⁺ influx into *Kcc1*^{-/-}; *Kcc3*^{-/-} mouse red cells in chloride media. ***P* < 0.008 vs. baseline NDA; #*P* < 0.04 and ##*P* < 0.008 vs A23187 NDA by Mann–Whitney test. *B*: SCP-sensitive basal and A23187-stimulated K⁺ influx into *Kcc1*^{-/-}; *Kcc3*^{-/-} mouse red cells. *C*: DIOA-sensitive basal and A23187-stimulated K⁺ influx into *Kcc1*^{-/-}; *Kcc3*^{-/-} mouse red cells. Data in *B* and *C* are derived from data in *A*. **P* < 0.04; ***P* < 0.008 vs baseline by Mann–Whitney test. DIOA, [(dihydroindenyl)oxy]acetic acid; NDA, no drug added; SCP, senicapoc.

In contrast, A23187 failed to stimulate K⁺ influx in red cells from mice with genetically inactivated expression of the *Kcnn4* gene, as we previously reported (2, 17). Moreover, neither senicapoc nor DIOA acted as inhibitors of K⁺ influx in the absence (Fig. 5A) or presence of A23187 (Fig. 5B). Unexpectedly, DIOA in the absence of A23187 slightly increased K⁺ influx into *Kcnn4*^{-/-} red cells from chloride and from sulfamate solutions (Fig. 5A), an effect magnified two- to threefold in the presence of A23187 (Fig. 5B and D). Curiously, senicapoc also increased K⁺ influx into *Kcnn4*^{-/-} red cells in chloride solution in the presence of A23187 (Fig. 5C).

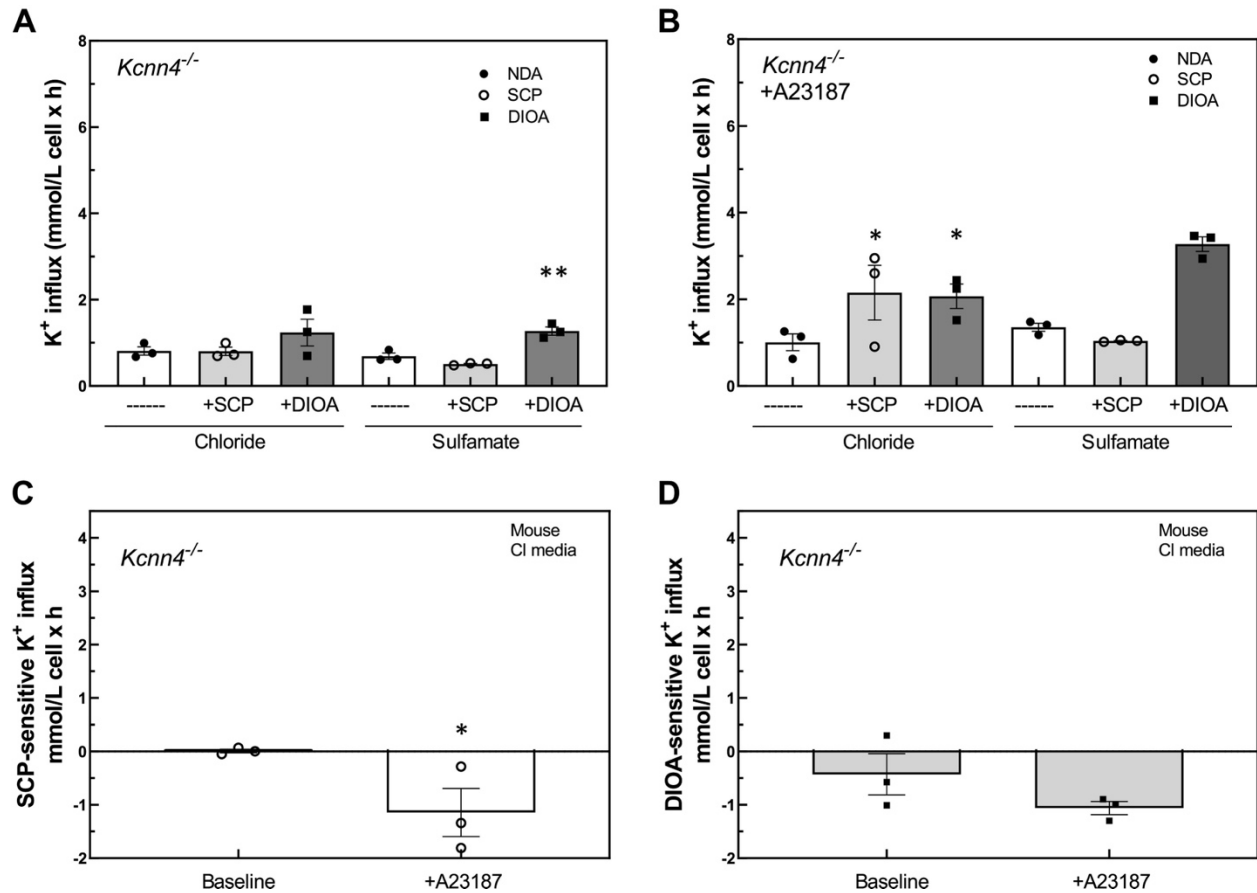


Figure 5. Gardos channel Inhibitory activity of DIOA is absent in red cells from mice in which *Kcnn4* has been genetically inactivated. *A*: basal K⁺ influx into *Kcnn4*^{-/-} mouse red cells in chloride or sulfamate media in the absence of A23187. Cells were exposed to no drug (dash), to SCP (200 nM), or to DIOA (100 μ M). ***P* $\leq$ 0.007 vs. no drug or SCP (both in sulfamate) by Mann–Whitney test. *B*: A23187-stimulated K⁺ influx into *Kcnn4*^{-/-} mouse red cells in chloride or sulfamate media, exposed to no drug, senicapoc, or DIOA. **P* < 0.025 vs. no drug in chloride, by Mann–Whitney test. *C*: SCP-sensitive K⁺ influx into *Kcnn4*^{-/-} mouse red cells in the absence (baseline) and presence of A23187. **P* < 0.005 by Mann–Whitney test. *D*: DIOA-stimulated K⁺ influx into *Kcnn4*^{-/-} mouse red cells in the absence and presence of A23187. Data in *C* and *D* are derived from the chloride media data of *A* and *B*. DIOA, [(dihydroindenyl)oxy]acetic acid; NDA, no drug added; SCP, senicapoc.

DIOA inhibits YODA-stimulated K⁺ influx in a chloride-independent manner in normal human red blood cells

YODA1 is a small molecule activator of PIEZO1, the mechanosensitive Ca²⁺-permeable cation channel of red cells (24, 25). YODA1-stimulated K⁺ influx into normal human red cells was more variable (Fig. 6A) than A23187-stimulated K⁺ influx into human cells. However, YODA1-stimulated K⁺ influx into human cells was statistically indistinguishable in chloride or in sulfamate solutions (Fig. 6A and C). Although YODA1-stimulated K⁺ influx was considerably reduced in HX red cells, DIOA inhibition of that K⁺ influx measured in chloride solution achieved statistical significance (Fig. 6B and D). The data were consistent with inhibition of Gardos channel activity by DIOA and by senicapoc, each downstream of

PIEZO1 activation by YODA1. Apparently incomplete inhibition of K^+ influx by either senicapoc or DIOA likely represents YODA-stimulated K^+ influx directly mediated by PIEZO1.

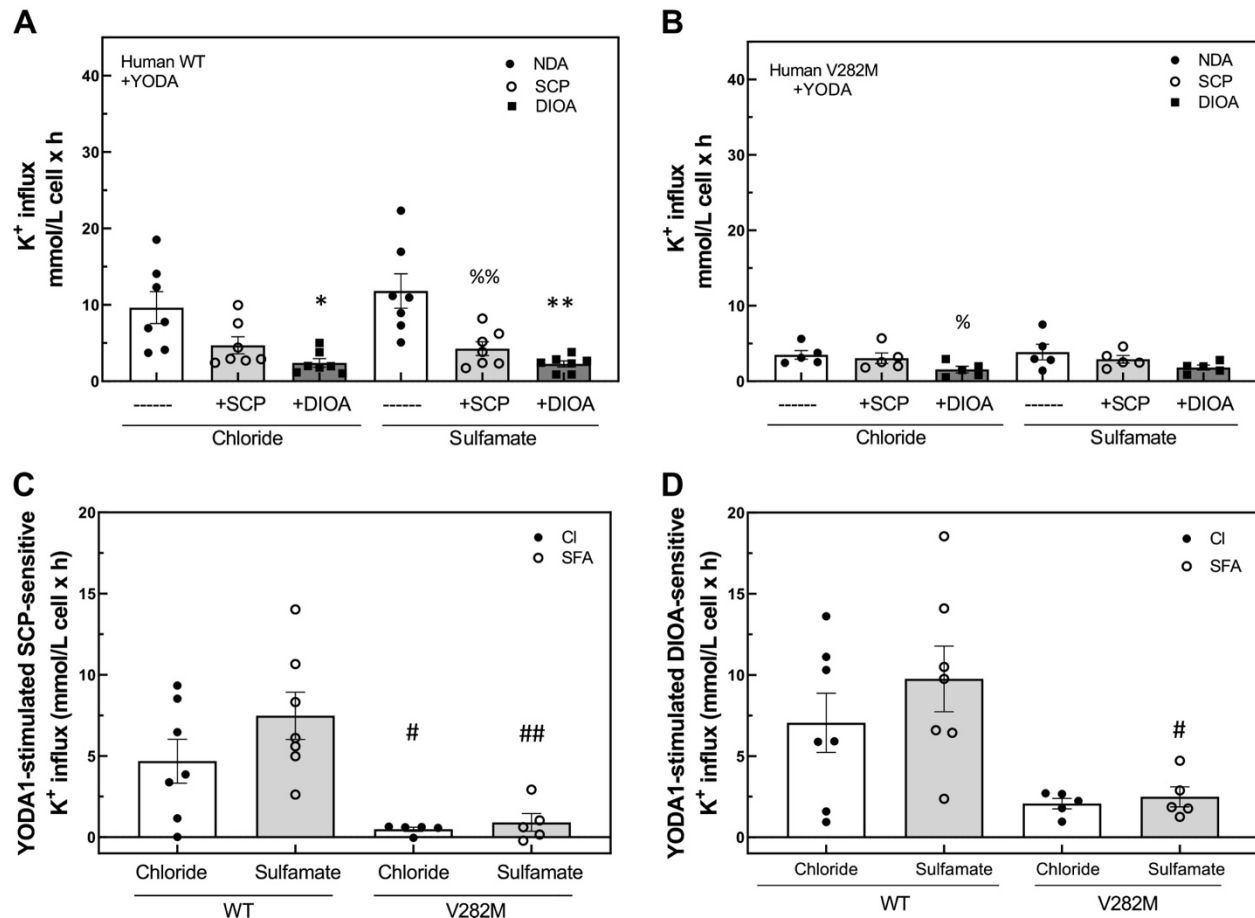


Figure 6. DIOA inhibits K^+ influx stimulated by the PIEZO1 activator YODA1 in human red cells. *A*: YODA1-stimulated K^+ influx into WT human red cells in chloride or sulfamate media, in the presence of no drug, SCP, or DIOA. * $P < 0.02$; ** $P \leq 0.004$ vs. WT in same solution, by Kruskal–Wallis test. % $P < 0.01$ by Mann–Whitney test. *B*: YODA1-stimulated K^+ influx into human HX V282M red cells in chloride or sulfamate media, in the presence of no drug, SCP, or DIOA. % $P = 0.032$ by Mann–Whitney test. *C*: SCP-sensitive, YODA1-stimulated K^+ influx into human WT and HX V282M red cells, in chloride and sulfamate media. *D*: DIOA-sensitive, YODA1-stimulated K^+ influx into human WT and HX V282M red cells, in chloride and sulfamate media. Data from *C* and *D* are derived from data in *A* and *B*. ## $P \leq 0.01$; # $P < 0.03$ vs. WT in same solution, by Mann–Whitney test. DIOA, [(dihydroindenyl)oxy]acetic acid; NDA, no drug added; HX, hereditary xerocytosis; SCP, senicapoc WT, wild type.

YODA1 also stimulated K^+ influx into mouse red cells (Fig. 7A), in a manner independent of expression of K-Cl cotransporters (Fig. 7B) or of the Gardos channel/KCNN4 (Fig. 7C). Senicapoc only modestly inhibited YODA1-stimulated K^+ influx in WT mouse red cells (Fig. 7A), likely reflecting a substantial fraction of PIEZO1-mediated K^+ influx. This was supported by undiminished YODA1-stimulated K^+ influx in the absence of KCNN4 function (Fig. 7C). YODA1-stimulated K^+ influx in red cells from WT mice and from *Kcc1*^{-/-}; *Kcc3*^{-/-} mice trended toward inhibition by DIOA, but this apparent decrease did not achieve statistical significance (Fig. 7A and B). However, DIOA significantly inhibited

YODA-stimulated K^+ influx into red cells of *Kcnn4*^{-/-} mice (Fig. 7C), suggesting possible upregulation of K-Cl cotransport and/or another K^+ transport pathway (22).

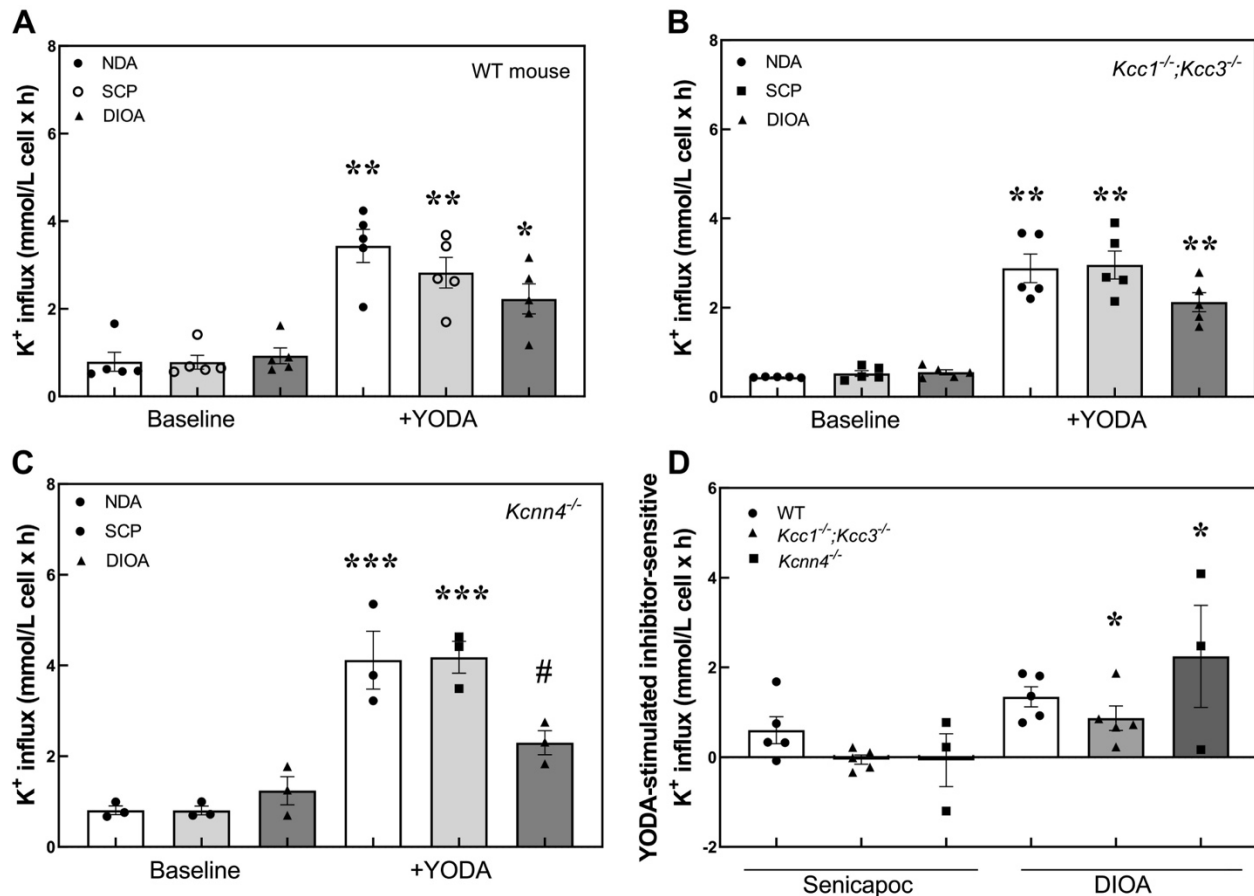


Figure 7. DIOA inhibits YODA1-stimulated K^+ influx in mouse red cells. *A*: basal and YODA1-stimulated K^+ influx into WT mouse red cells in chloride medium, in the presence of no drug, senicapoc, or DIOA. *B*: basal and YODA1-stimulated K^+ influx into *Kcc1*^{-/-};*Kcc3*^{-/-} mouse red cells in chloride medium in the presence of no drug, senicapoc, or DIOA. *C*: basal and YODA1-stimulated K^+ influx into *Kcnn4*^{-/-} mouse red cells in chloride medium in the presence of no drug, senicapoc, or DIOA. *** P < 0.0001; ** P < 0.008; * P < 0.04 vs. baseline in the presence of the same drug condition; # P < 0.05 vs. +YODA1-NDA, all by Mann-Whitney test. *D*: SCP-sensitive or DIOA-sensitive fractions of YODA1-stimulated K^+ influx into WT mouse red cells or red cells from *Kcc1*^{-/-};*Kcc3*^{-/-} or *Kcnn4*^{-/-} mice. Data in *D* is derived from data in *A–C*. * P < 0.025 vs. same genotype in senicapoc, by Mann-Whitney test. DIOA, [(dihydroindenyl)oxy]acetic acid; NDA, no drug added; HX, hereditary xerocytosis; SCP, senicapoc WT, wild type.

As shown in Fig. 8, DIOA inhibition of KCNN4 is similar in human and mouse red cells. The IC₅₀ values for DIOA inhibition of KCNN4 were 27 ± 4.2 μM in human red cells (n = 3) and 44 ± 13 μM (SE) in mouse red cells (n = 4, n.s.). The Hill coefficients for inhibition by DIOA were 1.5 ± 0.25 in humans and 2.0 ± 1.1 in mouse cells. Figure 9 demonstrates that DIOA at 100 μM inhibits A23187-stimulated K^+ influx (KCNN4 activity) in human sickle red cells to the same degree as in normal human red cells (Fig. 1). KCNN4 activity in red cells from the SAD mouse model of sickle cell disease is similarly inhibited by 100 μM DIOA (Fig. 9).

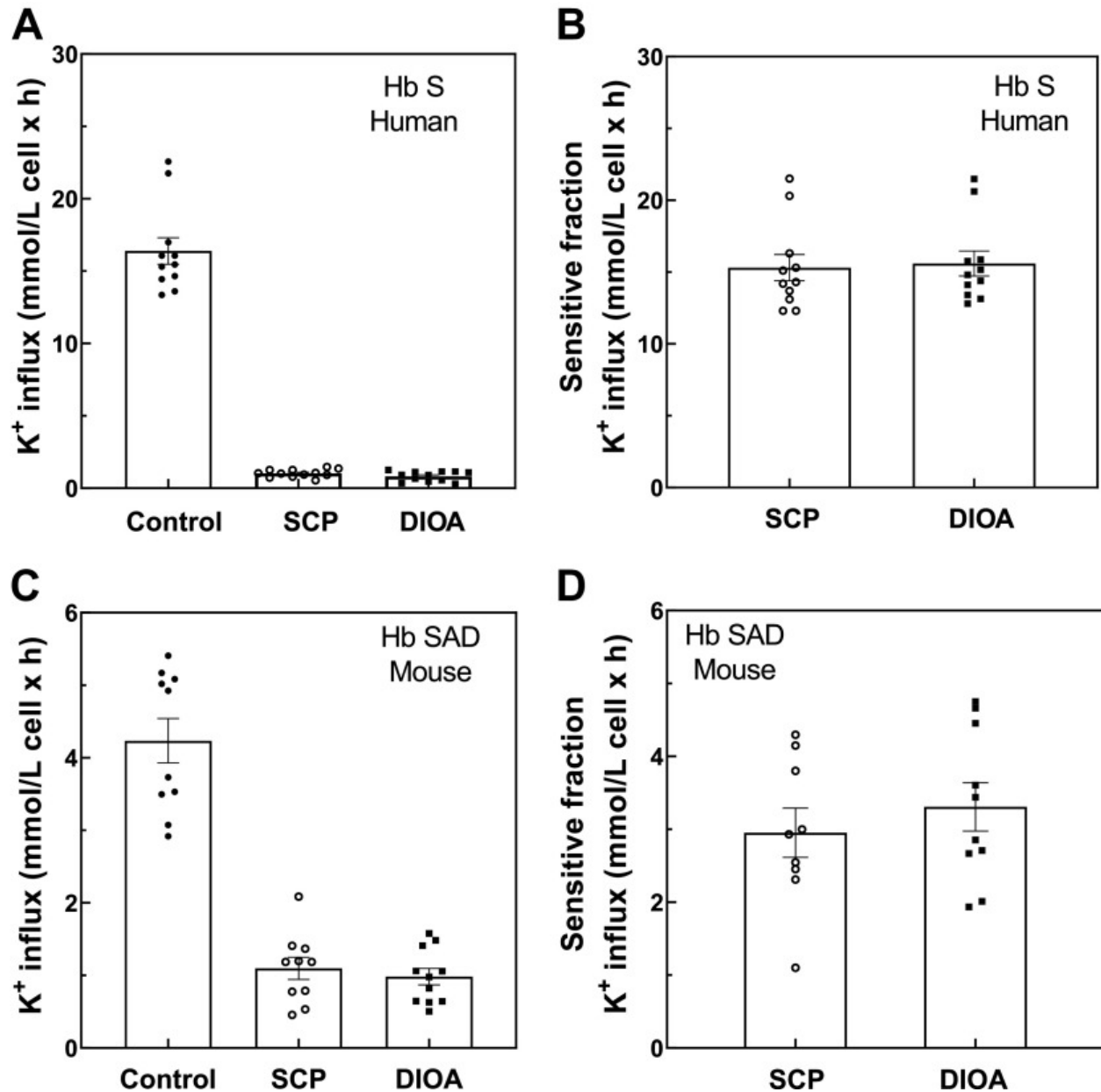


Figure 9. DIOA inhibits A23187-stimulated Gardos channel flux in human and mouse sickle red cells. *A*: DIOA and SCP inhibit A23187-stimulated K⁺ influx into human sickle cell disease (SS) red cells. *B*: inhibitor-sensitive fraction of A23187-stimulated K⁺ influx into human SS red cells. *C*: DIOA and SCP inhibit A23187-stimulated K⁺ influx into red cells of the SAD mouse model of sickle cell disease. *D*: inhibitor-sensitive fraction of A23187-stimulated K⁺ influx into red cells of the SAD mouse model of sickle cell disease. DIOA, [(dihydroindenyl)oxy]acetic acid; SCP, senicapoc.

DIOA inhibits currents from heterologously expressed KCNN4 channels

To confirm that DIOA directly interacts with KCa3.1, we performed whole cell patch clamp experiments on HEK-293 cells stably expressing human KCa3.1/KCNN4 (Fig. 10). DIOA blocked currents elicited by voltage ramps in the presence of 1 μ M free intracellular Ca²⁺ with IC₅₀ of 42 μ M ($n = 3$). The

Hill coefficient of the concentration-response curve was ~ 2 , consistent with the interaction of two or more DIOA molecules per tetrameric KCa3.1/KCNN4 channel.

Docking of DIOA into the KCa3.1/KCNN4 structure identifies two potential binding sites

The KCNN4 structure (20) contains several binding pockets for small molecules (Fig. 11). Inhibitors of higher affinity and specificity such as senicapoc and TRAM-34 bind in the inner pore lumen, whereas inhibitors of lower affinity and specificity such as the dihydropyridine nifedipine interact with residues in the fenestration region (21). Docking of DIOA in 10,000 random starting positions with the RosettaLigand method identified low energy binding poses in both the pore lumen (-12.4 REU) and the fenestration site (-15.8 REU) (As the Rosetta energy function is a combination of both physics-based and statistics-based potentials, Rosetta energies are reported on an arbitrary scale referred to as Rosetta energy units or REUs). These modeling results allow us to suggest that two DIOA molecules bind to KCNN4 in two of the tetrameric protein's four fenestration sites. Also possible would be one DIOA molecule bound in the single pore site, with a second DIOA engaging one of the fenestration sites. We note in this context that DIOA interacts with the KCC3 K-Cl cotransporter polypeptide with a 1:1 stoichiometry, as reflected in a recent cryo-EM structure of KCC3 in its inward-open state (26), in which two DIOA molecules are bound within the interprotomeric central cleft of the homodimeric transporter imaged as a single particle (26).

Discussion

We have reported here that DIOA used at $100\ \mu\text{M}$ nearly completely blocks erythroid KCNN4/Gardos channel activity maximally stimulated by A23187 in human and mouse red cells. This Gardos channel inhibitor activity of DIOA is chloride-independent and persists in mouse red cells genetically devoid of both the K-Cl cotransporters KCC3 and KCC1. The Gardos channel inhibitor activity of DIOA is appropriately abrogated in mouse red cells genetically devoid of the KCNN4/Gardos channel. The degree of inhibition is comparable in human sickle red cells and nearly so in SAD mouse red cells that model the red cell dehydration of sickle cell disease. The results are consistent with a previous report of DIOA blockade of a Cl^- -independent, Ca^{2+} -activated K^+ conductance in human lens epithelial cells, with apparent IC_{50} of $\sim 75\ \mu\text{M}$ (27).

DIOA inhibition of KCa3.1 is likely mediated through the binding of two DIOA molecules at two of the four fenestration sites present in the KCa3.1 tetramer. The binding of two inhibitor drugs per tetramer corresponds to the Hill coefficients of ~ 2 exhibited by DIOA inhibition of recombinant human KCa3.1 K^+ channel activity stimulated by $1\ \mu\text{M}$ free Ca^{2+} in whole cell patch clamp records of KCa3.1-

overexpressing HEK 293 cells, as well as by DIOA inhibition of A23187-stimulated K^+ influx into red cells of both mouse and human. Predicted DIOA binding to the KCa3.1 fenestration site resembles dihydropyridine binding to the fenestration sites of voltage-gated Ca^{2+} channels (28). Interestingly, DIOA binding to electroneutral cotransporter KCC3 is also located outside its ion translocation pathway (26). The inner pore lumen of KCa3.1, the nM affinity binding site for the specific and potent inhibitors TRAM-34 and senicapoc (20, 29), is predicted by the present study to serve as an energetically less favorable docking site for DIOA. The potential importance of this likely secondary DIOA binding site for DIOA inhibition of KCa3.1 activity remains to be determined.

K-Cl cotransport mediated predominantly by KCC3 and KCC1 is a major component of red cell volume regulation, along with the Ca^{2+} -activated K^+ channel activity of KCNN4/KCa3.1, also known as the Gardos channel. Inhibitor modulation of these volume regulatory activities in sickle erythrocytes is a therapeutic objective for adjunct treatment of sickle cell disease. Indeed, the potent Gardos channel inhibitor senicapoc has already been shown to swell red cells and decrease mean corpuscular hemoglobin concentration in mouse models of sickle cell disease (11), and in human red cells in clinical trials (30, 31).

The development of inhibitors of KCC3 and KCC1 with potency and specificity sufficient for clinical testing (12) has lagged behind the development of KCNN4 inhibitors. However, blockade of erythroid K-Cl cotransport is important during experimental tests of erythroid Gardos channel activity. Experimental K-Cl cotransport blockade is often accomplished by the use of 1 mM bumetanide, but bumetanide also inhibits the NKCC1 Na-K-2Cl cotransporter of the red cell. Garay et al. (13, 14) in their study therefore proposed the use of DIOA as an inhibitor of erythroid K-Cl cotransport. With a red cell IC_{50} of 10 μ M, 100 μ M DIOA blocked erythroid K-Cl cotransport nearly completely, without apparent inhibition of erythroid Na-K-2Cl cotransport. However, DIOA was later found to inhibit Na-K-2Cl cotransport activity mediated in HEK-293 cells by overexpressed recombinant human NKCC1 with IC_{50} of 23 μ M, at least as potently as DIOAs 57 μ M IC_{50} for *N*-ethylmaleimide-stimulated recombinant rabbit KCC1 similarly overexpressed in HEK-293 cells (32).

Since DIOA blocks neuron-specific KCC2-mediated K-Cl cotransport with similar potency (IC_{50} 13 μ M) (33) and KCC2 controls the equilibrium chloride potential of neuronal membranes, DIOA has also been useful for modulation of neuronal GABA(A) responses (34). Moreover, 100 μ M DIOA has been used to inhibit KCC3 in MCF7 mammary carcinoma cells (35). In lipopolysaccharide-stimulated neutrophils and other mononuclear cells, DIOA has been used to inhibit KCC3-mediated K-Cl cotransport (36) that in some conditions may contribute to the cellular K loss required for WNK1-regulated NLRP3 inflammasome activation (37).

The ability of DIOA to block the KCNN4/Gardos channel is not the first demonstration of “off-target” activities of DIOA. DIOA has been shown to inhibit the cystic fibrosis transmembrane regulator (CFTR) with IC_{50} of 17 μ M, a potency indistinguishable from the 16 μ M IC_{50} of traditional chloride channel blocker, 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) (38), which also happens to block KCa3.1 at a concentration of 100 μ M (39). DIOA at 50–100 μ M concentrations has also been shown to block swelling-activated chloride currents in retinal pigment epithelial cells (40) and in osteoblast-like cells (41), as well as NPPB-sensitive chloride currents in human glioma cells (42) and anion exchange activity in lamprey red cells (43). Moreover, DIOA has also been shown to inhibit P-type ATPases at comparable or lower potency, including dog kidney Na^+,K^+ -ATPase (IC_{50} 53 μ M), hog gastric H^+,K^+ -ATPase (IC_{50} 97 μ M), and rabbit muscle Ca^{2+} -ATPase (IC_{50} 127 μ M).

Low-K sheep red cells lacking Gardos channel activity (44) were originally reported to be insensitive to intracellular Ca^{2+} (45). However, subsequent observations detected biphasic regulation of K-Cl cotransport activity by A23187 in low-K sheep red cells, such that reduction of intracellular $[Ca^{2+}]$ further increased anisosmotically stimulated K-Cl cotransport, but not cotransport stimulated by *N*-ethylmaleimide. In contrast, elevated intracellular $[Ca^{2+}]$ in low-K sheep red cells nearly completely inhibited K-Cl cotransport stimulated either anisosmotically or by *N*-ethylmaleimide (46, 47). Both inhibitory and activating effects of A23187-mediated manipulation of intracellular $[Ca^{2+}]$ were fully reversible, and reversibly abolished in the setting of cellular ATP depletion (47). Later studies confirmed K-Cl cotransport inhibition by elevated intracellular $[Ca^{2+}]$ in Gardos channel-expressing red cells from humans with sickle cell disease (48).

Tests of erythroid K-Cl cotransport during periods of deoxygenation should be accompanied in a consistent fraction of the erythroid population by elevated intracellular $[Ca^{2+}]$, reflecting the deoxygenation-activated cation conductance known as Psickle (49). Ca^{2+} entry into red cells mediated by Psickle should in turn activate KCNN4/Gardos channel. Use of DIOA to monitor oxygen-sensitive K-Cl cotransport (50) during cyclic oxygenation/deoxygenation may thus include a fraction of DIOA-sensitive K^+ transport mediated by KCNN4/Gardos channel, although possibly not in conditions of continuous deoxygenation (51). Knowledge of the ability of DIOA to block KCNN4 may prevent inadvertent inhibition of KCNN4 during experiments in red cells or other pertinent cell types designed with the intention of specific inhibition of K-Cl cotransporters, or may modify interpretation of experimental results.

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Author contributions

A.R. and S.L.A. conceived and designed research; A.R., J.A.N., H.S., and B.E.S. performed experiments; A.R., J.A.N., H.S., B.E.S., and H.W. analyzed data; A.R., J.A.N., H.S., J.K., H.W., and S.L.A. interpreted results of experiments; A.R., J.A.N., H.S., and H.W. prepared figures; S.L.A. drafted manuscript; A.R., J.G.W., J.S.D., L.M.S., C.B., H.W., and S.L.A. edited and revised manuscript; A.R., J.G.W., J.S.D., L.M.S., C.B., H.W., and S.L.A. approved final version of manuscript.

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CHAPTER 5: AI is a viable alternative to high throughput screening: a 318-target study

Atomwise AIMS Program

Abstract

High throughput screening (HTS) is routinely used to identify bioactive small molecules. This requires physical compounds, which limits coverage of accessible chemical space. Computational approaches combined with vast on-demand chemical libraries can access far greater chemical space, provided that the predictive accuracy is sufficient to identify useful molecules. Through the largest and most diverse virtual HTS campaign reported to date, comprising 318 individual projects, we demonstrate that our AtomNet® convolutional neural network successfully finds novel hits across every major therapeutic area and protein class. We address historical limitations of computational screening by demonstrating success for target proteins without known binders, high-quality X-ray crystal structures, or manual cherry-picking of compounds. We show that the molecules selected by the AtomNet® model are novel drug-like scaffolds rather than minor modifications to known bioactive compounds. Our empirical results suggest that computational methods can substantially replace HTS as the first step of small-molecule drug discovery.

Authorship Contribution

This research was a large-scale collaboration involving hundreds of different academic laboratories, spanning 318 different protein targets of interest. The Wulff laboratory submitted KCa3.1 as a target and requested screening in the inner pore, using both the closed and open state of the channel as a template (PDB ID: 6CNO and 6CNN, respectively). Following the screen Atomwise provided 165 compounds for experimental testing, which I tested on KCa3.1 using automated and manual whole-cell patch-clamp.

Introduction

Despite present interest in AI/ML and thirty years of case studies^{1,4}, computational screening techniques have achieved limited adoption within the pharmaceutical industry. A recent investigation into the origins of 156 clinical candidates⁵ found that only 1% came from virtual screening; in contrast, over 90% of clinical candidates were derived from patent busting or high throughput screening (HTS). Unfortunately, these sources are increasingly challenged, given the pharmaceutical industry's shift to novel target classes, such as proximity-induced protein degradation⁶, protein-protein interactions⁷, and RNA targeting⁸.

Currently, HTS is the critical tool in drug discovery, providing most novel scaffolds of recent clinical candidates^{5,9,10}. These initial starting points crucially shape the course of downstream medicinal chemistry efforts, as most drugs preserve at least 80% of the scaffold of the initially identified lead¹¹. Despite these foundational contributions, HTS suffers from practical limitations. Principally, HTS, like all physical experiments, requires that the compounds exist. However, with the advent of synthesis-on-demand libraries, most commercially-available molecules have yet to be synthesized. Still, they can be made and delivered for testing in a matter of weeks^{12,14}. These libraries comprise trillions of molecules^{14,15} that exemplify millions of otherwise-unavailable scaffolds¹², providing an opportunity to substantially expand the scope and diversity of available chemical space explored in the standard drug discovery process.

Computational approaches unlock this opportunity by reversing the requirement to make molecules before testing them. When computational experiments replace HTS as the primary screen, molecules are tested *before* they are made, and the results from these experiments can inform which molecules are worth synthesizing. Computational experiments further promise to improve upon HTS in terms of cost, speed, need to produce significant quantities of protein¹⁶, effort of miniaturizing assay formats while maintaining experimental integrity^{17,19}, and reducing false-positive and false-negative rates^{16,20,23} including artifacts from aggregation, covalent modification of the target, autofluorescence, or interactions with the reporter

rather than the target^{20,24,25}. Historical computational techniques such as ligand-based QSAR^{26,28}, structure-based docking^{29,30}, and machine learning^{31,32} purport to address these limitations of physical screening methods. Unfortunately, these techniques have not replaced HTS; in fact, despite increasing interest in ML, the proportion of drugs discovered with computational techniques has remained steady over the past decades^{5,10}.

Because there will always be individual targets for which one screening technique can identify more hits than another, the key question governing if computation is ready to be the default hit discovery technique is whether computational screens can identify hits successfully across a broad range of diverse targets. Unfortunately, despite excellent benchmark accuracies^{33,35}, prospective discovery accuracy remains modest^{33,36,37}. For example, Cerón-Carrasco³⁸ reported over 700 virtual screens against the SARS-CoV-2 main protease. However, when the author sought to validate the computational predictions via physical experiments, the identified compounds were barely active (800 μ M). Computational approaches have also been limited by a need for extensive target-specific training data^{31,39,41}, a requirement for high-quality X-ray crystal structures^{42,43}, dependence on human adjudication (so-called ‘cherry-picking’)¹², or a limited domain of applicability^{44,48}. Even recent systems have demonstrated utility only in identifying minor variants of known molecules for well-studied proteins with tens of thousands of known binders in their training data^{49,50}. Figure 1 exemplifies the striking similarities between recently ML-developed compounds and their preceding published chemical matter. This is particularly concerning, as a myopic focus on well-studied proteins has been identified as a cause of low productivity in pharmaceutical discovery⁵¹.

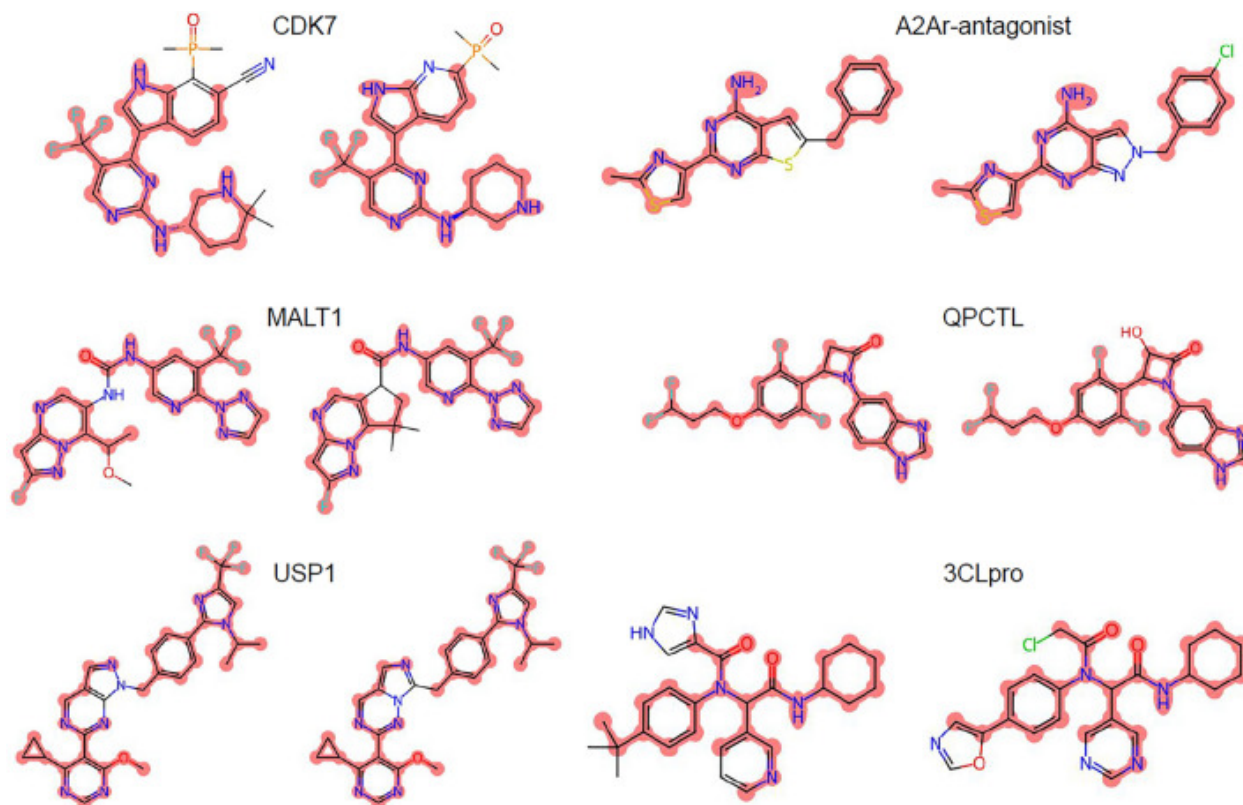


Figure 1. Pairs of representative compounds extracted from AI patents (right) and corresponding prior patents (left) for clinical-stage programs (CDK7^{92,93}, A2Ar-antagonist^{94,95}, MALT1^{96,97}, QPCTL^{98,99}, USP1^{100,101}, and 3CLpro^{102,103}). The identical atoms between the chemical structures are highlighted in red.

Nevertheless, we have observed that deep learning approaches are not as limited as these historical examples would imply. Using our AtomNet^{52,54} screening system, we have previously reported success in finding novel scaffolds for targets without known ligands^{55,57}, X-ray crystal structures^{56,60}, or both^{56,57}, as well as challenging modulation via protein–protein interaction^{59,61} or allosteric binding⁶⁰ (see Supplementary Table ^{S1} for examples). However, individual examples do not demonstrate the overall success of such deep learning systems. We therefore report our internal discovery efforts against 22 targets of pharmaceutical interest. We then attempted to further assess the generalizability and robustness of deep learning predictive systems by identifying bioactive molecules for a diverse set of targets. We partnered with 482 academic labs and screening centers, from 257 different academic institutions across 30 countries, through our academic collaboration program, the Artificial Intelligence Molecular Screen (AIMS). This collaboration afforded an opportunity to prospectively evaluate the utility of the AtomNet model as a primary screen across a broad range of diverse, challenging, and realistic targets. In aggregate, we report

successes and failures from 318 prospective experiments and evaluate our AtomNet machine-learning technology's ability to serve as a viable alternative to physical HTS campaigns.

Methods

Aims Screening Protocol

We began by evaluating screening libraries of millions of catalog compounds from commercial vendors MCule (10 M)⁷⁶ and Enamine in-stock (2.5 M)⁷⁷. We then selected a drug-like subset via algorithmic filtering by applying Eli Lilly medicinal chemistry filters⁷⁸ and removing likely false positives, such as aggregators, autofluorescers, and PAINS⁷⁹ (see Fig. 2 for the distributions of drug-like properties of the SD hits). The resulting library was virtually screened against the target of interest, removing any molecules with greater than 0.5 Tanimoto similarity in ECFP4 space to any known binders of the target and its homologs within 70% sequence identity. For kinase targets, we extend the exclusion to the whole kinome. The binding site was defined using co-complexes, mutagenesis studies, co-complexes of homologs, or by identifying potential sites using ICM Pocket Finder⁸⁰ or Fpocket⁸¹. Some were orthosteric, while others were allosteric, or as yet unestablished biological functions. In 64 cases, we built homology models using the closest sequence, with an average sequence similarity of 54%. We clustered the top 30,000 molecules using the Butina⁸² algorithm with a Tanimoto similarity cutoff of 0.35 in ECFP4 space, selecting the highest-scoring exemplars. Additional computed physico-chemical property filters were applied as needed. At no point were compounds cherry-picked. We purchased, on average, 85 compounds, quality controlled by LC-MS to > 90% purity, generally dispensed as 10 mM DMSO stocks plated in a single 96-well plate. In addition, two vials of DMSO-only negative controls were included before scrambling the compound locations on the plate, by the supplier, for blinded experimental testing. To further control for potential artifacts, we removed compounds that showed measurable activity toward more than one target from the analysis.

Dose-response and Analoging Validation Screening Protocol

We considered advancing AIMS projects to additional validation studies based on the ability to reorder at least some of the initial SD hits, the availability of chemical analogs in the screening library to the initial hits, the capability to perform dose–response experiments, and the ability of the collaborators to perform additional screens and return results promptly.

We performed two sets of experiments: DR validation of the SD hits from AIMS and analoging with DR readouts. We performed DR measurements using the same assays and protocols as SD.

We performed an analoging round by identifying, for each AIMS hit, its 1000 nearest neighbors from the Mcule library⁷⁶, using molecular fingerprints similarity⁶⁸. We augmented the set with additional analogs using substructure⁸³ or FTrees⁸⁴ searches, if needed. We used an AtomNet regression model, trained to predict quantitative bioactivities (e.g., IC50 or Ki), to score and rank the analogs. A set of 20—35 compounds from the analogs space of an initial hit were then obtained based on similarity and top scores from the AtomNet model for testing.

Internal Portfolio Screening Protocol

We followed a protocol similar to the AIMS screen with a few deviations. First, we used the Enamine REAL library of over 16 billion compounds⁶². Second, we used an ensemble of six AtomNet models for the screens. Last, on average, we selected a set of 440 compounds for testing.

The analoging protocol is similar to the AIMS validation studies, with the following deviations. First, we used the Enamine REAL library for analog search. Second, we selected an average of 676 analogs per project. Third, the analog search protocol was more complex, pulling nearest neighbors based on maximum common substructure and graph edit distance in addition to the ECFP4-based one.

AtomNet® model architecture

We previously published in detail^{52,53,55,58,59,61,85,86} during the course of the AIMS program, and we described the most recent version of the AtomNet model architecture in detail elsewhere⁵³. We provide a brief description below.

The AtomNet model is a Graph Convolution Network architecture with atoms represented as vertices and pair-wise, distance-dependent, edges representing atom proximities. The input is a graph network of features characterizing the atom types and topologies of an ensemble of protein–ligand complexes. Receptor atoms more than 7 Å away from any ligand atom are excluded from the complexes, and each node in the graph is associated with a feature vector representing the atom type using Sybyl typing⁸⁷.

The network has five graph convolutional blocks. In the first two graph convolution blocks, all ligand and receptor atoms 5 Å apart from each other are considered, and 64 filters per block are used. In the third block, the cutoff radius and filters are increased to 7 Å and 128, respectively. Only ligand features in the last two blocks are considered without changing the threshold cutoff or the number of filters. Finally, the sum-pool of the ligand-only layer creates a 3-task layer on top of the network. That multi-task layer predicts three endpoints: bioactivity, pose quality, and a physics-based docking score⁸⁸.

We trained an ensemble of 6 models, splitting the training data into sixfold cross-validation sets based on a protein sequence similarity cutoff of 70%. Then, each model in the ensemble was trained on a different fold for 10 epochs, using the ADAM optimizer⁸⁹ with a learning rate of 0.001, and targets were sampled with replacement, proportional to the number of active compounds associated with that target.

Results

We investigated the ability of deep learning-based methods to identify novel bioactive chemotypes by applying the AtomNet model to identify hits for 22 internal targets of pharmaceutical interest. We also explored the breadth of applicability of this approach by attempting to identify drug-like hits in single-dose

screens for 296 academic targets, of which 49 were followed up with dose–response experiments, and 21 were further validated by exploring analogs of the initial hits. The average hit rate for our internal projects (6.7%) was comparable to the hit rate for our academic collaborations (7.6%).

Internal Portfolio Validation

As part of Atomwise’s internal drug discovery efforts, we used the AtomNet model instead of high-throughput or DNA-encoded library (DEL) screening. We screened a 16-billion synthesis-on-demand chemical space⁶², which is several thousand times larger than HTS libraries and even exceeds the size of most DELs without suffering limitations of DNA-compatible chemistry^{16,23}. Each screen requires over 40,000 CPUs, 3,500 GPUs, 150 TB of main memory, and 55 TB of data transfers. We describe the protocol in detail in the Methods section; briefly, we computationally scored each catalog compound after removing molecules that were prone to interfere with the assays or were too similar to known binders of the target or its homologs. The neural network analyzes and scores the 3D coordinates of each generated protein–ligand co-complex, producing a list of ligands ranked by their predicted binding probability. Our workflow then clusters the top-ranked molecules to ensure diversity and algorithmically selects the highest-scoring exemplars from each cluster. At no point are compounds manually cherry-picked. The molecules were synthesized at Enamine (<https://enamine.net>) and quality controlled by LC–MS to purity > 90%, in agreement with HTS standards⁶³. Hits were further validated using NMR. We then physically tested, on average, 440 compounds per target at reputable contract research organizations (CROs), while attempting to mitigate assay interferences such as aggregation and oxidation with standard additives (*e.g.*, Tween-20, Triton-X 100, and dithiothreitol (DTT)). We describe the assay protocols in detail in the Supplementary Data ^{S1}.

We describe the results of the 22 experiments in Table Table1.1. In 91% of the experiments, we identified single-dose (SD) hits that were reconfirmed in dose–response (DR) experiments. The average target DR hit rate was 6.7% compared to 8.8% from the SD screens. Only 16 of the 22 projects were

structurally enabled with X-ray crystallography; one used a cryo-EM structure, while five used homology models with an average sequence identity of 42% to their template protein. The DR hit rate for the cryo-EM project was 10.56%, while the average hit rate for the homology models was a similar 10.8%.

Table 1: Results from 22 Atomwise internal programs

Gene name	# of compounds tested	SD hit rate (%)	DR hit rate (%)	Potency range (IC ₅₀ /K _i , uM)	# of analog tested	SD analog hit rate (%)	DR analog hit rate (%)	Analog potency range (IC ₅₀ /K _i , uM)
ASAH1	376	10.64	7.71	0.3–102	—	—	—	—
AXL	597	12.06	8.21	0.181–71	3200	35.59	33.56	0.079–86
BCL2	422	3.08	0.00	—	—	—	—	—
CBLB	422	1.66	0.00	—	—	—	—	—
CDK5	786	10.69	10.43	0.049–79	587	47.53	43.61	0.43–76
CDK7	786	10.69	10.56	0.099–60	735	28.44	27.35	0.191–10
GFPT1	384	6.51	2.34	31–86	734	24.93	24.11	1–194
KCNT1	416	9.62	7.69	1.1–30	—	—	—	—
KDM6A	356	3.93	1.12	24–58	—	—	—	—
LATS1	418	18.18	17.94	0.077–82	841	51.72	45.78	0.034–98
MC2R	208	11.54	9.62	16–68	419	39.38	38.42	2.4–97

MDM4	422	2.37	0.47	5.9–29.8	192	18.23	18.23	4.4–90
NT5E	335	1.49	0.30	176	221	9.95	1.81	8.3–65
PARG	334	7.78	7.78	15–250	–	–	–	–
PARP14	576	5.38	2.95	3–96	616	26.46	26.30	0.2–95
POLQ	330	11.82	11.52	1.2–49	559	11.27	8.77	1.5–42
PPARA	422	4.03	0.24	131	211	14.22	3.79	59–95
PPM1D	530	11.89	6.98	4.5–98	–	–	–	–
PRMT5	422	4.03	0.95	7.2–79	415	7.95	5.54	19–114
PRODH2	542	2.77	1.11	15–84	–	–	–	–
TYK2	189	38.10	34.39	0.016–9	457	71.33	60.39	0.006–10
VCP	416	4.81	4.81	2.4–64	738	–	–	–

SD and DR denote single-dose and dose-response, respectively

We then advanced 14 projects with at least one dose-responsive scaffold to a round of analog expansion. We found new bioactive analogs in the SD screen for all projects, with an average hit rate of 29.8%. Further validation with DR resulted in an average hit rate of 26% per project, which compares favorably with typical HTS hit rates ranging from 0.151 to 0.001%^{64,65}. We note that the size and chemical diversity within and between physical⁶⁶ and virtual¹⁴ HTS libraries prevent an explicit evaluation of the methods over the same chemical space. The most potent analogs ranged from single-digit nanomolar, against a kinase, to double-digit micromolar, against a transcription factor (Supplementary Table ^{S2}).

Additionally, we present two internal studies in detail. For Large Tumor Suppressor Kinase 1 (LATS1), we identified potent compounds despite the lack of a crystal structure or known active compounds. For ATP-driven chaperone Valosin Containing Protein (VCP) we identified novel allosteric and orthosteric modulators.

Academic Validation

In addition to our internal discovery efforts, we performed virtual screens for 296 targets, comprising more than 20 billion individual neural network scores of generated protein–ligand co-complexes. We purchased, on average, 85 off-the-shelf commercially available compounds, quality controlled by NMR and LC–MS to > 90% purity⁶³, and plated in a single 96-well plate. The compounds were then physically screened for activity against the target of interest in single-dose assays (see Supplemental Data ^{S1} for assay protocols). As with HTS primary screens, additional characterization studies are required to validate the initially identified hits so, in 49 projects, we performed dose–response studies and analog expansion. We present a summary of our results in Supplementary Table ^{S3}.

Figure 2 illustrates the distributions of projects across therapeutic areas, protein families, and assay types. Every major therapeutic area is represented, with the most frequent area being oncology, comprising 35% of projects, followed by infectious diseases and neurology, comprising 27% and 9% of projects, respectively. Breaking down the projects by protein families reveals that all major enzyme classes are represented, with enzymes comprising 59% of the targets and membrane proteins such as GPCR, transporters, and ion channels, representing 12% of the targets. Working on a large and diverse set of therapeutic targets requires a heterogeneous collection of biological assays; 20% of the assays measured direct binding, whereas 56% and 20% were functional and phenotypic.

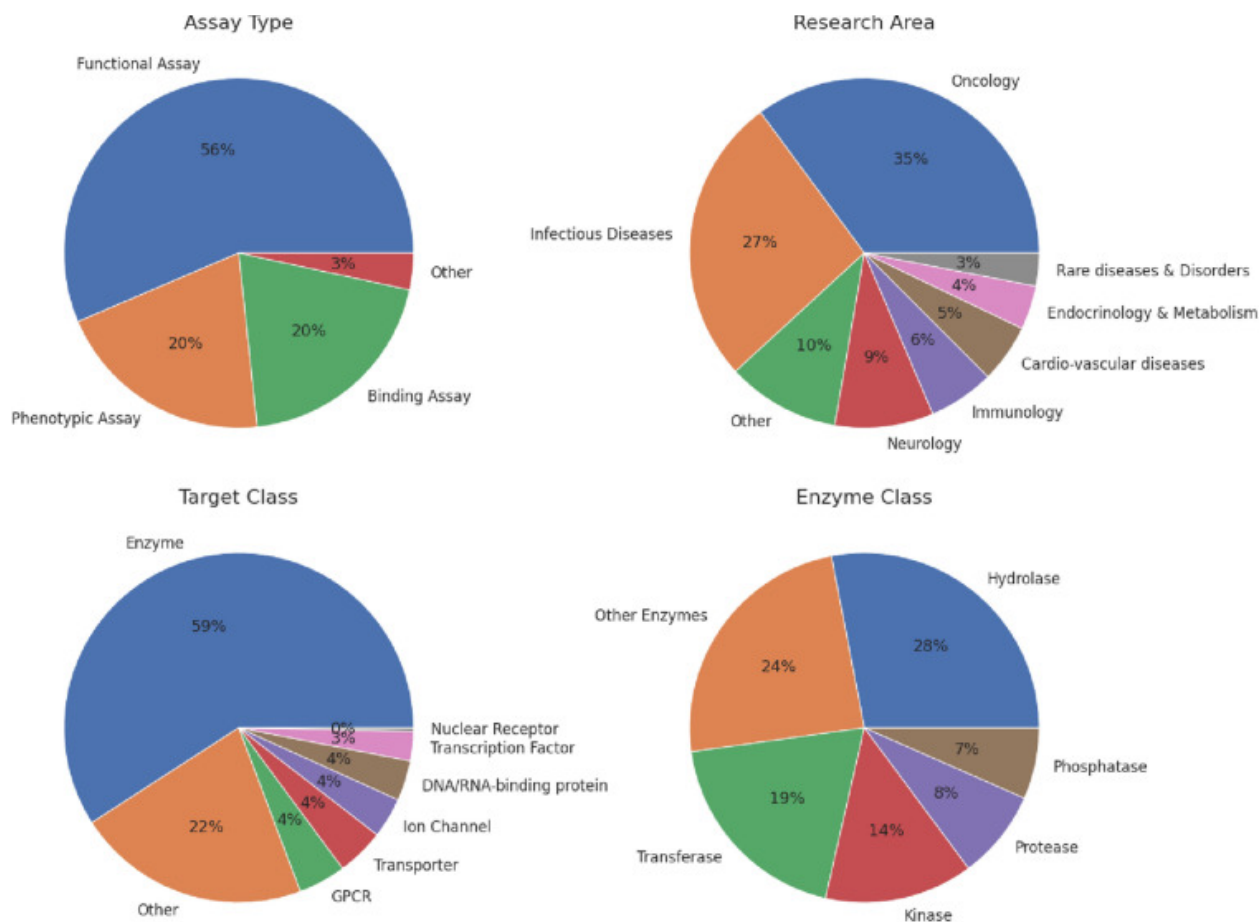


Figure 2. The distributions of 296 AIMS projects across assay types used in the primary screen, research areas, target classes, and further breakdown to enzyme classes when applicable.

In 215 projects, we identified at least one bioactive compound for the target in a biochemical or cell-based assay. This 73% success rate substantially improves over the ~50% success rate for HTS^{21,67}. On average, we screened 85 compounds per project and discovered 4.6 active hits, with an average hit rate of 5.5%. For the subset of targets where we found any hits, the average was 6.4 hits per project. Thus, we achieved an average hit rate of 7.6%, which again compares favorably with typical HTS hit rates. See Supplementary Material^{S1} for all assay definitions and conditions. Supplementary Table^{S4} shows a representative bioactive compound from each of the 215 successful projects, and Supplementary Fig.^{S2} shows that the physicochemical properties of the identified hits are largely druglike and Lipinski-compliant.

The AtomNet technology robustly identified active molecules, even for targets that lacked prior on-target bioactivity data. This ability to identify hits for previously undrugged targets is critical if machine learning-based approaches are to replace HTS as the default primary screening approach. For 207 out of the 296 targets (70%), the training data available for AtomNet models lacked a single active molecule for that target or any closely related protein (i.e., proteins with sequence identity greater than 70%). We interpret this as evidence of the ability of properly-architected machine learning systems to extrapolate to novel biological space. Figure 3A illustrates the hit rate versus the number of training examples available to our model. Although previous computational approaches typically require thousands of on-target training examples^{31,39,42}, the lack of correlation between training examples and hit rate ($R^2 = 0.0021$, $p\text{-value} = 0.43$) shows that our ML algorithm is agnostic to the availability of such data. We achieved an average success rate of 75% and hit rates of 5.3% when no training data was available, comparable to the 67% and 6.1% success and hit rates achieved when binding data was available in the training set. Interestingly, we also do not see a significant increase in hit rate attributable to the proportion of binding data available for a target ($R^2 = 0.008$, $p\text{-value} = 0.39$). This reflects the robustness of the screening protocol and the chemical dissimilarity of scaffolds identified by AtomNet models to previously known bioactive compounds.

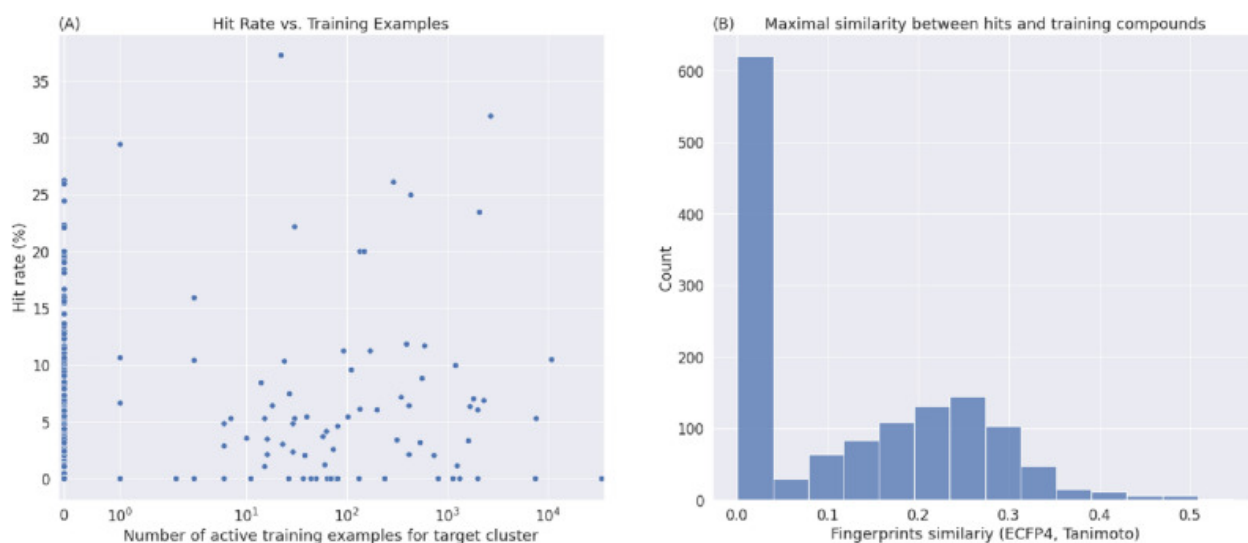


Figure 3. (A) An illustration of the hit rate versus the number of training examples available to our model. Each point represents a project, with the x-axis denoting the number of active molecules in our training for the target protein or homologs and the y-axis denoting the hit rate of the project (the percentage of molecules tested in the project that were active). The model shows no dependence on the availability of on-target training examples. For 70% of the targets, the AtomNet model training data lacked any active molecules for that target or any similar targets with greater than 70% sequence identity, yet the model achieved a hit rate of

5.3% compared to 6.1% when on-target data was available. **(B)** The distribution of similarities between hits and their most-similar bioactive compounds in our training data. Our screening protocol ensures that the compounds subjected to physical testing are not similar to known active compounds or close homologs (<0.5 Tanimoto similarity using ECFP4, 1024 bits). Because 70% of the AIMS targets had no annotated bioactivities in our training dataset, hits identified in these projects have a similarity value of zero.

Next, we assessed the ability of the AtomNet models to identify novel scaffolds. This is a critical capability for primary screens, as follow-up assays tend to work within the chemical space uncovered in the initial screen. The task of novel scaffold identification appears in two distinct scenarios: (1) when no scaffold is known for the target and we wish to identify the first scaffold, and (2) when some scaffolds are known but we wish to identify dissimilar scaffolds because novel chemical matter can yield improved selectivity, toxicity, pharmacokinetics, or patentability. Performance of AtomNet models for the first scenario, when no scaffolds for the target existed in the AtomNet model training data, was evaluated on 70% of the targets, where the training data contained no active molecules for the target or its homologs (*vide supra*). We achieved an average hit rate of 5.3% for targets with no training data. For the second scenario, we analyzed the similarity of the identified hits to known bioactive compounds in our training data (Fig. 3B). Our screening protocol ensures that the compounds subjected to physical testing are not similar to known active compounds or close homologs (<0.5 Tanimoto similarity using ECFP4⁶⁸, 1024 bits). We interpret this as evidence of the ability of properly-architected machine learning systems to extrapolate to novel chemical space as well. For cases where training data was available (i.e., the Tanimoto similarity is above zero), the similarity distribution is close to the one expected by random compound pairs⁶⁹. The novelty of the small-molecule structures is striking because target-specific machine-learning algorithms tend to uncover highly similar analogs for known bioactive molecules^{50,70,71}. The superior performance of the AtomNet model is expected, considering the bias-variance tradeoff⁷² in machine learning algorithms. Because the AtomNet convolutional neural network is a global model, concurrently trained on millions of bioactivities, hundreds of thousands of small molecules, and thousands of protein binding sites, it can reduce both bias and variance of the model compared to target-specific ones³³. Specifically, our global model can benefit from multiple levels of information captured in the structures of

the small molecules, the sequences of the target proteins, and the three-dimensional interactions between the two.

AtomNet also successfully identified active molecules when there was no X-ray crystal structure of the receptor. Figure 4A compares the hit rates obtained with 3-dimensional crystal structures, cryo-EM, and homology modeling. We did not attempt to select targets based on the similarity to the template but rather used the best template available. We observe no substantial difference in success rate between the three, in contrast to the common challenges in using homology models or low-precision structures for structure-based discovery^{42,43,73}. We achieved average hit rates of 5.6%, 5.5%, and 5.1% for crystal structures, cryo-EM, and homology modeling. We also successfully identified active compounds in projects with NMR structures, but the number of such targets is too small to make statistically-robust claims.

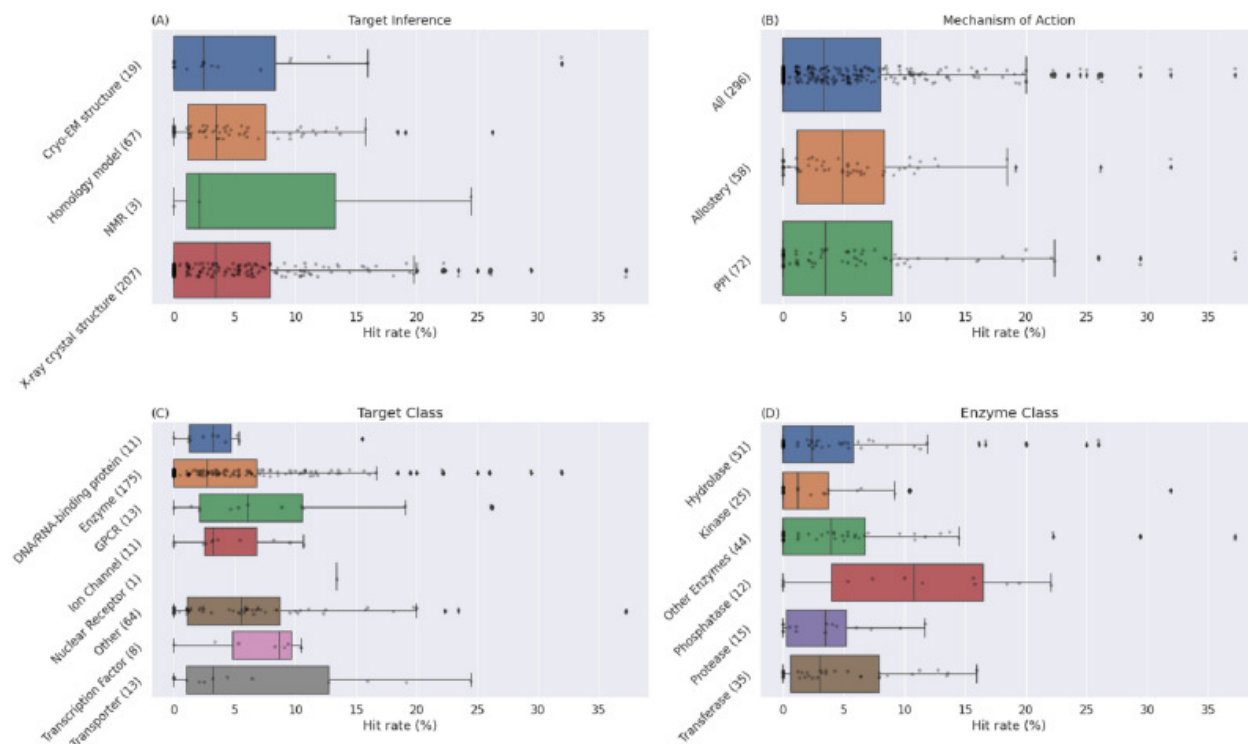


Figure 4. Hit rates obtained for the 296 AIMS projects. **(A)** A comparison of hit rates using X-ray crystallography, NMR, Cryo-EM, and homology for modeling the structure of the proteins. Each point represents a project with the x-axis denoting the hit rate of the project (the percentage of molecules tested in the project that were active). The number of projects of each type is given in parentheses. We observed no substantial difference in success rate between the physical and the computationally inferred models. We achieved average hit rates of 5.6%, 5.5%, and 5.1% for crystal structures, cryo-EM, and homology modeling, respectively. The number of projects using NMR structures is too small to make statistically-robust claims. **(B)** A comparison of hit rates observed for traditionally challenging target classes such as protein–protein interactions (PPI) and allosteric binding. Of the 296 projects, 72 targeted PPIs and 58 allosteric binding sites. The average hit rates were 6.4% and 5.8% for PPIs and allosteric binding, respectively.

(C) Comparison of hit rates observed for different target classes and (D) enzyme classes. No protein or enzyme class falls outside the domain of applicability of the algorithm.

An interesting demonstration of the robustness of the AtomNet model to low data and poorly characterized protein structure is its ability to identify novel hits for traditionally challenging target classes such as protein–protein interaction (PPI) sites and allosteric binding sites (Fig. 3B). Of the 296 projects, 72 targeted PPIs and 58 allosteric binding sites. We identified hits for 53 (74%) PPI sites and 46 (79%) allosteric sites, with 13 projects representing allosteric sites at PPI interfaces. The average hit rate was 6.4% and 5.8% for PPIs and allosteric binding sites, respectively. The algorithm's success in these target classes, which often suffer from poorly characterized binding sites and a lack of bioactivity training data, is not surprising because Fig. 2A shows that our model is largely not dependent on the availability of on-target training data.

Finally, we investigated whether the algorithm exhibits domain of applicability limitations regarding different protein classes. Figures 4C and 3D illustrate the hit rate observed for each protein and enzyme class. No protein or enzyme class falls outside the domain of applicability of the algorithm, demonstrating that machine learning-based approaches are well-suited as a default technology for new scaffold identification. The hit rate for nuclear receptors is an outlier, with seemingly better accuracy than other classes, but a single data point is not statistically meaningful.

Dose-response Validation Studies

We performed additional validation studies for 49 AIMS projects with at least one reported hit. The objective of the validation studies was to establish dose–response (DR) relationships for the single-dose (SD) hits. We describe the protocol of the DR experiments in the Methods section. Briefly, we performed dose–response measurements for the reported hits from the single-dose primary screens. DR was determined using the same assay and screening protocol as the single-dose screens, at the same lab, and with the same personnel. Full dose response curves were obtained in most cases, however in some instances

a full curve was not obtained, or concentration dependent activity was qualitatively determined by testing at concentrations other than that for the primary screen. The distribution of assay types and target classes for the projects selected for DR validation also was similar to that of the AIMS projects (Supplementary Fig. ^{S3}).

We describe the results of the DR experiments in Supplementary Table ^{S5}. In 84% of the experiments, we validated at least one SD hit and got a DR readout. The median activity for the total of 144 DR measurements was 15.4 μ M (which compares favorably with HTS^{25,74}), of which 13% showed sub- μ M potency. Overall, we achieved an average of 2.8 hits per validation study, resulting in a hit rate of 51%. The false positive rate of 49% observed in these experiments is favorably compared to HTS' which can be as high as 95%^{20,75}. This difference in false positive rates may stem from the comparative ease and robustness of the low-throughput assay format we employed versus high-throughput assay. Representative dose–response curves for each of the 49 projects are shown in Supplementary Table ^{S6}.

KCNN4 Screening Methods and Results (Wulff Lab Contribution)

The inner pore of KCa3.1 was screened virtually in two different states; the open state (PDB ID: 6CNN) and the closed state (PDB ID: 6CNO) (Figure 5).

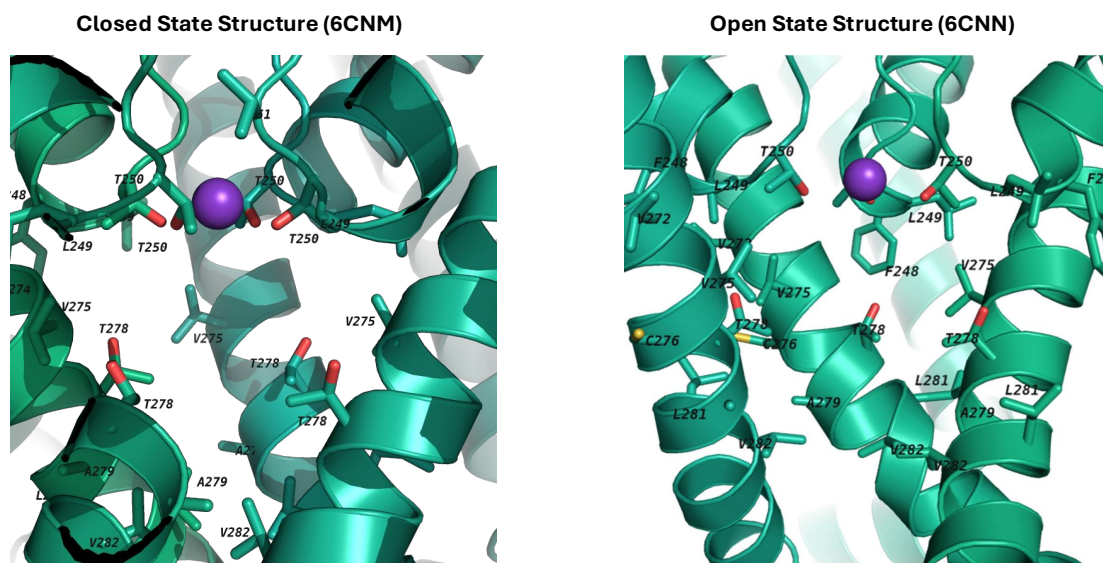


Figure 5. Close up view of the inner pore of KCa3.1 in the closed state (PDB ID: 6CNM) and open state (PDB ID: 6CNN), with key residues labeled and a potassium ion included as a purple sphere at the bottom of the channel's selectivity filter.

165 total compounds were identified as a result, 82 from the open state and 83 from the closed state screening. All compounds were then tested on HEK293 cells stably expressing hKCa3.1 (these cells were obtained by the Wulff lab from Khaled Houamed, University of Chicago, IL, in 2002 and maintained since then at the University of California, Irvine & Davis). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 25mM glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1mM Na⁺ pyruvate, 100 units/mL penicillin, 100 mg/mL streptomycin, and 1 mg/mL puromycin to maintain selection pressure. Cells were maintained at 37°C in a 5% CO₂ atmosphere and typically passaged when they were 70%–80% confluent. Whole-cell patch-clamp experiments were carried out on a QPatch-16 automated electrophysiology platform (Sophion Biosciences) using disposable 16-channel planar patch chip plates (QPlates; patch hole diameter*1 mm, resistance 2.00 – 0.02 MΩ). Cell positioning and sealing parameters were set as follows: positioning pressure - 70 mbar, resistance increase for success 750%, minimum seal resistance 0.1 GΩ, holding potential - 80 mV, holding pressure - 20 mbar. To avoid rejection of cells with large KCa3.1 currents, the minimum seal resistance for whole-cell requirement was lowered to 0.001 GΩ. Following establishment of the whole-cell configuration, cells were held at - 80mV and

KCa3.1 currents elicited by a voltage protocol that held at -80 mV for 20 ms, stepped to -120 mV for 20 ms, ramped from -120 to +40 mV in 200 ms, and then stepped back to -120 mV for 20 ms. This pulse protocol was applied every 10 s. The external solution was Na⁺-Ringer's and contained (in mM): 160 NaCl, 10 HEPES, 4.5 KCl, 1 MgCl₂, 2 CaCl₂ (pH 7.2, 310 mOsm). The internal solution contained (in mM): 120 KCl, 10 HEPES, 1.75 MgCl₂, 1 Na₂ATP, 10 EGTA and different amounts of CaCl₂ (4.6 for 150 nM; 5.4 for 200 nM and 8.6 for 1 μ M) to achieve different concentrations of free Ca²⁺ (pH 7.4, 300 mOsm). Current slopes (in amperes per second) were measured using the Sophion QPatch software and exported to Microsoft Excel and GraphPad Prism for analysis. Each current slope is the average of three measurements taken at the end of the respective liquid period. Increases or decreases of slopes between -85 and -65 mV were used to calculate KCa3.1 inhibition or activation. Data fitting to the Hill equation for EC₅₀ and IC₅₀ calculation was performed via GraphPad Prism, with all data expressed as mean $\pm$ SD.

Of the 165 compounds tested, 30 were found to inhibit KCa3.1 channel conductance by at least 30% at 10 μ M concentrations (Figure 6, 7 & 8).

Data Summary of KCa3.1 Compound Screening

- Closed State: 83 compounds tested

Total Hits	19
30%>	10
50%<	9

- Open State: 82 compounds tested

Total Hits	12
30%>	8
50%<	3
Activator	1

Figure 6. Table summarizing the observed activity (or lack thereof) of all 165 most promising compounds selected via Atomwise's screen of KCa3.1's inner pore, in both the closed and open state conformations. All compounds were tested at a concentration of 10 μ M.

Compound Chart for Closed State Screening of KCa3.1

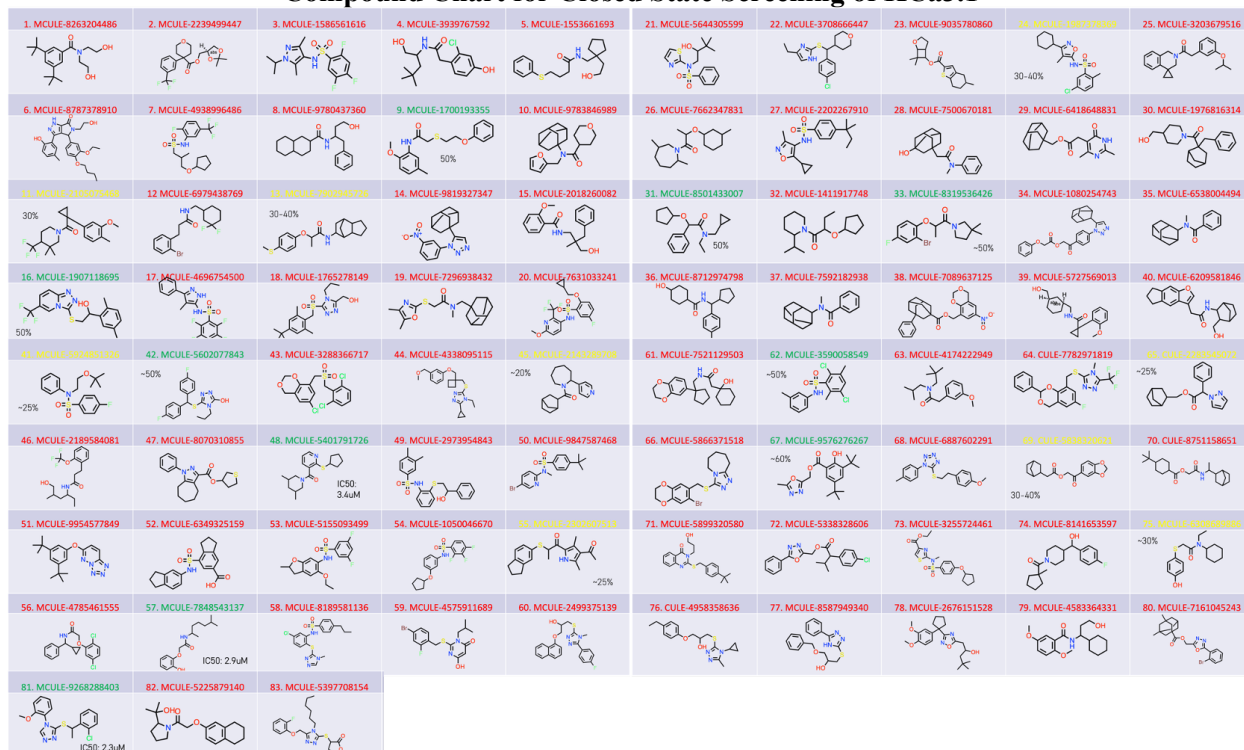


Figure 7. Chart of all 83 compounds screened within the inner pore of KCa3.1 in the channel's closed state conformation (PDB ID: 6CNM). Compound IDs are color coded based on activity or lack thereof, with green highlighted compounds blocking at least 50 percent of KCa3.1 activity at 10 uM, yellow compounds blocking between 25-40 percent, and red compounds being completely inert.

Compound Chart for Open State Screening of KCa3.1

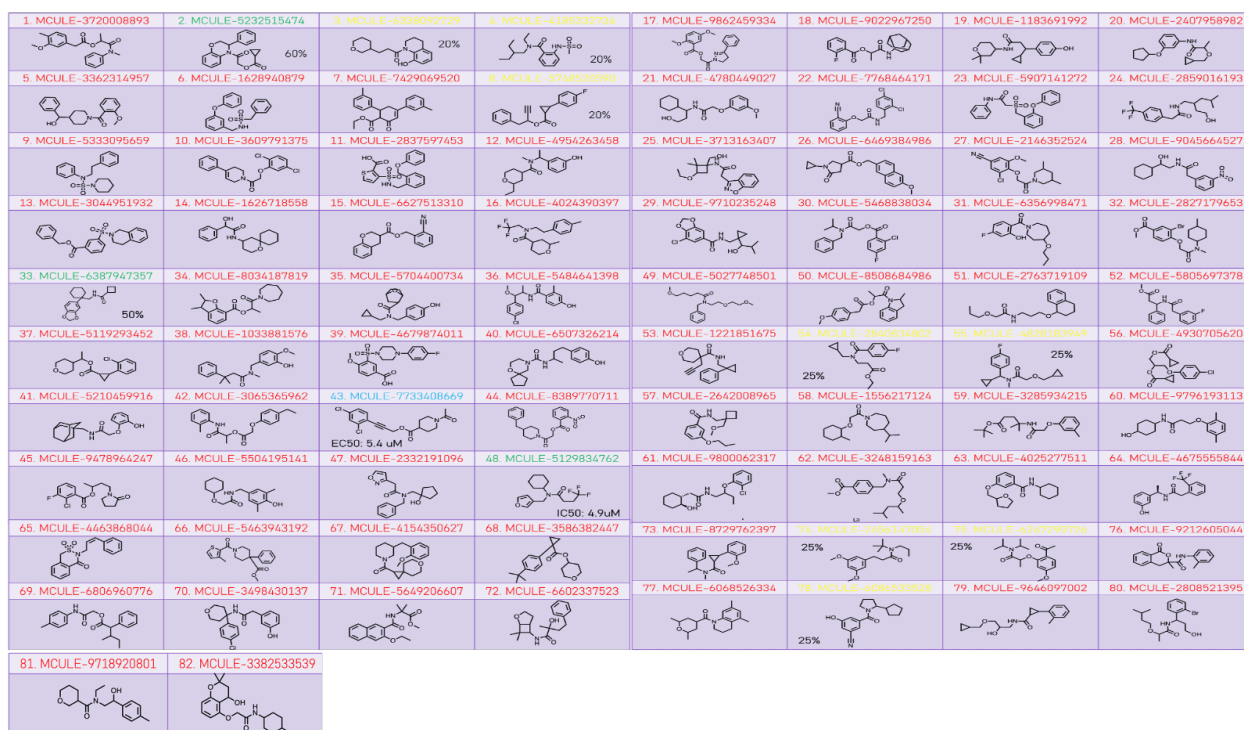


Figure 8. Chart of all 82 compounds screened within the inner pore of KCa3.1 in the channel's open state conformation (PDB ID: 6CNN). Compound IDs are color coded based on activity or lack thereof, with green compounds blocking at least 50 percent of channel activity at 10 μ M, yellow compounds blocking between 25-40 percent total current, red compounds being inert, and the compound highlighted in blue surprisingly representing an activator of KCa3.1 rather than a blocker.

Of those 30 inhibitory compounds, the four most potent and chemically attractive compounds were selected for further concentration response data generation (Figure 9). While these compounds were fairly diverse structurally, they all possessed similar IC_{50} values ranging from 2.3 to 4.5 μ M, and Hill slope values of 1 ± 0.2 .

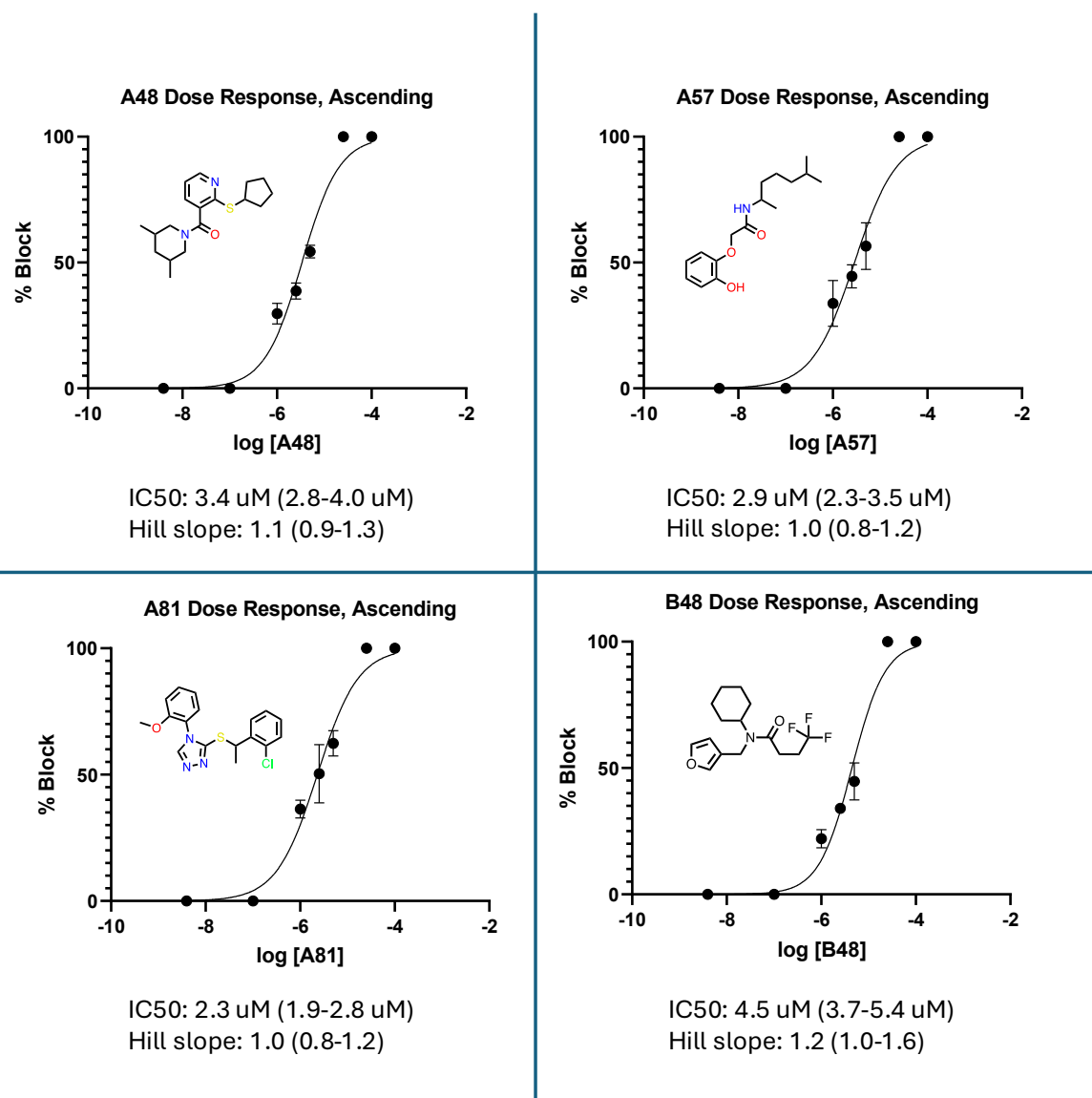


Figure 9. Concentration response curves of the top 4 most potent inhibitors found in AtomNet screen of KCa3.1's inner pore (designated as compounds A48, A57, A81, and B48 respectively), including IC₅₀ and Hill slope values, with 95% confidence intervals in parenthesis.

In addition, a positive gating modulator was found among the compounds from the open state screening of the inner pore (highlighted in blue of Figure 8). A concentration response curve was also generated for this compound (B43), which was found to have an EC₅₀ of 5.9 μ M and was capable of producing 10-fold increases in current amplitude at saturating concentrations (Figure 10). Unfortunately, due to time and budget constraints, none of these active compounds were pursued further through

derivatization. However, all of them constitute new chemical matter that bears no similarity of existing KCa3.1 inhibitors or activators.

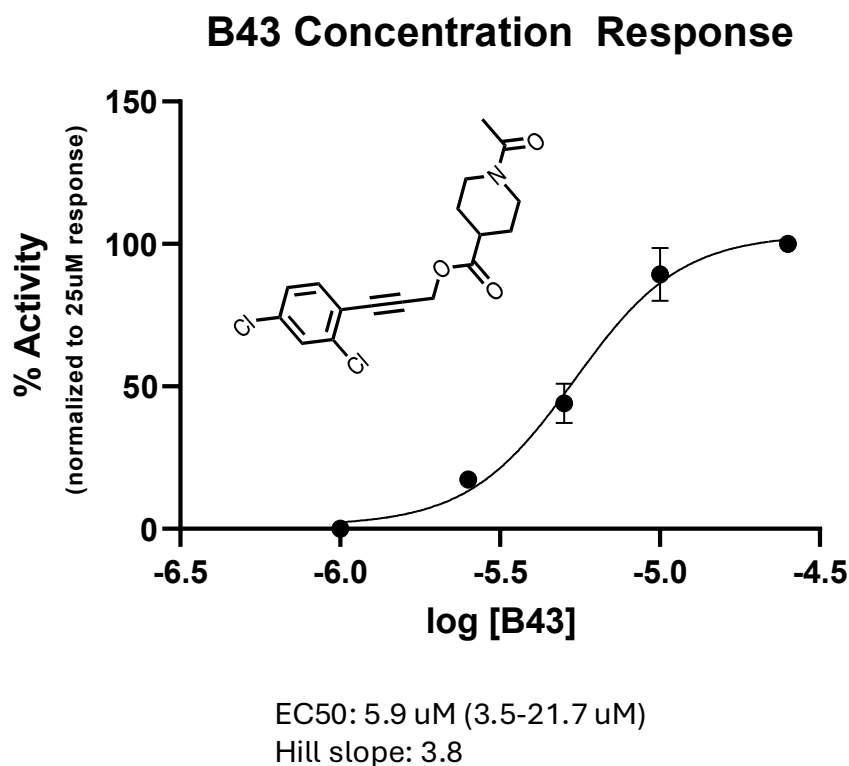


Figure 10. Concentration response curve for compound B43, a positive gating modulator of KCa3.1 with an EC₅₀ of ~5.9 uM and a Hill slope of 3.8.

Conclusion

HTS is the most widely-used tool for hit discovery for new targets. Unfortunately, all physical screening methods share the critical limitation that a molecule must exist to be screened. Computational methods enable a fundamental shift to a test-then-make paradigm. In this work, we report on 318 projects (22 internal projects and 296 collaborations) where we used the AtomNet platform as the primary screening tool coupled with low-throughput physical screens as validation. The AtomNet technology can identify bioactive scaffolds across a wide range of proteins, even without known binders, X-ray structures, or

manual cherry-picking of compounds. Our empirical results suggest that machine learning approaches have reached a computational accuracy that can replace HTS as the first step of small-molecule drug discovery.

Supplemental Data

Can be found at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10987645/>

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CHAPTER 6: Concluding remarks and future directions

The pharmacological manipulation of ion channels has emerged as a critical tool for both understanding cellular mechanisms of action and, arguably more importantly, developing therapeutic strategies. Calcium-activated potassium channels are no exception. In this dissertation, we have extensively explored the intricate relationship between calcium-activated potassium channels and their pharmacological modulation by both inhibitors and activators.

Inhibiting potassium conductance through key channels such as KCa3.1 can interfere with calcium influx/calcium signaling pathways in immune cells, slowing down their inflammatory effects and showing therapeutic promise in treating disease states such as Alzheimer's disease and Multiple Sclerosis. Even now, a phase-2 clinical trial (NCT#04804241) is underway at the UC Davis Alzheimer's Disease Center with the aim of treating Alzheimer's disease via the potent KCa3.1 inhibitor Senicapoc. Inhibition of that same channel in erythrocytes has been looked at as a potential treatment for Sickle cell anemia, stemming from the theory that a reduction in potassium efflux would result in a corresponding osmotic influx of water, effectively leading to an increase of the shrunken red blood cell volume. This was observed in a clinical trial applying Senicapoc to patients with sickle cell anemia. Unfortunately, however, this correction in cell morphology was not accompanied by a reduction in pain crises, leading the clinical trial to fail in phase 3. Inhibitors have also been proposed to treat complications resulting from COVID-19 and stroke reperfusion injury, following the same anti-inflammatory mechanism of action leading to the ongoing trial in Alzheimer's disease.

Amplifying calcium-activated potassium channel activity via positive gating modulators can also be therapeutically relevant. Given the KCa3.1 channel's expression in vascular endothelial cells, one of the early proposed uses of KCa3.1 channel "activators" was for the treatment of hypertension, through the process of enhancing endothelium-derived hyperpolarization of the underlying smooth muscle cells and subsequent vasodilation. This channel is also expressed in secretory bronchial epithelial cells, where

increasing channel activity via positive gating modulators might aid in clearance of mucus build-up in cystic fibrosis patients by stimulating chloride secretion. Positive gating modulators of KCa2.x channels, which unlike KCa3.1 are expressed in the heart and CNS, have been proposed as potential drugs to treat muscle tremors, ataxia and epilepsy, while KCa2.x inhibitors are currently undergoing clinical trials for atrial fibrillation. Muscle relaxation already possesses a KCa2.x modulator on the market in the form of Chlorzoxazone, although the exact mechanism underlying its effects is still a mystery.

While not quite as pressing given their relative rarity, both loss and gain-of-function genetic mutations of the calcium-activated potassium channels exist and have been directly linked to severe disease morphologies such as Zimmerman-Laband syndrome and hereditary xerocytosis. Whether KCa channel dysregulation is genetic in nature or a result of some other pre-existing disease state like Alzheimer's or diabetes, channel modulators targeting this family of proteins could certainly be of enormous therapeutic benefit, highlighting the importance of the research described within this dissertation.

In Chapter 2, we explored the binding site and mechanism of action of the most potent small molecule activator of KCa3.1, NS309. This compound amplifies KCa3.1 activity at low nanomolar concentrations, while also possessing similar effects on the small-conductance KCa2.x channels at high nanomolar concentrations. Based on the molecular modeling, mutagenesis and electrophysiological experiments performed for this dissertation, we demonstrate that NS309 binds within the interface between calmodulin's N-lobe and the S4-S5 intracellular linker of the channel itself. Additionally, we also discovered through the use of constitutively open KCa3.1 mutations and molecular dynamics simulations that NS309 seems to operate by stabilizing the channel in its open-state conformation and prevents the hydrophobic gate located at V282 from closing. This channel modulator is even capable of amplifying channel activity beyond what the endogenous ligand (calcium) is capable of on its own, and we have therefore termed it a "superagonist".

In Chapter 3, we revisited the pharmacology of apamin, one of the most potent and most widely used peptide inhibitors of the small-conductance calcium-activated potassium channels, confirming its exceptional selectivity for KCa2.1, 2.2 and 2.3 and complete lack of promiscuity. In Chapter 4, we looked at off-target effects of the erythroid K-Cl cotransport inhibitor [(dihydroindenyl)oxy] acetic acid (DIOA), demonstrating that the compound's effects on red blood cell volume may actually be driven through KCa3.1 inhibition rather than its originally perceived effect on K-Cl cotransporters.

Apamin, NS309 and DIOA may never be more than tool compounds, but everything we have learned about how they interact with calcium-activated potassium channels can potentially be used in our ongoing search for more selective or more potent KCa-targeting channel modulators. This can be seen from our work in Chapter 5 where, in collaboration with the drug discovery company Atomwise, we used the KCa3.1 cryo-EM structure and its central pore binding site to conduct a large-scale virtual screen featuring millions of compounds, ultimately leading to the discovery of several novel channel modulators suitable for investigation as new therapeutics. The more well-characterized a channel modulator and/or binding pocket, the easier the task of finding new modulators, or even modifying existing ones in such a way as to increase potency, selectivity, or both. And this was the overarching premise of this dissertation.

However, there is still much work to be done, and many questions to be answered. In the near future, we would like to take a closer look at some of the novel modulators of KCa3.1 found during our collaboration with Atomwise, including a positive gating modulator that may or may not be binding in the central pore; a binding site which typically accommodates channel inhibitors rather than activators. Using molecular dynamics simulations, we would like to explore in depth constitutively open KCa3.1 mutants V282G and A279G, investigating how these single substitutions can alter open probability so significantly and what more they might tell us about KCa channel function and pharmacology. Finally, given the discovery of KCa channel superagonists, it is essential to explore the therapeutic differences in vivo regarding superagonistic vs non-superagonistic positive gating modulators. It has already been shown in slice recordings that superagonists targeting KCa2 channels result in a much more complete silencing of

neuronal firing when compared to standard positive gating modulators, suggesting that a superagonist might be more effective for specific indications like ataxia but also more sedating than positive gating modulators that do not enhance channel activity beyond the endogenous ligand, calcium. What could this mean for the treatment of muscle tremors, or even pain? We still have plenty of work ahead, but our lab remains passionate about continuing our dig in this exciting sandbox.