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Pharmacology of Small- and Intermediate-Conductance Ca^{2+}-Activated K^+ Channels

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Abstract

The three small-conductance calcium-activated $K_{Ca}2$ channels and the related intermediate-conductance $K_{Ca}3.1$ channel are voltage-independent K^+ channels that mediate calcium-induced membrane hyperpolarization. When intracellular calcium increases in the channel vicinity it calcifies the flexible N-lobe of the channel-bound calmodulin, which then "swings over" to the S4-S5 linker and opens the channel. $K_{Ca}2$ and $K_{Ca}3.1$ channels are highly druggable and offer multiple binding sites for venom peptides and small molecule blockers as well as for positive and negative gating modulators. In this review we will first briefly summarize the physiological role of K_{Ca} channels and then discuss the pharmacophores and the mechanism of action of the most commonly used peptidic and small molecule $K_{Ca}2$ and $K_{Ca}3.1$ modulators. Finally, we will describe the progress that has been made in advancing $K_{Ca}3.1$ blockers, and $K_{Ca}2.2$ negative and positive gating modulators towards the clinic for neurological and cardiovascular diseases, and discuss the remaining challenges.

Keywords

Calcium-activated potassium channel; $K_{Ca}2.2$; $K_{Ca}2.3$; $K_{Ca}3.1$; gating modulation

INTRODUCTION

Potassium (K^+) channels are critically involved in regulating fundamental physiological processes such as cellular volume, membrane potential, hormone secretion, calcium signaling, and action potential firing (1). To allow for fine tuning of these processes the human genome contains 78 K^+ channels. According to the IUPHAR (International Union of Basic and Clinical Pharmacology) *Guide to Pharmacology* (2) these channels have been grouped based on sequence similarity and the number of their transmembrane domains (TM), which can be 2, 4, 6 or 7. The small- and intermediate-conductance Ca^{2+}-activated K^+ channels belong to the 6TM family and thus resemble the voltage-gated K^+ channels, with a 4TM voltage sensor domain (VSD) and a 2TM pore domain (3). Like K_V channels, functional K_{Ca} channels are tetramers and, at least in expression systems, the

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different family members are able to form heteromultimers in addition to the more common homotetramers (4). However, unlike K_V channels, K_{Ca} channels have fewer positively charged residues in the S4 segment of their VSD, and are therefore unresponsive to changes in transmembrane voltage and have essentially linear current voltage-relationships at physiological ion gradients (3; 5). Before their cloning, $K_{Ca}2$ and $K_{Ca}3.1$ channels were referred to as small-conductance (SK) or intermediate-conductance (IK) Ca^{2+}-activated K^+ channels based on their unitary conductance of ~10 pS or ~40 pS in symmetrical K^+ solutions to differentiate them from the large-conductance BK channel (~200 pS), which is now called $K_{Ca}1.1$ (3). This phenomenological nomenclature is still widely used together with the IUPHAR and HUGO nomenclatures, which is why we wanted to introduce it upfront to avoid confusion.

The three $K_{Ca}2$ channels, $K_{Ca}2.1$ (SK1, *KCNN1*), $K_{Ca}2.2$ (SK2, *KCNN2*) and $K_{Ca}2.3$ (SK3, *KCNN3*) were cloned in 1996 by the group of John Adelman and are highly homologous across their transmembrane cores (80–90%) but diverge in sequence and length in their N- and C-termini (5). Two years later, the same group demonstrated that the calcium-sensor of the SK channels is calmodulin, which is constitutively bound to the calmodulin binding domain (CaM-BD) in the C-terminus and thus functions as a β-subunit that endows these channels with Ca^{2+} sensitivity (6). The $K_{Ca}3$ family only contains a single member, $K_{Ca}3.1$ (IK, SK4, *KCNN4*), which was cloned in 1997 and delegated to its own subfamily because it only is ~40% identical to the three $K_{Ca}2$ channels (7; 8). Similar to the $K_{Ca}2$ channels, the Ca^{2+}-dependent activation of $K_{Ca}3.1$ is mediated by calmodulin (9). The reported EC_{50} values for Ca^{2+} range from 100 to 400 nM for $K_{Ca}3.1$ and from 300 to 750 nM for the $K_{Ca}2$ channels. This submicromolar Ca^{2+}-sensitivity together with their lack of voltage-dependence enables K_{Ca} channels to be open at relatively negative membrane potentials when intracellular Ca^{2+} is raised in their immediate vicinity and to hyperpolarize towards the K^+ equilibrium potential of −90 mV. K_{Ca} channels are accordingly expressed in cells that need to be able to prevent premature action potential generation or sustain Ca^{2+} influx through inward-rectifier Ca^{2+} channels.

EXPRESSION AND PHYSIOLOGICAL FUNCTION OF $K_{Ca}2$ CHANNELS

$K_{Ca}2$ channels are widely expressed on neurons of the central and peripheral nervous system (10). $K_{Ca}2$ currents underlie the so-called medium afterhyperpolarization (mAHP), the second phase of neuronal hyperpolarization following an action potential, and thus regulate intrinsic excitability and spike firing rates. $K_{Ca}2$ currents are activated by calcium entering neurons via Ca_V channels activated during the action potential, but may also functionally couple to post-synaptic calcium sources such as NMDA receptors and nicotinic acetylcholine receptors as well as calcium released from intracellular ryanodine or IP_3 receptors (10).

The mAHP is absent in $K_{Ca}2.2$ knock-out mice, but not mice lacking $K_{Ca}2.1$ or $K_{Ca}2.3$, revealing that $K_{Ca}2.2$ is the dominant subtype responsible for this current (11). Reversely, 10-fold overexpression of $K_{Ca}2.2$ increases the mAHP and dampens excitatory postsynaptic potentials (EPSP) making postsynaptic neurons less likely to fire, resulting in mice with impairments in hippocampal learning and memory (12). $K_{Ca}2.3$ channels are strongly

expressed on dopaminergic substantia nigra neurons, where they maintain regular firing and pace-making control (13) and serotonergic raphe neurons, where they regulate burst firing (14). Pharmacological modulation confirms the role $K_{Ca}2$ channels in neurons (Figure 1). The selective $K_{Ca}2$ blocker apamin increased intrinsic excitability and firing frequency, while $K_{Ca}2$ activators enhance the magnitude of the mAHP and slow down firing rates (10; 15). These altered responses have consequences for long-term potentiation (LTP), the increase in synaptic strength following high-frequency stimulation, which underlies some forms of learning. Inhibiting $K_{Ca}2$ activity with apamin improves learning and memory encoding in rodents (16; 17), while increasing $K_{Ca}2$ activity with an activator impairs associative learning (18). In the peripheral nervous system, $K_{Ca}2$ channels are expressed in DRG primary sensory neurons, where they play a role in nociception (19; 20).

While all three $K_{Ca}2$ channels are found in the nervous system, $K_{Ca}2.3$ is the only member found in the endothelial cells of blood vessels, where, together with $K_{Ca}3.1$ (21), $K_{Ca}2.3$ underlies the endothelium derived hyperpolarization (EDH), a phenomenon that, together with prostacyclin and nitric oxide, controls vessel tone (22). Activation of K_{Ca} currents on the endothelium leads to hyperpolarization and relaxation of the underlying smooth muscle cells, ultimately reducing blood pressure (Figure 1). $K_{Ca}2$ channels are further expressed in the heart (Figure 1) and have been shown to play an important a role in the repolarization of the cardiac action potential, specifically in atrial myocyte and atrioventricular nodes (23; 24). Multiple single nucleotide polymorphisms (SNPs) in $K_{Ca}2.3$ have been found to be associated with lone atrial fibrillation (AF) (25; 26). Increasing $K_{Ca}2.3$ activity significantly shortens cardiac action potentials resulting in increased susceptibility to AF (27). A $K_{Ca}2.3$ splice variant is expressed in liver hepatocytes, where $K_{Ca}2.3$ plays a role in cellular responses to metabolic stress (28).

EXPRESSION AND PHYSIOLOGICAL FUNCTION OF $K_{Ca}3.1$ CHANNELS

A calcium-activated K^+ efflux, which was later demonstrated to be mediated by $K_{Ca}3.1$ and to contribute to volume regulation and hydration state (29; 30), was fist described in erythrocytes in 1958 (31), which is why $K_{Ca}3.1$ is also called the Gárdos channel after the scientist who first described the phenomenon. Heterozygous gain of function mutations in $K_{Ca}3.1$ are responsible for erythrocyte dehydration in a subset of patients with hereditary xerocytosis, a disease characterized by hemolytic anemia associated with erythrocyte dehydration (32; 33). In addition to red blood cells (Figure 1), $K_{Ca}3.1$ is also widely expressed in cells of the immune system such as T cells (34), B cell (35), mast cells (36), macrophages (37), and microglia (38). The primary role of $K_{Ca}3.1$ in the immune cells is to hyperpolarize the cell membrane and create the driving force for the calcium entry that is necessary for activation, proliferation and cytokine production (39). Most, if not all, $K_{Ca}3.1$ expression in the brain seems to be localized to microglia, which upregulate $K_{Ca}3.1$ after activation *in vitro* and *in vivo* (40; 41). Although two studies recently reported that $K_{Ca}3.1$ may also be expressed in neurons and contribute to the slow afterhyperpolarization (42; 43), another study presented data that $K_{Ca}3.1$ does not contribute to the slow AHP (44). A potential role for $K_{Ca}3.1$ in neurons therefore currently remains uncertain. The phenotype of the $K_{Ca}3.1^{-/-}$ mouse reinforces the channel's role in the immune system. T cells from $K_{Ca}3.1^{-/-}$ mice show reduced T-cell receptor mediated calcium influx and

inflammatory cytokine production and the mice develop less severe colitis (45) and arthritis (46). $K_{Ca}3.1^{-/-}$ mice further display blunted IgE-mediated anaphylactic reactions and reduced infarction and neuroinflammation after ischemic stroke (40).

$K_{Ca}3.1$ deletion in mice also reduces the EDH response, raises mean arterial blood pressure by 7–9 mm Hg (47) and causes subtle erythrocyte macrocytosis and progressive splenomegaly (48). In secretory epithelia of the lung and gastrointestinal tract $K_{Ca}3.1$ works in concert with the Na-K-2Cl cotransporter to facilitate chloride and fluid secretion (49). While $K_{Ca}3.1$ channels are not expressed in normal vascular smooth muscle cells, expression is turned on in dedifferentiated vascular smooth muscle cells where $K_{Ca}3.1$ activity promotes proliferation and migration, while $K_{Ca}3.1$ inhibition reduces atherosclerosis in mice (37) and restenosis in rats and pigs (50; 51). Likewise, $K_{Ca}3.1$ drives proliferation and migration in many cancers such as glioblastoma (52; 53), breast or prostate cancer (54; 55), which is why $K_{Ca}3.1$ blockers have been proposed to treat diseases that have a proliferative component.

CHANNEL STRUCTURE

The K_{Ca} channel field recently obtained some tremendous structural insights when the group of Roderick MacKinnon solved the full-length cryo-EM structures of $K_{Ca}3.1$ in the absence and presence of calcium (56). Unlike $K_V1.2$, $K_{Ca}3.1$ is non-domain swapped and the structure showed four CaMs per channel tetramer, with the CaM C-lobe of each CaM tightly bound to the CaM-BD of each subunit. The CaM N-lobes were only visible in the two open, Ca^{2+}-bound states and poorly resolved in the closed, Ca^{2+}-free structure suggesting that they are flexible in the absence of Ca^{2+}. When Ca^{2+} binds to the N-lobe, it “swings over” to the S4-S5 linker of another subunit and pulls part of the S4-S5 linker, namely the $S_{45}A$ helix downward, thus expanding the S6 helices and opening the pore (56). This structure solved the long-standing conundrum of the K_{Ca} channel gating symmetry. In 2001, Schumacher *et al.* had crystallized the $K_{Ca}2.2$ channel C-terminal CaM-BD in complex with CaM. This 1.6 Å resolution structure had shown an elongated, anti-parallel dimer of two $K_{Ca}2.2$ C-terminal fragments with CaM tightly bound with its C-lobe to two alpha helices connected by a turn from the same channel subunit (57). The CaM N-lobe was “grabbing” the free end of CaM-BD from the other subunit in the dimer suggesting that CaM-BD dimerization might gate $K_{Ca}2$ channels (57). However, the 2-fold symmetry suggested by this dimer-of-dimers was difficult to reconcile with the 4-fold symmetry of the pore (58) and the new full-length structure now makes it clear that $K_{Ca}3.1$ in fact gates with a more common 4-fold symmetry.

Interestingly, the dimeric crystal has proven very resilient in that it was repeatedly observed in subsequent crystallographic studies addressing the mechanism of action of small molecule K_{Ca} channel activators and of PIP_2 on $K_{Ca}2.2$ channel function (59–62). Several K_{Ca} channel activators were shown to bind in the interface between the CaM N-lobe and the CaM-BD in this dimeric crystal and the interaction was even confirmed by solution state NMR experiments (62). However, the full-length $K_{Ca}3.1$ structure demonstrated that the dimeric crystal is an artefact and suggested that the existing ideas about the binding site of $K_{Ca}2$ and $K_{Ca}3.1$ activators need to be revised (56). We therefore here show a Rosetta

refined (63) $K_{Ca}3.1$ model, which is based on open state-1 of the $K_{Ca}3.1$ cryo-EM structure, and a $K_{Ca}2.2$ homology model to illustrate and discuss the binding sites of the commonly used pharmacological tool compounds in context of this structure.

$K_{Ca}2$ AND $K_{Ca}3.1$ CHANNEL PHARMACOLOGY

In 1982 the neurotoxic peptide apamin was shown to inhibit some Ca^{2+}-activated K^+ channels (64) and K_{Ca} channels were therefore typically differentiated into “apamin-sensitive” and “apamin-insensitive” in the 1980s and 1990s. Following their cloning, K_{Ca} channel pharmacology developed relatively rapidly and the field now has quite a range of peptidic and small-molecule inhibitors as well as positive and negative gating modulators available. Since K_{Ca} channel pharmacology has been reviewed by us and others a decade ago in great detail (65–67), we here concentrate on the most commonly used modulators and their mechanisms of action.

Venom peptides

The most widely used $K_{Ca}2$ channel blocker is the 18-amino acid honey bee venom peptide apamin (64), which is remarkably selective for $K_{Ca}2$ channels. Apamin is most potent on $K_{Ca}2.2$ (IC_{50} ~200 pM) and blocks $K_{Ca}2.1$ and $K_{Ca}2.3$ with 10–50-fold lower affinity (5; 10), while it has no effect on $K_{Ca}3.1$. *In vitro* application of apamin to neurons or brain slices has been instrumental for demonstrating the crucial role of $K_{Ca}2$ channels in neuronal excitability (10). While low concentrations of apamin improve cognitive performance in rodents, higher concentrations induce seizures (67; 68). Apamin was initially assumed to be a simple pore blocker but was later found to inhibit $K_{Ca}2$ channels through an allosteric mechanism involving an outer pore histidine (69) and residues in the S3-S4 extracellular loop (70), a binding configuration that is recapitulated in our docking pose in the $K_{Ca}2.2$ homology model (Figure 2). The larger scorpion toxins scyllatoxin, which is also called leiurotoxin I (71; 72), and tamapin (73) have roughly the same potency as apamin and show comparable preference for $K_{Ca}2.2$. A less potent, but highly $K_{Ca}2.2$ selective blocker, is the scyllatoxin derivative Lei-Dab^7 (74), in which one residue is replaced by the unnatural amino acid diaminobutonoic acid (Dab). While Lei-Dab^7 thus constitutes an even more selective tool to block $K_{Ca}2.2$ channels in physiological studies (75), there currently are no natural toxins or analogs that selectively inhibit $K_{Ca}2.1$ or $K_{Ca}2.3$.

The best known peptidic blocker of $K_{Ca}3.1$ is the 37-amino acid scorpion toxin charybdotoxin, which anchors itself in the outer vestibule of $K_{Ca}3.1$ by two salt-bridges while inserting its central lysine residue into the selectivity filter (76) as shown in Figure 3. However, charybdotoxin never was an ideal $K_{Ca}3.1$ blocker because it also inhibits $K_{Ca}1.1$ (BK) and the voltage-gated Kv1.3 channels, both cross-reactivities, which initially caused confusion concerning the role of $K_{Ca}3.1$ in the cardiovascular system and in T cells. Another, somewhat more potent scorpion toxin is maurotoxin (77), which unfortunately cross-reacts to $K_V1.2$. While these toxins are sometimes used *in vitro* to obtain a complete biophysical and pharmacological signature of $K_{Ca}3.1$, they have not been used as $K_{Ca}3.1$ blockers *in vivo*.

Small molecule $K_{Ca}2$ Channel Blockers

The key structural feature of selective $K_{Ca}2$ blockers is that they carry one or two positive centers, either permanently charged or strongly basic nitrogens, or in some cases acquire a charge by complexing divalent cations. All known $K_{Ca}2$ blockers work from the extracellular side and competitively displace radioactively labeled apamin in binding assays. The positive charges are reminiscences of the essential arginines of apamin (dark blue in the apamin structure in Figure 2). Intriguingly, this requirement for positive charges in the pharmacophore parallels classical anti-cholinergic drugs, many of which like *d*-tubocurarine and dequalinium (Figure 2), also block $K_{Ca}2$ channel (78; 79). Dequalinium was used as a starting point for several structure-activity-relationship studies focusing on both the nature of the permanently charged ring and the distance between the positive charges (80; 81), efforts which ultimately lead to the discovery of the high-affinity bis-quinolinium cyclophane $K_{Ca}2$ blockers UCL1684 and UCL1848 (Figure 2), which are as potent as apamin in blocking $K_{Ca}2.2$ (82; 83). Interestingly, the commonly used permanently charged derivative of the $GABA_A$ receptor antagonist bicucculine, bicucculine methiodide, also blocks $K_{Ca}2$ channels as potently as $GABA_A$ receptors (84).

These highly selective but permanently charged $K_{Ca}2$ blockers have been mostly used in academia, while there have only been modest activities in the pharmaceutical industry, probably due to the expected complications of permanently charged molecules, such as low permeability across biological membranes. Icagen published a patent on bis-benzimidazoles (USOO7482373B2), which block $K_{Ca}2$ channels and displace apamin. These molecules are not permanently charged, but form charged complexes with divalent cations, which probably constitute their active form. Bristol Myers Squibb published a series of 2-aminothiazoles, which also chelate divalent cations (85). Recently, the $K_{Ca}2$ channel blocking effect of 2,6-bis(2-benzimidazolyl)pyridine (BBP) (Figure 2), a molecule belonging to this class (86), has been shown to depend on H491 in the extracellular S5-P linker, the same histidine which is also a determinant for apamin binding (69). Despite these quite intriguing approaches, no $K_{Ca}2$ blockers have yet entered clinical development. However, due to their potency and good selectivity several of these molecules have been extremely valuable tools for elucidating the role of $K_{Ca}2$ channels in cardiac arrhythmias (87; 88) or the endothelium derived hyperpolarization response (89).

Small molecule $K_{Ca}3.1$ Blockers and their Preclinical and Clinical Applications

Apart from low affinity inhibitors like the β-blocker cetiedil (90), a compound that been reported to affect erythrocyte K^+ fluxes in 1981 (90) and therefore inspired some early medicinal chemistry (67; 91), two compound classes spurred a real interest in finding therapeutically useful small molecule $K_{Ca}3.1$ inhibitors in the late 1990s: Dihydropyridines (92; 93) and triaryl-methanes (94). Both pharmacophores had previously been successfully developed as L-type Ca^{2+} channel antagonists for hypertension and as P450 inhibitors for topical antifungals. Following the cloning of $K_{Ca}3.1$, scientist at Bayer worked on dihydropyridines (95; 96), a lead optimization resulting in a series of very potent and selective phenyl-pyrans and cyclohexadienes (Figure 3) that showed *in vivo* efficacy in animal models of traumatic brain injury (97), but never entered clinical development for undisclosed reasons. Following up on work performed by the group of Carlo Brugnara at

Harvard showing that clotrimazole reduced erythrocyte dehydration and exerted anti-sickling effects in transgenic mice and in patients with sickle cell disease (98; 99), Icagen pursued the triaryl-methanes and developed the clotrimazole derivative senicapoc. In parallel, one of us used clotrimazole as a template for the design of TRAM-34 (100). In both TRAM-34 and senicapoc (Figure 3) clotrimazole's toxic effect on the human P450 system have been avoided by substitution of the imidazole ring with either a pyrazole ring or an amide group. While TRAM-34 was not suitable for development, it has become a widely used academic tool compound based on its selectivity and its acceptable pharmacokinetic properties when administered intraperitoneally. For example, TRAM-34 has been utilized to validate $K_{Ca}3.1$ as a potential target for vascular restenosis (50), atherosclerosis (37), asthma (101), allograft vasculopathy (102), inflammatory bowel disease (45), ischemic stroke (40), as well as renal (103; 104) and cardiac fibrosis (105). In many of these disease models the pathophysiological relevance of $K_{Ca}3.1$ was confirmed by parallel experiments in $K_{Ca}3.1^{-/-}$ mice.

Senicapoc, which is orally available and has a long 12-day half-live in humans, entered clinical trials for sickle cell anemia but despite showing good effects on several haematological parameters, it unfortunately failed to meet the predefined primary endpoint, which was reduction in the number of painful crisis, in phase-III clinical studies (106). Based on senicapoc's efficacy in an asthma model in sheep (107), Icagen subsequently tested senicapoc in two small Phase-II trials for asthma (108) and demonstrated encouraging results in allergic asthma. However, senicapoc did not improve lung function in exercise induced asthma. Following these failures Icagen was purchased by Pfizer and senicapoc deposited in the 2012/13 NIH National Center for Advancing Translational Research (NCAT) library as PF-05416266 making it theoretically available for investigator initiated clinical trials. Senicapoc is currently being "repurposed" by the Pfizer spin-out SpringWorks Therapeutics for the hemolytic anemia disease hereditary xerocytosis, a rare condition caused by gain of function mutations in $K_{Ca}3.1$ (109). Another "repurposing" Phase-IIa clinical trial with senicapoc will be conducted by the Alzheimer's Disease Center at the University of California, Davis. This trial, which is anticipated to start in Fall of 2019, is based on findings that the expression of $K_{Ca}3.1$ is increased on microglia in brains from patients with Alzheimer's disease (AD) and that $K_{Ca}3.1$ inhibition with senicapoc reduces inflammation and amyloid-β deposition in mouse models of AD (110).

More recently, several new classes of $K_{Ca}3.1$ inhibitors were reported by the pharmaceutical industry. Using a so-called "scaffold hopping" approach (e.g. tetrazole derivatives, US9556132B2) and high-throughput thallium-flux (111) NeuroSearch, a biopharmaceutical company in Denmark, identified a completely new series of benzothiazinone-based $K_{Ca}3.1$ blockers in 2013. One of the exemplary compounds, NS6180 (Figure 3), showed efficacy in an animal model of inflammatory bowel disease despite low *in vivo* exposure (112). Boehringer Ingelheim pursued the closely related fused thiazine-3-ones (US 2015/0232484) and Roche published a patent on 3,4-disubstituted oxazolidinones (WO 2014/067861), which constitute an interesting variation on the triaryl-methane motive (Fig. 3). Nothing specifically was reported on therapeutic indications by the two companies, but a paper co-authored by Boehringer Ingelheim scientists focused on the role of $K_{Ca}3.1$ in the process

of multinucleation of macrophages and osteoclasts (113) suggesting a possible focus on chronic inflammation or bone-diseases.

Atomistic Mechanism of Action of $K_{Ca}3.1$ Blockers

Overall, the various compound classes (Figure 3), which have been reported as potent and selective $K_{Ca}3.1$ inhibitors during the last 20 years, are quite remarkable for their chemical diversity. However, on closer inspection a unifying characteristic is the absence of acidic or basic moieties and the presence of two or usually three substituted aryl groups making most of these compounds quite “greasy”, insoluble and prone to suboptimal pharmaceutical properties, such as high plasma protein binding. Remarkably, the majority of the $K_{Ca}3.1$ blockers shown in Figure 3 are binding to the same site in the inner pore of $K_{Ca}3.1$, just below the selectivity filter. While this canonical site is often touted as not suitable for obtaining subtype selective inhibitors, this seems to be possible for $K_{Ca}3.1$, because it is “alone” in its family. Indeed, none of the currently known $K_{Ca}3.1$ blockers, cross-react to $K_{Ca}2$ channels and the compounds typically also exhibit between 200–1000-fold selectivity over other ion channels.

The triaryl-methane type $K_{Ca}3.1$ blockers clotrimzole, TRAM-34 and senicapoc interact with threonine 250 in the pore loop and valine 275 in S6 as demonstrated by the fact that mutations of these residues completely abolish the sensitivity of $K_{Ca}3.1$ to triaryl-methanes (114). Based on a study using the Rosetta molecular modeling suite (115), TRAM-34 anchors itself through hydrophobic interactions with the V275 residues from all four subunits and forms a hydrogen bond to the T250 side chain from one subunit with its pyrazole nitrogen and thus blocks ion conduction by filling the site that would normally be occupied by a K^+ ion before it enters the selectivity filter. Senicapoc is assuming a similar binding pose (Figure 3) but instead of acting as a hydrogen-bond acceptor like TRAM-34, its amide group functions as a hydrogen-bond donor and interacts with T250 side chains from two subunits (115). Interestingly, the same two mutations that “knock off” triaryl-methane binding, also drastically reduce the affinity of the benzothiazinone NS6180 (112). However, in contrast to TRAM-34 and senicapoc, which are positioned directly under the selectivity filter and interact with all four subunits, NS6180 interacts with the T250 and V275 side chains from only two adjacent subunits (115). However, although NS6180 is “sitting” differently is still overlaps with the pore lumen potassium site (116) and thus seems to act by preventing ion permeation.

In contrast to the triaryl-methanes, the binding site of the dihydropyridine nifedipine has been localized to the fenestration region of $K_{Ca}3.1$ (Figure 3), where it binds between the side-chains of T212 in S5 and V272 in S6 from adjacent subunits and has been suggested to stabilize the channel in a non-conducting conformation without directly occluding the pore (115). While this fenestration binding site constitutes a very attractive alternative to the pore site for future design efforts directed towards the identification of $K_{Ca}3.1$ inhibitors with improved pharmaceutical properties, a completely unexpected and somewhat “shocking” observation from our group was that the nifedipine isosteric 4-phenyl-pyran (Figure 3), which had been initially described by Bayer (95) and which we resynthesized (115), is binding in the inner pore at the triaryl-methane site and not in

the fenestration like its template nifedipine. As explained in detail elsewhere, this finding is consistent with the published structure-activity-relationship of the phenyl-pyrans (95) and the related carba-analogous cyclohexadienes (96). Medicinal chemists generally assume when making isosteric replacements to improve potency and selectivity that the template and the derivatives bind to the same site, which is clearly not the case here and a caution against making assumptions that are not experimentally tested.

Negative Gating Modulators

Negative gating modulation as applied to $K_{Ca}2$ channels means an inhibitor that shifts the calcium-activation curve towards higher Ca^{2+} concentrations (in contrast to the left-shifting by positive gating modulators), thereby reducing the apparent Ca^{2+}-sensitivity of the channel. The first molecule in this functional class was the aminobenzimidazolone NS8593 (117) and its analogues (118) which show high selectivity for $K_{Ca}2$ channels compared to $K_{Ca}3.1$. In contrast to most $K_{Ca}2$ blockers, this class of molecules is uncharged at physiological pH and therefore more likely to pass biological barriers. NS8593 does not displace radiolabeled apamin and its activity is not reduced by mutations of the extracellular amino acid residues mediating sensitivity to apamin and the small molecule $K_{Ca}2$ blockers (see above). Instead, the effect of NS8593 was shown through site directed mutagenesis to depend on the same amino acid positions in the pore region of $K_{Ca}2$ (Figure 2) that mediate sensitivity of $K_{Ca}3.1$ to TRAM-34 (119) and introduction of just two mutations into $K_{Ca}3.1$ could render this normally insensitive channel highly sensitive to NS8593 (119). A closer inspection of the binding pose, however, suggests, that unlike TRAM-34, NS8593 is not completely obstructing the permeation pathway. The fact that gating modulation is possible at this position was hypothesized to be a pharmacological reflection of the previously suggested deep pore gating in $K_{Ca}2$ channels (120). The basic characteristics of negative modulators including their pore binding site have recently been confirmed by using the drug candidate AP14145 (Figure 2) from Acesion Pharma, which belongs to the same general class of molecules as NS8593 (121). Interestingly, scientists from Bristol Myers Squibb published another series of molecules, 4-(aminomethylaryl)-pyrrazolopyrimidines, which inhibit $K_{Ca}2$ mediated Tl^{+} -fluxes at a site not involving the apamin site (122). Although, not rigorously shown in the paper, these compounds may also act via a negative gating modulatory mechanism. While the compounds described so far do not differentiate among the three $K_{Ca}2$ subtypes, subtype selective negative gating modulation was demonstrated for the triazolopyrimidine (−)-B-TPMF (Figure 2), which preferentially inhibited $K_{Ca}2.1$ over the other $K_{Ca}2$ members and $K_{Ca}3.1$, by interaction with Ser293 in $K_{Ca}2.1$, a position not previously identified for modulators of $K_{Ca}2$ (123). Notably, as described later, this site on $K_{Ca}2.1$ can also give rise to positive modulation. Negative gating modulation has recently also been shown to account for the effects of certain dibenzoates, such as RA2 (1,3-phenylenebis(methylene)bis(3-fluoro-4-hydroxybenzoate), which inhibit both $K_{Ca}2.x$ and $K_{Ca}3.1$ channels with similar potency (124), demonstrating that this mode of action is also possible for $K_{Ca}3.1$. Although its binding site has never been mapped, the inner vestibule of both $K_{Ca}3.1$ and the $K_{Ca}2$ channels is large enough to accommodate the molecule.

Based on proof-of-concept animal studies demonstrating that NS8593 or AP14145 can terminate atrial fibrillation in rats (125; 126) or even large animals such as pigs (127),

negative gating modulators have successfully progressed into clinical development for atrial arrhythmia. According to a press release from Acesion Pharma, a putative analogue of NS8593/AP14145 called AP30663 has recently passed Phase-I clinical trials in human volunteers.

Positive Gating Modulators

Most $K_{Ca}2$ and $K_{Ca}3.1$ activators are “clean” positive gating modulators meaning that they shift the calcium-activation curve concentration-dependently towards lower intracellular Ca^{2+}-concentrations, thereby increasing the apparent Ca^{2+} affinity, but are unable to activate the channels at “0” intracellular Ca^{2+}. However, there seem to be exceptions to this simple mechanism in that the existence to “true” activators (123) and potentially superagonists (128) has been suggested. The prototype activators of $K_{Ca}3.1$ and $K_{Ca}2$ channels are the benzimidazolone 1-EBIO (129) and its more potent derivative dichloro-EBIO (130), which have both played a significant role as pharmacological *ex vivo* tool compounds in brain slices, endothelia and epithelia, or smooth muscle preparations. However, several drugs that have been on the market for decades, such as the muscle relaxant chlorzoxazone and the ALS drug riluzole are also quite effective K_{Ca} activators (67; 131), which may well be their major therapeutic action. Dedicated search for more potent and selective activators led to NS309 (132), one of the most potent “pan”- $K_{Ca}3.1/K_{Ca}2$ activators and an important mechanistic tool compound, but not suited for *in vivo* studies, due to poor pharmacokinetic properties. With the aim of making more selective and potent riluzole-like compounds that could potentially be used *in vivo*, Sankaranarayanan *et al.* (133) identified a series of benzothiazoles including SKA-31 and SKA-121, which has improved selectivity for $K_{Ca}3.1$ and which has been used to demonstrate that selective $K_{Ca}3.1$ activation can lower blood pressure in mice (134).

Also pursuing subtype selectivity, NeuroSearch scientists discovered cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA) and the more potent analogue (4-chloro-phenyl)-[2-(3,5-dimethyl-pyrazol-1-yl)-9-methyl-9*H*-purin-6-yl]-amine) (NS13001), which activates $K_{Ca}2.3$ and $K_{Ca}2.2$ while being inactive on $K_{Ca}2.1$ and $K_{Ca}3.1$ (75; 135). A different selectivity profile was exemplified by CM-TPMF, which preferentially activates $K_{Ca}2.1$ (123) and like the negative gating modulator (−)-B-TPMF depends on a serine residue in S5 for its activity (Figure 2). These subtype selective compounds formed the basis for a collaboration between Saniona, a company continuing the research assets of NeuroSearch, and Ataxion (now Cadent Therapeutics), which finally resulted in selection of CAD-1883 for preclinical development for cerebellar dysfunction. According to the Cadent Therapeutics homepage, CAD-1883 is now in phase-II for essential tremor and for spinocerebellar ataxia. The idea of using $K_{Ca}2$ activators for these indications is based on the observations that genetic silencing of $K_{Ca}2$ channels in deep cerebellar neurons induces ataxia in mice (136), while treatment of mice with spinocerebellar ataxia type-2 (SCA2) with the $K_{Ca}2$ activator NS13001 alleviates motor symptoms and prevents neurodegeneration of Purkinje cells (75).

Atomistic Mechanism of K_{Ca} Channel Positive Gating Modulators

Mutational studies performed by Pedarzani *et al.* suggested in 2001 that the binding site of the benzimodazolone-type K_{Ca} activators is probably located in the C-terminus, close to or within the CaM-BD, since "swapping" the C-terminus of $K_{Ca}3.1$ into $K_{Ca}2.2$ made this channel as 1-EBIO sensitive as $K_{Ca}3.1$ (137). So, when 1-EBIO, NS309 and riluzole were later reported to bind at the interface between the CaM N-lobe and the $K_{Ca}2.2$ CaM-BD after being soaked into the above described C-terminal dimeric crystal using x-ray crystallography or in solution state NMR (59–62), this interface was widely assumed to be the binding site of this type of K_{Ca} activators in both $K_{Ca}2$ and $K_{Ca}3.1$ channels (62; 138). However, the recently published full-length cryo-EM structure of $K_{Ca}3.1$ demonstrated that the analogous segment of the $K_{Ca}3.1$ C-terminus, which is designated the C-terminal HC helix, actually forms a coiled coil at the center of the channel (56) and therefore is unlikely to constitute the binding site. In their study the MacKinnon group also proposed that the "real" binding pocket of 1-EBIO is located in the interface between the $S_{45}A$ helix and the CaM N-lobe, in which 1-EBIO is hypothesized to contact L185 in the $S_{45}A$ linker (56) instead of L480 in the C-terminal crystal complex (59), but did not experimentally test this very plausible alternative binding site hypothesis. Our own group recently picked up on this postulate and confirmed through mutagenesis that at least the SKA-type K_{Ca} channel activators as exemplified by SKA-111 (5-methylnaphtho[1,2-*d*]thiazol-2-amine) are binding in the interface between the CaM N-lobe and the $S_{45}A$ helix (139). In this interface pocket Rosetta modeling shows that SKA-111 makes van der Waals contacts with S181 and L185 in the $S_{45}A$ helix of $K_{Ca}3.1$ but interacts with the same CaM N-lobe residues (M51, E54, and M71) that were previously shown to be involved in binding of the aminothiazole riluzole in the C-terminal crystal dimer (62).

We here show SKA-111 docked into the $S_{45}A$ helix/CaM N-lobe interface of both $K_{Ca}2.2$ (Figure 2) and $K_{Ca}3.1$ (Figure 3), fully recognizing that the $K_{Ca}2.2$ binding pose is currently not supported by experimental data. Based on the high sequence similarity in the $S_{45}A$ helix between $K_{Ca}3.1$ and the three $K_{Ca}2$ channels, we would expect the CaM mediated gating and the putative stabilization of the interaction between the CaM N-lobe and the $S_{45}A$ helix by benzothiazole-type K_{Ca} activators to be similar, even if there are some sequence differences between $K_{Ca}3.1$ and $K_{Ca}2$ channels. However, in addition to the $S_{45}A$ helix/CaM N-lobe interface, which is present four times in the $K_{Ca}3.1$ channel, there are certainly more sites on the cytoplasmic surface of K_{Ca} channels that could accommodate small molecules and it is feasible that NS309 or the $K_{Ca}2.2/2.3$ selective CyPPA and NS13001 are binding at other sites or occupy a different number of sites. Gating modulation is also possible in the transmembrane domain as has been demonstrated by the fact, that a serine residue in S5 is crucial for the action of both the $K_{Ca}2.1$ selective positive gating modulator CM-TMPF and the negative modulator (−)-B-TPMF (123).

OUTLOOK

As described here, $K_{Ca}2/3$ channels have a relatively well-developed pharmacology and their therapeutic targeting for neurological and cardiovascular diseases is supported by ample preclinical data. The most advanced compound, the $K_{Ca}3.1$ blocker senicapoc

unfortunately failed in a phase-III clinical trial in sickle cell anemia (106). However, the trial certainly demonstrated that $K_{Ca}3.1$ inhibition is safe in humans, and, as described above, senicapoc is currently in the process of being repurposed for the treatment of hereditary xerocytosis and Alzheimer's disease. For Alzheimer's disease the therapeutic hypothesis is that $K_{Ca}3.1$ inhibition would reduce neuroinflammation by suppressing microglia activation (140). The same hypothesis is used to rationalize repurposing senicapoc for stroke (141) and neuropathic pain (142). Other indications for which repurposing of senicapoc might be worthwhile considering is idiopathic pulmonary fibrosis (143) and glioblastoma (144). While repurposing is an attractive short cut, senicapoc due to its high lipophilicity, low solubility, high plasma protein binding and very long half-live in humans, is not necessarily an "ideal" $K_{Ca}3.1$ blocker and it will be interesting to see if the now available full-length $K_{Ca}3.1$ structure (56) will revive the interest of the pharmaceutical industry in developing better $K_{Ca}3.1$ inhibitors.

The other K_{Ca} modulators that have recently entered clinical trials, the yet undisclosed negative $K_{Ca}2$ channel gating modulator AP30663 for atrial fibrillation and the positive $K_{Ca}2$ channel modulator CAD-1883 for cerebellar disorders, have passed phase-I and are currently being tested in patients, which demonstrates the feasibility of balancing benefits and side effects of $K_{Ca}2$ channel modulation by optimizing compound properties. AP30663 is intentionally made peripherally restricted presumably by increasing its polarity or by introducing structural elements favoring its extrusion across the blood brain barrier, thereby strongly reducing liability for inducing tremors and seizures that are observed with the brain-penetrant NS8593 in animal studies (121). In contrast, CAD-1883, which is designed for targeting $K_{Ca}2$ channels in pacemaker neurons of the cerebellar cortex and deep cerebellar nuclei (primarily $K_{Ca}2.2$), is optimized for selectivity and good brain exposure. Similarly, for positive gating modulators targeting peripheral diseases, it will be important to achieve subtype and ideally tissue selectivity. Unselective activators like SKA-31 are useful tool compounds but quickly demonstrated that central $K_{Ca}2$ channel mediated sedation and heart rate reduction (145) constitute undesirable side-effects when attempting to target endothelial $K_{Ca}3.1$ channels to lower blood pressure, even if the approach is effective in large animals such as dogs (146) and pigs (147). A useful $K_{Ca}3.1$ activator for improving endothelial function in hypertension and other cardiovascular disease should therefore ideally be $K_{Ca}3.1$ selective and peripherally restricted.

The very significant advances that have recently been made in elucidation the structure of $K_{Ca}3.1$ (56) and the resulting improvements in modeling have so far not been used for drug design. All current drug candidates in the K_{Ca} channel field have been identified by screening or classical medicinal chemistry approaches. However, as more high-resolution protein structures will become available, ideally with K_{Ca} channel modulators differing in structure, mode of action, and selectivity positioned at their respective pharmacological sites, there is no doubt that this information will be of increasing importance in future drug optimization programs. Immediate questions to solve are, for example, how to explain the already obtained $K_{Ca}2.3/K_{Ca}2.2$ versus $K_{Ca}2.1/K_{Ca}3.1$ selectivity of compounds in the CyPPA/NS13001 series at the atomistic level. Another item on the "wish-list" of the pharmacologist and the drug developer is to gain insights into how to design selective blockers or negative gating modulators for $K_{Ca}2.3$, an important channel in all

monoaminergic neurons, which could be an important step in developing new drugs for psychiatric diseases. While we believe that $K_{Ca}2.2$ or $K_{Ca}2.2/K_{Ca}2.3$ selective activators certainly are also promising for the treatment of dependence on alcohol (148) and other habit forming substances (149), it might be challenging to ever safely translate the beneficial effects of $K_{Ca}2.2$ inhibition on learning and memory into the clinic.

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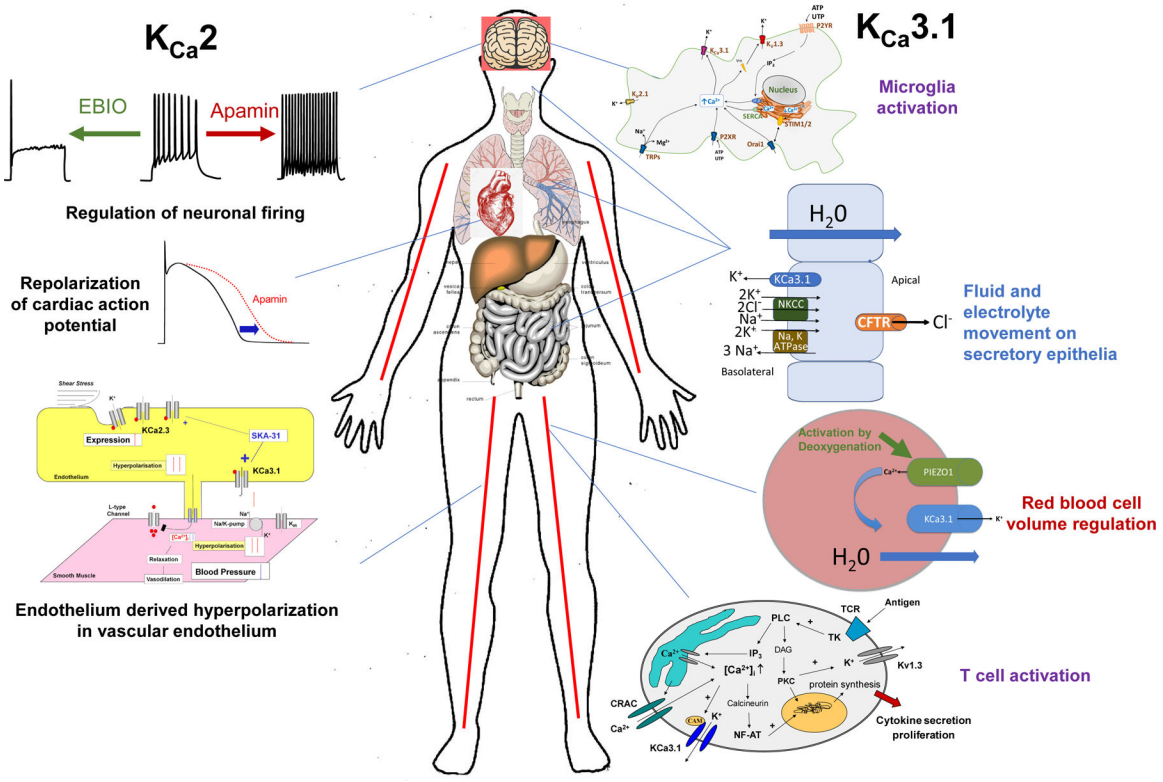

Figure 1.
Physiological role of $K_{Ca}2$ and $K_{Ca}3.1$ channels.

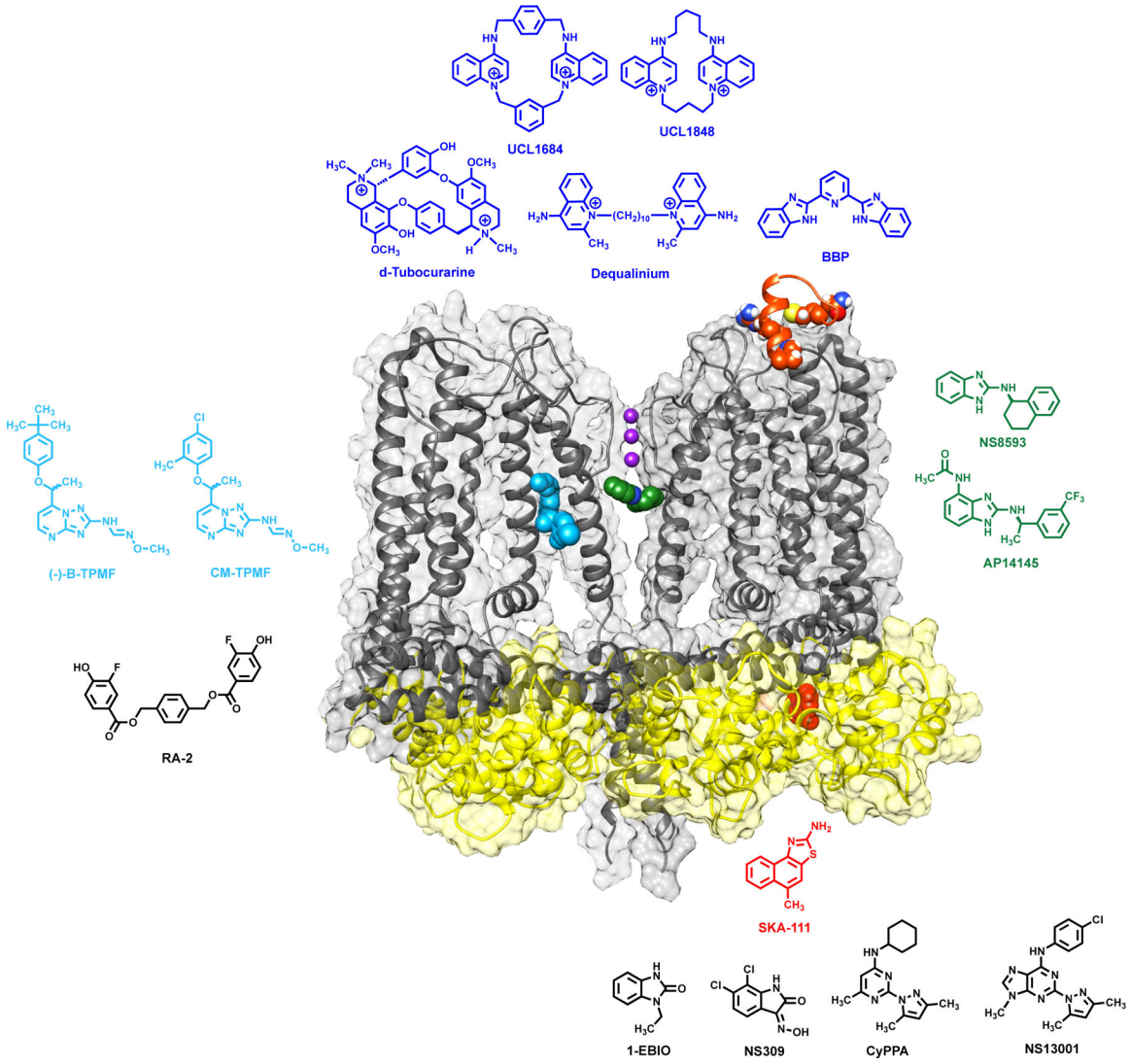

Figure 2.

Rosetta $K_{Ca}2.2$ homology model based on the $K_{Ca}3.1$ cryo-EM structure (open state 1, pdb: 6cnn). The longer N- and C-terminus of $K_{Ca}2.2$ was not modeled. For clarity only two of the four channel subunits are shown (dark gray). Calmodulin is shown in yellow. Potassium ions in the selectivity filter are colored dark purple. The bee venom apamin with its N-atoms colored dark blue and several small molecule modulators are docked where they have been shown to bind by mutagenesis: Apamin in the outer pore, NS8593 in the inner pore (dark green), CM-TMPF (sky blue) in the inner vestibule, and SKA-111 in the interface between the CaM N-lobe and the $S_{45}A$ helix in the S4-S5 linker. The chemical structures of other $K_{Ca}2$ blockers, negative gating modulators and activators are colored according to where they have either been shown to bind by mutagenesis or are suspected to bind.

Potencies (IC_{50}s for blockers and negative gating modulators; EC_{50}s for activators): Apamin 60–400 pM, d-tubocurarine 5 μM, dequalinium 200 nM, UCL1684 200 pM, UCL1884 110 pM, BBP 400 nM, NS8593 600 nM, AP14145 1 μM, (–)-B-TMPF 31 nM for $K_{Ca}2.1$ and

1 μM for $K_{Ca}2.2$, CM-TMPF 24 nM for $K_{Ca}2.1$ and 290 nM for $K_{Ca}2.2$, SKA-111 8 μM, RA-2 ~100 nM, SKA-31 2 μM, NS309 620 nM, CyPPA 14 μM, NS13001 2 μM.

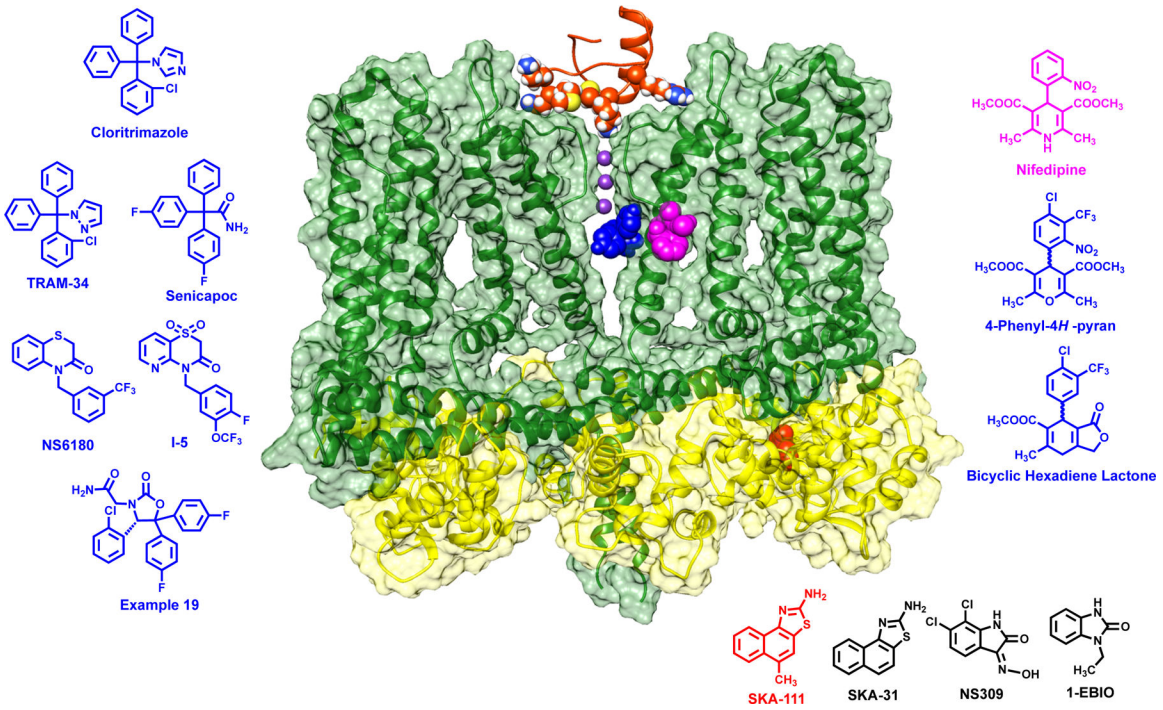

Figure 3.

Rosetta refined model of the $K_{Ca}3.1$ cryo-EM structure (open state 1, pdb: 6cnn). For clarity only two of the four channel subunits are shown (dark green). Calmodulin is shown in yellow. Potassium ions in the selectivity filter are colored dark purple. The scorpion toxin charybdotoxin (ChTX) is shown docked into the outer vestibule. Various small molecule modulators are docked were they have been shown to bind by mutagenesis: Senicapoc as a representative triaryl-methane in the inner pore (blue), nifedipine in the fenestration region (pink), and SKA-111 in the interface between the CaM N-lobe and the $S_{45}A$ helix in the S4-S5 linker. The chemical structures of other $K_{Ca}3.1$ blockers and activators are colored according to where they have either been shown to bind by mutagenesis or are suspected to bind.

Potencies (IC50s for blockers; EC50s for activators): ChTX 2–28 nM, clotrimazole 70–250 nM, TRAM-34 10–25 nM, senicapoc 11 nM, NS6180 11 nM, nifedipine 0.8–4 μM, 4-phenyl-4*H*-pyran 8 nM, SKA-111 150 nM, SKA-31 250 nM, NS309 10–30 nM, 1-EBIO 24–80 μM.