Current Topics

Chemical Tools for K^+ Channel Biology[†]

Christopher A. Ahern*[,‡] and William R. Kobertz*[,§]

Departments of Anesthesiology, Pharmacology and Therapeutics, and Cellular and Physiological Sciences, University of British Columbia, 2350 Health Science Mall, Vancouver, BC V6T 1Z3, Canada, and Department of Biochemistry and Molecular Pharmacology, Programs in Neuroscience and Chemical Biology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605-2324

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ABSTRACT: K^+ channels are revered for their universal action of suppressing electrical activity in nerve and muscle, as well as regulating salt and water transport in epithelial tissues involved in metabolism and digestion. These multisubunit membrane-embedded proteins carry out their physiological chore, selectively allowing the passage of potassium across the membrane, in response to changes in membrane voltage and ligand concentration. Elucidating the diverse gating properties of K^+ channels is of great biological interest since their molecular motions provide insight into how these structurally similar proteins function in a wide variety of tissues. Armed with patch clamps, chart recorders, and now high-resolution structures, electrophysiologists have been dipping into the top tray of the chemist's tool box: synthesizing cysteine-modifying agents and organic cations and grinding up insects, spiders, and other vermin to isolate natural products to poke, probe, and prod K^+ channels. Recently, there has been further cross-fertilization between chemists and K^+ channelologists, resulting in greater accessibility to more elaborate synthetic methodologies and screening approaches. In this review, we catalogue the evolution of chemical tools and approaches that have been utilized to elucidate the mechanistic underpinnings of K^+ channel biology.

NUTS AND BOLTS OF K^+ CHANNEL SUBUNITS

K^+ channels function as multiprotein complexes made up of conducting α-subunits and regulatory *β*-subunits. The α-subunits house the pore-forming domain that tetramerizes to form a K^+ selective aqueous pore, which is structurally conserved between the different families of K^+ channels (Figure 1A) (*1*). The inward rectifier family of K^+ channels (Kir)[1] has the simplest α-subunit design: two transmembrane (TM) helices (M1 and M2) connected by an reentrant pore loop that contains the K^+ selectivity sequence (*2*). The opening and closing of Kir channels is regulated by cytoplasmic ligands, including nucleotides, phosphatidylinositols, G-proteins, linear polyamines, and divalent cations (*3*). Accordingly, the cytoplasmic pore of these channels continues substantially beyond the lipid membrane (Figure 1A) (*4, 5*). The ATP-sensitive versions of inward rectifiers (Kir6.1 and Kir6.2) form obligatory membrane-embedded complexes with the sulfonylurea receptors (SUR1 and SUR2) in a 4:4 (α:*β*) stoichiometry (*6*). These complexes act as molecular sensors of cellular metabolism and are critical players in insulin secretion (*7*).

Voltage-gated K^+ channels (Kv-type) are involved in electrical excitability and accordingly are "gated"; that is, they open, close, and inactivate in response to changes in transmembrane voltage. Therefore, Kv α-subunits possess an extra module, the voltage-sensing domain, which is comprised of four TM segments (S1−S4) that are appended to the pore-forming domain (S5−P−S6) (Figure 1B). From the resting state, voltage sensors respond to membrane depolarization by shuttling positively charged arginine and lysine residues on the S4 segment through the electric field, with some charges traveling entirely from the cytoplasmic to the extracellular space (*8*). The absolute distance covered by these charges need not be substantial because the electric field is highly focused (∼5 Å) in the vicinity of the voltage sensor (*9*). Voltage sensor movement is coupled to the activation gate in the S6 segment via the S4−S5 linker, although the molecular interplay between these three K^+ channel parts is still being investigated (*10*). After channel

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* To whom correspondence should be addressed. C.A.A.: Departments of Anesthesiology, Pharmacology and Therapeutics, and Cellular and Physiological Sciences, University of British Columbia, 2350 Health Science Mall, Vancouver, BC V6T 1Z3, Canada; phone, (604) 827-4393; fax, (604) 822-2316; e-mail, cahern@interchange.ubc.ca. W.R.K.: Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St., Worcester, MA 01605-2324; phone, (508) 856-8861; fax, (508) 856-8867; e-mail, william.kobertz@umassmed.edu.

[‡] University of British Columbia.

[§] University of Massachusetts Medical School.

[1] Abbreviations: Kir, inward rectifier K^+ channel; Kv, voltage-gated K^+ channel; UAA, unnatural amino acid; TEA, tetraethylammonium; QA, quaternary ammonium; TM, transmembrane domain.

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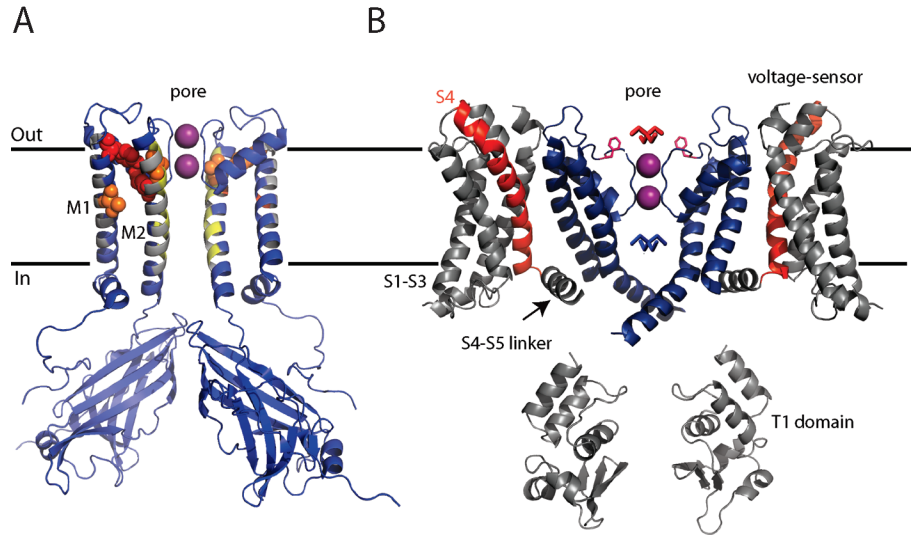

FIGURE 1: Structural models of inward rectifier Kir and voltage-gated Kv K^+ channels. High-resolution structures of the (A) Kirbac3.1 and (B) Kv2.1 paddle chimera K^+ channels (Protein Data Bank entries 1X6L and 2R9R). For the sake of clarity, the front and back subunits have been removed. For Kirbac3.1, the gray and yellow colors demark the lipid- and pore-facing residues determined by a mutagenic yeast screen; orange and red space-fill atoms are the pairs of intrasubunit interacting residues determined by second-site suppressor mutants. For Kv2.1, red identifies the positively charged S4 helix and pink residues have been morphed into aromatic groups to frame the extracellular tetraethylammonium (TEA) binding site. The red TEA molecule shows the extracellular binding site and the blue TEA molecule the internal binding site. Purple spheres denote K^+ ions in the selectivity filter for both structures.

activation, the flow of potassium through the channel is shut off through two distinct mechanisms to regulate the repolarizing phase of the action potential (*11*). Fast inactivation, also known as N-type, occurs on a millisecond time scale and is carried out by a "ball and chain" mechanism where a positively charged segment of the cytoplasmic N-terminus of the channel snakes through the open gate and into the inner vestibule where it binds and blocks the channel (*12, 13*). Alternatively, sustained depolarization can cause "slow" or C-type inactivation through a localized constriction or collapse in the vicinity of the selectivity filter (*14, 15*). The Kv channel structure (Figure 1B) shows the channel with the cytoplasmic gate open and the voltage sensor in the active state, i.e., with their charged Arg and Lys residues on the extracellular side of the membrane. Recent discoveries have shown that the voltage-sensing domain is truly an autonomous module, able to regulate enzymatic activity, proton flux, and cation movement across the membrane in a voltage-dependent manner (*16, 17*).

For Kv channels, a plethora of regulatory β-subunits have been identified (*18*), and for the sake of conciseness, we will cover only the regulatory domains examined by chemical approaches. Many Kv channels assemble with the five different KCNE type I transmembrane peptides, forming membrane-embedded complexes with varied voltage gating properties (*19*). Coassembly between Kv α-subunits and KCNE β-subunits provides the voltage sensing diversity to permit the same K^+ channel to function in a wide variety of tissue types (*20*−*23*). Results from the KCNQ1−KCNE1 K^+ channel complex demonstrate that only two KCNE peptides assemble with four K^+-conducting subunits, leading to complexes that are not 4-fold symmetric (*24, 25*). In addition to transmembrane β-subunits, Kv channels also associate with water soluble accessory Kvβ subunits (β1−β3), a family of cytoplasmic proteins that dock onto the tetramerization domain (T1) domain of Kv channels with a 4:4 α:β stoichiometry (*26*). Similar to the membrane-embedded KCNE β-subunits, Kvβ subunits modulate the voltage sensitivity, inactivation rates, and cell surface expression of K^+ channel α-subunits (*27*). Moreover, Kvβ subunits possess an aldo-keto reductase domain, which may link the metabolic state of the cell to electrical excitability (*28*).

Many of the nuts and bolts of K^+ channels have been ascertained using chemical probes in combination with electrical recordings. Here we briefly review some of the lessons learned from the original chemical probes, the quaternary ammoniums (QAs), and then progress to tethering reagents, unnatural amino acid incorporation, and screening technologies that have become a mainstay in K^+ channel structure−function investigations.

THE PLUNGERS: QUATERNARY AMMONIUMS

K^+ channels select for their namesake ion over other cations by creating a proteinaceous environment that attempts to replicate the hydrated state of potassium in solution (*29, 30*). In doing so, the selectivity filter of K^+ channels reduces the energetic penalty paid by the naked potassium ion, as it streaks through the bilayer while rejecting ions reluctant to fully dehydrate. This simple test allows for the selection of potassium over sodium, two featureless cations that differ by less than half an angstrom in diameter, by roughly 100 to 1. K^+ channel blockers that successfully masquerade as their permeant cousins bind to sites near either entrance of the selectivity filter where they ultimately fail to pass due to steric and energetic mismatches. However, in failing to permeate, a blocker will modify ionic conductance or gating (or both) and, through biophysical analysis of these effects, will provide details about their binding site and often insight into channel function. Of the wide range of K^+ channel blockers, simple QAs have been and continue to be essential probes in the ongoing efforts to refine the understanding of K^+ channel structure−function relationships.

External application of the blocker tetraethylammonium (TEA) inhibits potassium current by occupying a site near the entrance to the selectivity filter, which is highlighted in

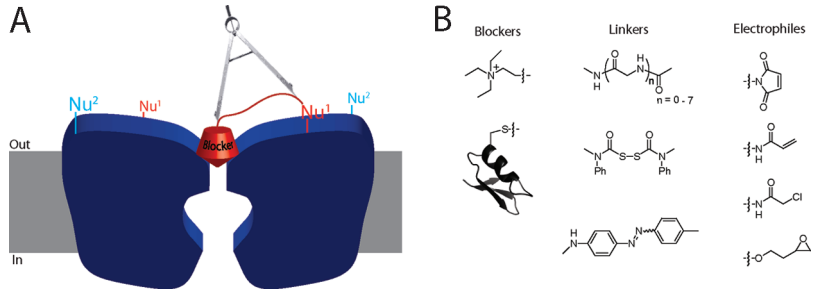

FIGURE 2: Tethered blockers as molecular calipers. (A) At low reagent concentrations, tethered blockers first bind to the channel pore and then react with the target nucleophile. The cartoon depicts nucleophile 1 (Nu^1) reacting with the electrophile on the tethered blocker, whereas the linker of tethered blocker is too short to permit reaction with nucleophile 2 (Nu^2). Upon modification with a tethered blocker, subsequent reactions with unreacted nucleophiles are inhibited by the covalently tethered blocker. (B) Chemical components of tethered blockers (listed from top to bottom). The blockers were tetraethylammonium (TEA) and charybdotoxin (CTX). The linkers were polyglycine (synthetic control of tether length), bis(*N*-phenylcarbamoyl)disulfane (can be cleaved by reductant), and azobenzene (can be controlled by light). The electrophiles were maleimide (thiol specific), acrylamide, chloroacetamide, and epoxide (nonspecific).

Figure 1B as pink side chains. TEA blockade is rapidly reversible and has little voltage dependence, consistent with its shallow binding site and modest affinity. However, relative high TEA affinity can be found, or produced through a single mutation, in channels that possess an aromatic phenylalanine or tyrosine residue in the blocker's binding site. This difference in affinity can be used to identify different channel isoforms in native tissues, and the simple transportability of high-affinity TEA binding by a single mutation suggests that diverse isoforms share significant structural homology. Furthermore, an electrostatic, cation$-\pi$ interaction between TEA and the four aromatic side chains forms the basis for high-affinity inhibition (*61*). Extracellular TEA, like permeant ions, can speed or slow the pore collapse associated with slow inactivation depending on the channel isoform and side chain present in the blocker site, suggesting that TEA is closely associated with the selectivity filter. Unfortunately, modest chemical alterations to TEA result in very poor channel blockers, limiting the potential use of compounds with longer or shorter branches to further explore channel$-$blocker interactions. However, the requirements for internal blockade of K^+ channels are more generic, expanding the potential for compounds with variable hydrophobicity and alkyl chain length. For instance, systematic lengthening of the alkyl chains produces a more hydrophobic blocker that has a substantially diffuse positive charge painted on its surface. These compounds exhibit enhanced block with added carbons until the length of the alkyl chain bars their entry into the inner vestibule, thus providing an estimate of the physical dimensions of the open channel while simultaneously demonstrating the hydrophobic nature of the binding site. Variance of a single alkyl chain can also be informative. QA derivatives with such additions show a trend of enhanced occupation of their binding site near the selectivity filter. Analysis of the blocker's prolonged dwell times demonstrates that the compounds inhibit potassium conductance by "starving" the selectivity filter of permeant ions, leading to a C-type inactivation-like structural collapse of this region (*31*). This particular insight into channel$-$blocker interactions stretches beyond the biophysical realm and has potential clinical ramifications. For instance, the distinction between a simple blocker and a drug that inhibits K^+ channel conductance by promoting or stabilizing nonconducting conformations of the channel has important mechanistic implications for both channel gating and the design of novel therapeutics. Further derivatization of QA compounds promises to expand the capabilities of these valuable probes of K^+ channel gating, permeability, and architecture.

CALIPERS: TETHERED BLOCKERS

Tethered blockers have three basic biophysical properties that are useful for the study of ion channels (Figure 2A). [1] *Affinity labeling*: Most tethered blockers modify ion channels as selective affinity labeling reagents, reversibly binding to the channel first, followed by covalent modification of a target nucleophile via the reagent's electrophile. [2] *Accelerated modification rates*: By binding to the channel through the inhibitor portion of a tethered blocker, chemical modification rates are directly proportional to linker length. Tethers that are too short to reach the target nucleophile do not enhance the bimolecular reaction, and the reaction rate depends solely on reagent concentration. In contrast, an ideal length tether can increase the local concentration of the electrophile into the millimolar range, greatly accelerating the reaction between tethered blocker and ion channel. In theory, the effective molarity is lower for tethers that are too long because the electrophile samples more three-dimensional space than is necessary to react with the nucleophile; however, in practice, the modification rates for these tethered blockers are also significantly enhanced. [3] *Intramolecular inhibition*: Modification of a channel with a tethered blocker creates a localized concentration of inhibitor near its site of attachment. If the inhibitor can reach its binding site, the tethered blocker acts as an intramolecular inhibitor whose concentration is dependent on linker length.

Tethered blocker design starts with the inhibitor or blocking portion of the molecule since this is the primary source of K^+ channel specificity (Figure 2B). Two different blocking moieties have been used for tethered blockers of K^+ channels: QAs and the peptide toxin, charybdotoxin. QAs are convenient blocking moieties since they inhibit a wide range of K^+ channels and are compatible with standard organic chemistry transformations. Because of their relatively low micro- to millimolar affinity for K^+ channels (*33*), tethered QAs are highly dependent on the effective concentration generated by the length of the linker. Therefore, QA-tethered blockers have been advantageous in experiments that require a strong dependence on linker length (*34*$-$*36*). In contrast to QA, charybdotoxin binds to K^+ channels with

nanomolar (or higher) affinity (*24, 37, 38*). Because the lifetime of charybdotoxin bound to the outer vestibule of a K^+ channel is on the order of tens of seconds, the electrophile on the backside of a charybdotoxin-tethered blocker typically reacts with its target nucleophile before the toxin moiety unbinds from the channel (*39*). Once covalently tethered to the channel, the charybdotoxin moiety prevents subsequent inhibitors from binding to the outer vestibule of the K^+ channel since its effective concentration is orders of magnitude above its dissociation constant (*25*). Consequently, intramolecular inhibition by charybdotoxin-tethered blockers is essentially insensitive to linker length. One practical disadvantage of charybdotoxin is the slow off rate of the untethered molecules, which take minutes to fully wash out. Although only two K^+ channel blockers have been used for tethered blockers, the modularity of the syntheses allows for the incorporation of many different K^+ and other ion channel inhibitors for investigation of a variety of ion channels.

Of the three components of a tethered blocker (reversible blocker, linker, and electrophile), the linker is the most synthetically tunable. Since both modification rates and irreversible block are highly dependent on linker length, distances in K^+ channels radiating from the inhibitor binding site can be measured using a library of different length tethered blockers. To synthesize different length tethered blockers as molecular tape measures, Blaustein and coworkers adapted solid-phase peptide synthesis to synthesize maleimido-QAs with glycines as spacer residues to incrementally lengthen the tether (*34*). Their solid-phase approach enabled the rapid synthesis of tethers that range from 20 to 45 Å in length (Figure 2B); however, the longer polyglycine linkers become increasingly harder to purify to homogeneity. Since the K^+ channel complex landscape grows larger with the discovery of additional regulatory subunits, the need for tethered blockers with longer, defined lengths may arise. By using a longer spacer unit (β-amino acids or Fmoc-protected amino acid polyethylene glycols, etc.), standard peptide synthesis would yield longer tethered blockers with fewer repeating units and ameliorate purification issues of the longer polyglycine linkers.

Although solid-phase peptide synthesis offers discrete control over the length of an individual linker, altering the linker length after the tethered blocker is covalently attached to the K^+ channel would afford control of K^+ permeation. Incorporation of an azobenzene moiety (Figure 2B) into the linker of a tethered blocker allows for this "on the fly" change of linker length. Long-wave UV light converts the azobenzene into predominately the *cis* isomer, shortening the linker by ~7 Å, whereas 500 nm visible light restores the linker to its original length in the *trans* conformation. By judicious attachment of a tethered blocker with an azobenzene linker, many ion channels can be turned on or off with the flip of a (light) switch. Consequently, the azobenzene linker has found widespread usage, originally pioneered by Lester and co-workers in activating the acetylcholine receptor (*40*), and more recently in activating ionotropic glutamate receptors, controlling K^+ channels in neurons, and blocking gramicidin channels (*41*−*43*).

Tethered blockers with orthogonally cleavable linkers provide another chemical approach to manipulating K^+ channel function. Chemical cleavability is routinely utilized in ion channel structure function studies with the substituted cysteine accessibility method (SCAM) (*44*) since the resultant disulfide bond made by methanethiolsulfonate (MTS) modifying reagents can be reversed with chemical reductants. Recently, Morin and Kobertz developed a maleimido-toxin-tethered blocker with a bis(*N*-phenylcarbamoyl)disulfane linker (Figure 2B) that can be readily cleaved with cell compatible concentrations of reductant (*25*). The advantage of this linker over a traditional disulfide bond is that chemical cleavage gives rise to secondary amines and does not regenerate thiol groups. This unique property of the linker allowed the KCNE β-subunits in functioning K^+ channels to be labeled and counted by iteratively applying and cleaving out the tethered blocker. Furthermore, cleavage of the linker leaves behind a small chemical remnant, allowing for the potential to affinity label K^+ channel subunits with two (or more) different biophysical probes. This sequential labeling strategy could find use in studies that require the exact placement of acceptor/donor or quencher pairs for resonance energy transfer experiments.

The electrophilic components of tethered blockers have been thiol specific modifying agents since cysteine mutagenesis has had a long, storied history with K^+ and other ion channels. Maleimide has been the workhorse electrophile since it forms an irreversible thioether bond and the reagent hydrolyzes relatively slowly. In contrast, MTS reagents are reversible by reductant, but the fast rates of thiol modification and hydrolysis make these electrophiles challenging to use in tethered blocker experiments. Recently, Kramer and colleagues have utilized less specific electrophiles (Figure 2B) in reactions with nucleophiles that natively reside on the surface of K^+ channels (*45*). Here, the rationale is that the specificity does not come from the electrophile, but from the tethering process itself. By taking advantage of affinity labeling, QAs linked to chloroacetamide, epoxide, or acrylamide through an azobenzene linker were used to selectively control K^+ conductance in transfected cells. Moreover, in untransfected isolated hippocampal neurons and cerebellar slices, the endogenous K^+ channels could be controlled by light using the acrylamide-based tethered blocker. The exploration of different electrophiles, in particular, ones that can modify nucleophiles in the hydrophobic confines of the membrane, would provide access to the protein surfaces of K^+ channels that have been previously protected by the lipid bilayer.

SOUPED-UP CHANNELS: UNNATURAL AMINO ACID INCORPORATION

Site-directed mutagenesis allows one to sample the available palette of 20 amino acids. This simple exchange will simultaneously impact the chemical attributes of the side chain (size, shape, hydrophobicity, etc.) and, with luck, alter a measurable attribute of the channel in an informative way (permeability, gating, drug block, etc.). Studies adopting this approach have been successful in the identification of amino acids that contribute to gating, voltage sensing, and selectivity, in addition to side chains that engage therapeutics, blockers, and toxins. Despite these significant gains, the method has its drawbacks. First, site-directed mutagenesis rarely gives insight into the role that the original side chain played, aside from the fact that it did contribute in some way, because it is difficult to isolate the chemical property

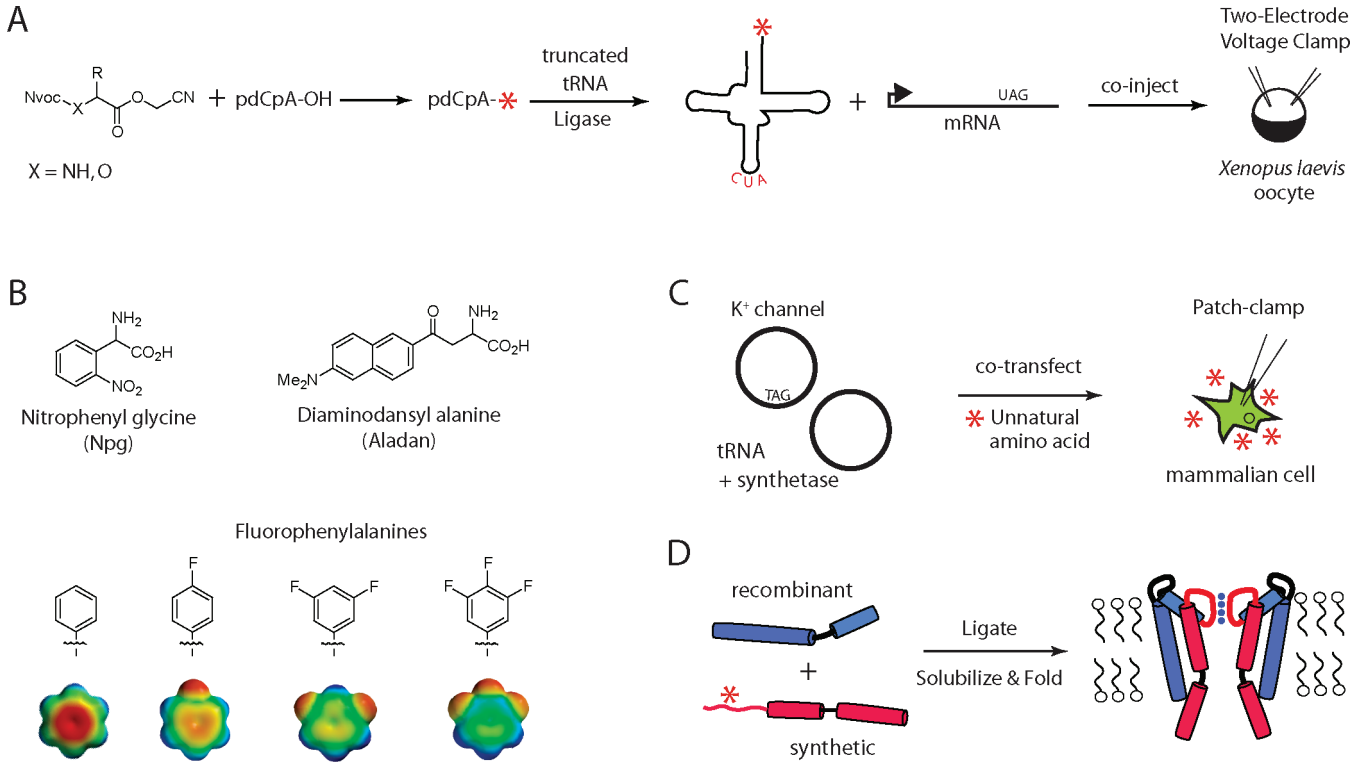

FIGURE 3: Incorporation of unnatural amino acids into K^+ channels. (A) In vivo nonsense suppression in *Xenopus laevis* oocytes. An appropriately protected (NVOC), cyanomethyl ester-activated unnatural amino acid is coupled to either the 2′- or 3′-hydroxyl of dinucleotide pdCpA. This product, shown here with the unnatural amino acid (UAA) as a red asterisk, is ligated enzymatically to a truncated, amber suppressor tRNA from *T. thermophilia*. The full-length, aminoacylated tRNA and the UAG-mutated K^+ channel mRNA are coinjected into oocytes. UAG denotes the site of UAA incorporation. K^+ channel function is measured using two-electrode voltage clamp. (B) Examples of UAA incorporated into K^+ channels. The top structures are photocleavable (Npg) and fluorescent (Aladan). The bottom row consists of fluorophenylalanines. The effect of serial fluorination is shown with color images of the 6-31G** electrostatic potential with red and blue corresponding to −20 and 20 kcal/mol, respectively. (C) Incorporation of UAA into mammalian cells via DNA transfection of cDNA plasmids carrying coevolved tRNA/synthetase pairs and K^+ channel cDNA with a TAG mutation. Successful UAA incorporation requires the prolonged exposure to the free UAA (red asterisks). K^+ channel function is assayed by electrophysiological (patch−clamp) approaches. (D) Schematic of the expressed protein ligation (EPL) method as applied to K^+ channels. UAA-containing K^+ channel complexes can be probed biochemically, functionally in lipid bilayers, and by structural methods.

or properties that were perturbed with mutation. This is especially true in cases where the mutation is lethal. In general, mutations exert their effects for mostly unknown reasons. Second, chemical modification of targeted amino acids, through site-directed mutation to cysteine for instance, is limited to water accessible surfaces outside of the membrane, leaving the hydrophobic nether regions of the channel uninvestigated. Third, site-directed mutagenesis is limited to amino acids available in the cell that are compatible with the ribosomal machinery, ruling out experiments that may require lines of questioning that go beyond naturally existing amino acids. As a remedy for these shortcomings, serial breakthroughs in protein chemistry have brought within the grasp of ion channel researchers methods that allow for the site-directed introduction of so-called "unnatural" amino acids into a protein sequence. To date, three distinct methods have been used to incorporate both subtle and extreme variants of naturally occurring side chains into K^+ channels: (1) in vivo nonsense suppression in *Xenopus* oocytes, (2) directionally evolved tRNA/aminoacyl-tRNA synthestase pairs that permit the study of unnatural amino acid containing proteins in mammalian cells and cultured neurons, and (3) expressed protein ligation (EPL) and protein-*trans*-splicing (PTS) for studying semisynthetic channels with artificial bilayers and structural biology.

In vivo nonsense suppression allows for the site-directed incorporation of tailor-made unnatural amino acids into proteins upon the successful completion of a series of biochemical and chemical steps (*46, 47*). The first is to enlist conventional site-directed mutagenesis to introduce a TAG codon (nonsense) at the site where the unnatural amino acid will be targeted, shown as the resulting UAG on the mRNA transcript in Figure 3A. The second step is the construction of the tRNA loaded with the unnatural amino acid. To do this, a dinucleotide, pdCpA, is acylated with a cyanomethyl-activated, NVOC-protected unnatural amino acid, which is subsequently ligated to a truncated amber suppressor tRNA lacking its two terminal residues. A tRNA from *Tetrahymena thermophila* THG73 is used, which also carries a G73 mutation to obscure the recognition by the *Xenopus* Glu amino-acyl-tRNA synthetases to prevent reacylation of the uncharged tRNA. Generation of the acylated dinucleotide requires the synthesis of pdCpA and the NVOC-protected, activated ester of the desired unnatural amino acid. For experimentalists lacking direct access to chemical synthesis resources, several commercial operations are willing, for a price, to manufacture the appropriately derivatized amino acid and dinucleotide building blocks. With both RNA molecules in hand, the third step is coinjection into *Xenopus* oocytes for expression and characterization, often by two-

electrode voltage clamp. Unwanted counterfeit proteins produced by either TAG codon "bleed-through" or tRNA manipulation by endogenous tRNA synthetases are controlled for by examining oocytes coinjected with the cRNA and an "uncharged" THG73 tRNA that lacks an appended amino acid. This general approach has been used successfully to express numerous side chain and backbone mutations into a variety of ligand and voltage-gated ion channels.

The nonsense suppression method was first applied to *Shaker* K^+ channels to introduce the photocleavable amino acid (2-nitrophenyl)glycine (Npg) into the cytosolic N-terminal tether that connects the inactivation ball to the channel. Photocleavage of the peptide backbone and the concomitant loss of the "inactivation ball" and fast inactivation demonstrate both the unnatural amino acid incorporation and the potential for the method (*48*). Next, Lu and co-workers targeted ester carbonyl backbone variants of Phe and Tyr into the selectivity filter of Kir1.1 and Kir2.1 inward rectifier channels (*49*). This region of the channel is especially resistant to traditional site-directed mutagenesis, as side chain replacement strategies often fail to produce functional channels. Here the mutant channels were characterized via single-channel analysis, a truly heroic undertaking, demonstrating that the peptide backbone connecting this stretch of highly conserved amino acids contributes to K^+ channel gating. Lastly, the environmentally sensitive fluorescent side chain, 6-dimethylamino-2-acylnaphthalene (AlaDan) has been incorporated into *Shaker* and Kir2.1. Although K^+ channels harboring this fluorescent side chain are functional, fluorescence signals from the resultant channels were not reported (*50*). Each of these examples (Figure 3B) serves to push the chemical diversity that can be introduced into K^+ channels using nonsense suppression.

While nonsense suppression allows for the incorporation of a wide variety of amino acids with structurally diverse side chains, modest side chain alterations are equally informative. Serial fluorination of aromatic phenylalanine (Figure 3B) or tryptophan residues has been used for the investigation of putative cation$-\pi$ interactions between the candidate aromatic side chains and cationic molecules. This manipulation titrates the negative electrostatic potential off the face of the aromatic and leaves the size, shape, polarizability, and, importantly, hydrophobicity unchanged (*51*). As opposed to the limited predictive capability of standard mutagenesis, here each added fluorine reduces the affinity for the ligand or blocker in a stepwise manner for a legitimate cation$-\pi$ pair. Plotting the relative lost binding energy ($\Delta\Delta G$) per fluorine versus the ab initio calculated energies for the equivalent theoretical system gives an approximation of the strength of the interaction. Using this approach, cation$-\pi$ interactions have been demonstrated to underlie ligand binding to nicotinic acetylcholine, 5-HT3 (serotonin), $GABA_C$, and glycine receptors (*52*−*57*). Energetically significant cation$-\pi$ interactions have also been shown to support the extracellular block by either tetrodotoxin (TTX) or calcium ions of voltage-gated sodium channels and their inhibition by local anesthetics (*58*−*60*). Lastly, such interactions underlie the external tetraethylammonium (TEA) blockade of *Shaker* K^+ channels where four aromatic phenylalanine residues near the selectivity filter coordinate a single blocking molecule (*61*). Moreover, the cation$-\pi$ interaction is geometrically constrained such that the blocker must interact with the face, not the edge, of the aromatic. Thus, evidence of a cation$-\pi$ interaction provides direct information about side chain orientation. In addition to the blockers, drugs, and ligands noted here, cation$-\pi$ interactions may also play a role in the structure−function relationships throughout biology. A theoretical study of high-resolution PDB files with a data set of more than 200000 side chains concluded that roughly 1 in every 77 residues in any given protein has an energetically significant cation$-\pi$ interaction, roughly half the incidence of predicted salt bridges (*62*). The role, if any, of putative cation$-\pi$ interactions in *Shaker* K^+ channels for voltage sensing or gating mechanisms has yet to be empirically determined, but the tools to do so are within reach.

The current iteration of the in vivo nonsense suppression method is streamlined for the study of proteins in the *Xenopus* oocyte. Therefore, the recent demonstration of a suppressed UAG codon in a green fluorescent protein (GFP) expressed in cultured neurons expands the potential for more sophisticated study of the biology of ion channels in the context of a mammalian cell (*63*). The method, advanced by the Schultz laboratory, employs coevolved orthogonal tRNA/amnioacyl-tRNA synthetase pairs from the archea *Methanococcus jannaschii* (recently reviewed in ref *64*). This approach (Figure 3C), like in vivo nonsense suppression, uses a UAG nonsense mutation in the cRNA channel transcript to guide the tRNA to the site of incorporation, but instead of chemically preacylating the tRNA in vitro, a coexpressed tRNA synthetase serves to append the tRNA with the unnatural cargo. The method relies on a specialized tRNA synthetase that has been evolved to recognize the appropriate tRNA. As with nonsense suppression, the tRNA synthetase pair should be unrecognized by the host cell so that the tRNA is not mislabeled or the tRNA synthetase labels endogenous tRNA molecules inappropriately. Unlike nonsense suppression, the tRNA synthetase continues to recycle (and recharge) the tRNA molecule and therefore, in theory, produces a higher density of mutant channels. Once a pair is developed for a particular unnatural amino acid, the plasmids can be readily shared, reducing the synthetic chemistry component of the approach, but because the tRNA synthetase continuously reacylates the tRNA, the unnatural amino acid has to readily enter the cell and be present at millimolar levels.

This method has been used to incorporate a number of side chains, and those with fluorescent (*65*) or chemically reactive characteristics (*66*) may be of special interest for structure−function studies in ion channels. For K^+ channels, tRNA-synthetase pairs have been used to target side chains with increasing volumes in the N-terminal inactivation particle of Kv1.4 channels (*63*). In this particular study, each side chain variant produced a high density of potassium current with gating behavior similar to that of wild-type channels except that larger side chains inactivate on a slower time course. This difference was attributed to the steric effects of larger side chains having trouble accessing their binding site in the inner vestibule (Figure 1B). Although these experiments provide an insightful proof of principal, the true potential for this approach lies in its application to the home turf of K^+ channels: neurons, cardiac myocytes, lymphocytes, pancreatic beta cells, and the myriad of cultured mammalian

systems where its application will allow for a more nuanced approach to ion channel experimentation in a physiological setting.

Two potential caveats are worth noting when using nonsense suppression to incorporate unnatural amino acids into ion channels. First, both methods generate truncated proteins because, for the time being, the stop codon is essential for guiding the charged tRNA to the site of incorporation. For K^+ channel expression, these protein fragments are particularly deleterious since they can coassemble with full-length mutant monomers, resulting in heterotetrameric proteins that are either nonfunctional or localized to intracellular compartments. In contrast, voltage-gated sodium channel expression is relatively unaffected by truncated proteins since the entire channel protein is formed from a single polypeptide (*60*). Second, in a cellular environment, ribosomal compatibility of the unnatural amino acid is a limiting factor when considering unconventional side chains. In the in vitro realm, however, advances in protein ligation chemistry have made possible the large-scale synthesis of polypeptide chains that contain amino acid variants that would be otherwise difficult, if not impossible, to derive in a cellular setting.

Biochemical approaches to unnatural amino acid incorporation allow for the ligation of two proteins, one of which can be synthetic, thus expanding the chemical possibilities of the amino acids used while simultaneously avoiding the stringent control of the ribosome (Figure 3D). A variant of the technique, expressed protein ligation or EPL, takes advantage of a novel family of proteins that catalytically ligate recombinant and/or synthetic polypeptides to produce semisynthetic polypeptides (*67*). The general approach, reviewed recently in ref *68*, has been applied successfully to a number of soluble proteins, yet its application to an ion channel is complicated by the substantial experimental hurdles presented by the postligation solubilization and folding of these integral membrane proteins. These twin challenges not withstanding, the mechanisms of selectivity and gating in the pore region have been investigated with semisynthetic KcsA K^+ channels (*69*−*73*). Motivation for the use of EPL in this example can be found upon close inspection of KcsA pore region structures that show the "signature" TVGYG residues alternating between left-handed and right-handed α-helical angles, as if constructed from D- and L-amino acids, with the two Gly residues adopting dihedral angles typically reserved for D-amino acid counterparts. To test the possibility that Gly-77 truly mimics a D-amino acid, Valiyaveetil and co-workers employed EPL to introduce D-alanine into the KcsA selectivity filter at this site and show that these channels not only are capable of selecting potassium over lithium but can bind TEA and charybdotoxin with wild-type affinity, all indicators that the D-alanine is completely tolerated (*73*). An additional benefit of the semisynthetic approach is the potential for the biochemical production of significant quantities of purified unnatural amino acid-containing protein, a side benefit that lends itself to structural studies. The ensuing high-resolution crystal structure of D-Ala-77 KcsA was captured under conditions that would normally promote the collapse of the selectivity filter, in the absence of potassium for instance, yet the D-Ala-containing channels show a seemingly normal and conducting pore region (*70, 72*). These experiments suggest that the endogenous Gly-77 is capable of rotation when the pore region collapses, but the methyl side chains of D-Ala-77 prevent this motion due to the 4-fold steric clash that occurs between the other D-Ala methyl groups from other subunits. In sum total, these results explain why this site is unwelcoming to traditional amino acid replacement: glycine is the only naturally occurring residue capable of morphing into the contorted requirements of D-amino acids. The common characterization of the selectivity filter as a protein domain that catalyzes the transmembrane flow of potassium rings true when considering that similarly contorted amino acids can also be found in the active sites of enzymes where they sample similar forbidden dihedral space (*74*). It seems that K^+ channels, like those who study them, will use any tool to get the job done.

THE KITCHEN SINK: SCREENING LIBRARIES

Much of the structural and functional investigation of K^+ channels has come from the methodical work of biophysicists equipped with a small set of novel tools to pick apart the molecular motions of K^+ channels. While the appeal of exploring chemical and genetic space through screening technologies has been tempting, most of these techniques have been more successful in studying the water soluble accessory proteins that latch onto the cytoplasmic portions of K^+ channels (*75*). Nonetheless, some progress has been made using screening approaches to examine the membrane-embedded regions of K^+ channels and to find small molecule modulators of K^+ channel function.

The first foray into screening technologies was made possible with the cloning of two high-affinity K^+ transporters from yeast, proteins which allow for growth at very low K^+ concentrations (*76, 77*). Deletion of both genes results in yeast cells that cannot grow at low K^+ concentrations (<7 mM) but can survive on permissive K^+ concentrations of 100 mM (*78*). Initially, this yeast strain was used for expression cloning of K^+ transport proteins (*79*). Subsequently, Minor and colleagues made libraries of mammalian Kir2.1 channels with mutagenzied transmembrane domains and found a large number of mutant K^+ channels that could rescue the K^+ transport deficient yeast under selective conditions (*80*). Mapping the mutations onto helical wheel diagrams resulted in the tolerant and intolerant positions clustering to different faces of the helices. Relying on the assumption that lipid-facing and pore-lining residues would be more tolerant to hydrophobic and hydrophilic mutations, respectively, the researchers identified the lipid- and protein-facing residues of M1 and M2 segments and the pore-lining residues of the M2 segment. In addition, Fourier transform analysis of the data provided the first unbiased evidence that these two segments were indeed α-helices. To identify interacting residues between the M1 and M2 helices, a second genetic screen was employed to uncover second site suppressor mutants. In this screen, two pairs of putatively M1−M2 interacting residues were identified and two semilethal residues could be rescued with a doublet of mutations in the opposite transmembrane domain. These results led to the conclusion that one M1 helix interacts with two M2 helices, a structural motif that did not map out well onto the then, only high-resolution structure of a K^+ channel, KcsA.

The recently published high-resolution structures of bacterial inward rectifier K^+ channels (*4, 5*) afford the opportunity

to map the identified residues on bona fide inward rectifier channels. Using these structural scaffolds, the tolerant and intolerant positions nicely map onto the lipid, protein, and pore-lining residues of both bacterial inward rectifiers. In addition, the pairs of predicted M1−M2 interacting residues are reasonably close to each other in all of the structures. Moreover, the packing arrangement of the M1 helix in the open structure of Kirbac3.1 crosses over two M2 helices. However, the second site suppressor mutations that were used to predict this unique scaffold make intrasubunit contacts and do not sit at the intersubunit interface. Part of the discrepancy may be due to the fact that many of the intersubunit contacts predicted by the yeast screen required a double mutation in one helix to rescue a single mutation in the opposite helix. In spite of this minor glitch, the major structural conclusions from the yeast screen were correct, demonstrating that a screening approach can produce useful structural data in a post-crystal structure era.

One disadvantage of yeast is their hyperpolarized resting potential (*81*). Thus, many Kv channels are closed at these voltages and cannot rescue the K^+ transport deficient yeast. There are a handful of mammalian Kv channels and complexes that are open at hyperpolarized potentials; however, in practice, yeast cells transformed with these K^+ channels have not been able to grow under the restrictive conditions (W. R. Kobertz and K. J. Swartz Laboratories, unpublished data). In contrast, yeast cells expressing the hyperpolarization-activated K^+ channels from plants are fully capable of growing at low K^+ concentrations, as these channels were cloned using this yeast strain (*79*). Hyperpolarization-activated channels possess the same voltage-sensing domain as standard Kv channels; however, the coupling between the voltage sensor and cytoplasmic gate is apparently reversed: at negative potentials the voltage sensor is at "rest" with its charges occupying intracellular space, but the cytoplasmic gate is open and the channel is conducting (*82, 83*). Jan and colleagues exploited this inverted relationship to generate a structural model of a voltage sensor in the resting state utilizing yeast screens and molecular dynamics simulations (*84, 85*). A more stringent K^+ medium (0.2−0.4 mM) was used with the hyperpolarization-activated plant K^+ channel, KAT1, to identify mutations in the voltage sensor (S1−S4) that could be rescued with complementary mutations on the backside of the pore-forming domain (S5−S6). The identified pairs of suppressor mutants were then used as constraints in molecular dynamics simulations starting with the rKv1.2 crystal structure. The generated model predicts a 12−15 Å movement of S4 perpendicular to the membrane, which would coincide with 10−12 charges moving across the membrane on the basis of electrostatic calculations. These predictions are copacetic with most of the functional data that have been generated to map S4's trajectory from the resting to active state in canonical Kv channels (*86*−*88*). The structural model was then validated by using it to predict and test pairs of interacting residues in second site suppressor screens. These model-predicted interacting residues yielded two unique pairs of mutants that could rescue the K^+ transport deficient yeast, indicating the structural model derived from these more stringent conditions appears to mimic the structure of the voltage sensor at rest. On the basis of the physical separation of the second site suppressor mutants in the structural model, it was proposed that the S4 helix will interact with an S5 helix in an adjacent subunit. Given that second site suppressor screens did not faithfully predict inward rectifier K^+ channel intersubunit interactions (vide supra), verification of these finer structural details with further functional experiments or visualization with a high-resolution structure of a voltage sensor in the resting state would be welcomed.

To circumvent the associated caveats of yeast screens, two different high-throughput approaches have been used to discover small molecule modulators of K^+ channels in mammalian cells. The first approach exploits the fact that K^+ channel function can change the membrane potential of the cell. Using a commercially available membrane-potential sensitive fluorescent dye, more than 20000 compounds were screened, and specific Kv1.3 K^+ channel inhibitors were identified (*89*). The second approach utilizes a nonradioactive rubidium (Rb^+) flux assay (*90*). The advantage of this approach is that most K^+ channels conduct Rb^+, which provides a direct measurement of function that can be readily detected by atomic absorption (*91*). Using a Rb^+-based approach, zinc pyrithione, the active ingredient in dandruff shampoo, was identified as a KCNQ K^+ channel activator (*90*). Unlike inhibitors and blockers, small molecule activators of voltage-gated K^+ channels are rare (*92*). Encouragingly, zinc pyrithione activates all homotetrameric KCNQ channels (except KCNQ3) and rescues the function of mutant KCNQ2 channels linked to benign familial neonatal convulsions. However, formation of the physiologically relevant KCNQ1−KCNE complexes abrogates zinc pyrithione's effect on the ion-conducting KCNQ1 subunit (*93*). A similar reduction of efficacy has been previously observed with another activator of homomeric KCNQ1 channels (R-L3) (*94*) and indicates that KCNE β-subunits may occlude the binding sites of activators of K^+ channels. Although washing your hair with dandruff shampoo will (thankfully) not activate neuronal K^+ channels, finding molecules like zinc pyrithione provides new tools for studying voltage-gated K^+ channel opening, which will ultimately lead to the discovery of promising new pharmacophores for the treatment of K^+ channelopathies.

FUTURE DIRECTIONS

The chemical approaches described here allow for the molecular interrogation of structure−function relationships in K^+ channels, but it is important to consider that these protein complexes are also targets of clinically relevant drugs that effect signaling in the endocrine, cardiovascular, and nervous systems. Accordingly, defects in these channels result in diabetes, epilepsy, cardiac arrhythmia, and neuropathic pain, conditions that will benefit substantially from tailor-made therapeutics that modulate tissue specific K^+ channel isoforms. Therefore, future studies that focus on elucidating the molecular details of drug−K^+ channel interactions, either intended or not [see hERG (*84*)], will provide details about both K^+ channel structure and function and lead to the design of safer and more specific drug therapies. Increasing the cadre of chemical tools for probing K^+ channel complexes, whether it is the inclusion of orthogonal chemoselective chemistries, small molecule dimerizers, new screening and protein labeling technologies, or

old-fashioned pharmacology, will provide valuable assistance on both scientific fronts.

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