Pharmacological Approaches to Studying Potassium Channels

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Abstract

In this review, we consider the pharmacology of potassium channels from the perspective of these channels as therapeutic targets. Firstly, we describe the three main families of potassium channels in humans and disease states where they are implicated. Secondly, we describe the existing therapeutic agents which act on potassium channels and outline why these channels represent an under-exploited therapeutic target with potential for future drug development. Thirdly, we consider the evidence desired in order to embark on a drug discovery programme targeting a particular potassium channel. We have chosen two "case studies": activators of the two-pore domain potassium (K_{2P}) channel TREK-2 ($K_{2P}10.1$), for the treatment of pain and inhibitors of the voltage-gated potassium channel $K_V1.3$, for use in autoimmune diseases such as multiple sclerosis. We describe the evidence base to suggest why these are viable therapeutic targets. Finally, we detail the main technical approaches available to characterise the pharmacology of potassium channels and identify novel regulatory compounds. We draw particular attention to the Comprehensive in vitro Proarrhythmia Assay initiative (CiPA, https://cipaproject.org) project for cardiac safety, as an example of what might be both desirable and possible in the future, for ion channel regulator discovery projects.

Keywords

CiPA · hERG · Kv1.3 · Patch-clamp electrophysiology · Potassium channel · TREK-2

1 Potassium Channel Families

Potassium selective ion channels are pore-forming proteins that allow the flow of potassium ions across membranes, primarily, but not exclusively, the plasma membrane. Potassium channels regulate cell excitability, control cell resting membrane potential and determine the shape of the action potential waveform in cells that use action potentials such as neurons and cardiac cells. However, potassium channels are present in virtually all cells within the body, influencing a wide range of diverse processes.

Potassium channels are the largest class of mammalian ion channel proteins. The human genome contains over 75 genes that encode for the primary (alpha)-subunits of potassium channel proteins. These genes are divided into either three or four (Taura et al. 2021) distinct families in mammals (see Fig. 1) that encode pore-forming subunits based on their structural and functional properties.

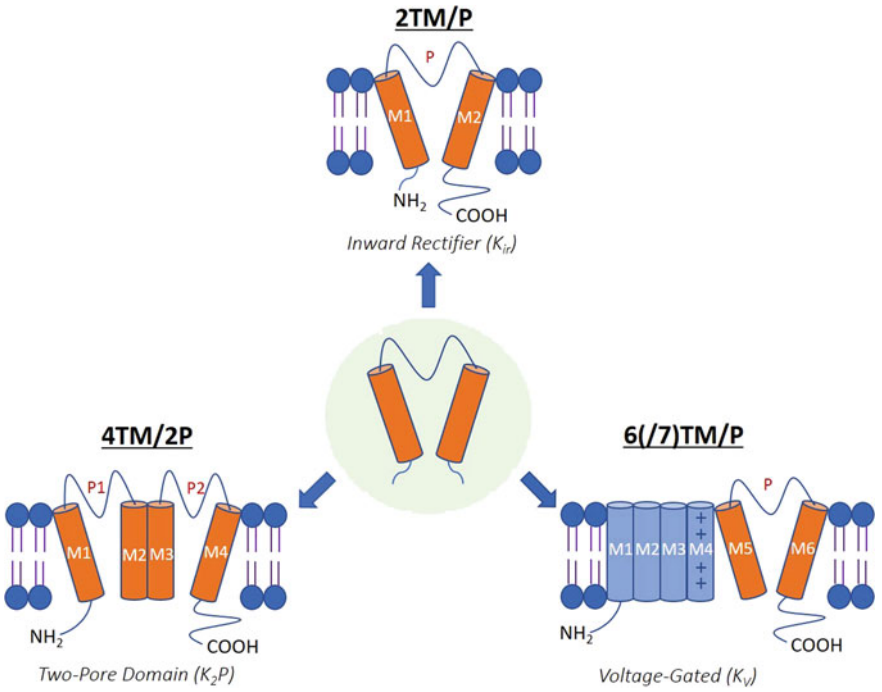

Fig. 1 Potassium channel families

The number of different, functioning ion channel proteins is, potentially, much greater than this because of the formation of heteromeric channel subunit combinations. Diversity is further enhanced by subunit variation due to alternative splicing, alternative translation initiation (ATI) and by co-assembly with accessory proteins. A standardised nomenclature for potassium channels has been proposed by NC-IUPHAR (Adelman et al. 2019; Aldrich et al. 2019; Attali et al. 2019; Bayliss et al. 2019). Nomenclature of K channels, however, remains a divisive topic. Whilst formal classification exists ($K_V1.x$, $K_{ir}1.x$, $K_{2P}1.x$, etc.,), there is an established and entrenched literature which utilises the more familiar potassium channel names such as hERG, BK_{Ca}, K_{ATP}, TWIK, KCNQ1, etc., that are often much more recognisable to researchers both within and outside the field. In this review, we have attempted to accommodate both positions by using either nomenclature where appropriate.

An introduction to potassium channels is given in Taura et al. (2021) and a comprehensive description of each channel subtype and its pharmacology is given by NC-IUPHAR's Guide to Pharmacology: (http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=696).

An up-to-date snapshot of current K channel pharmacology, in particular a tabulated summary of the important properties of each subfamily of K channels, is provided by the related Concise Guide to Pharmacology (Alexander et al. 2019).

1.1 6TM Potassium Channels

The six transmembrane domain (6TM), family of K channels is the largest of the K channel families and is made up of the voltage-gated K_V subfamilies, the KCNQ subfamily (which includes KCNQ1 channels, $K_V7.1$), the EAG subfamily (which includes hERG channels, $K_V11.1$), the Ca^{2+} activated K_{Ca} subfamilies and the Na^+ activated K_{Na} subfamilies. Sometimes, the latter two subfamilies are classified in a separate family (Taura et al. 2021). There are 40 human voltage-gated potassium channel genes belonging to 12 "K_V" subfamilies. In nerve cells and cardiac cells, K_V channels regulate the waveform and firing pattern of action potentials. They may also regulate the cell volume, proliferation, and migration of a wide range of cell types.

Each K_V channel contains four pore-forming α-subunits and may also contain auxiliary β-subunits that affect the channel function and/or localisation (Gonzalez et al. 2012; Vacher et al. 2008). Each pore-forming subunit of K_V channels contains six transmembrane segments (S1-S6, hence 6TM), with the first four transmembrane segments (S1-S4) constituting the voltage sensor and the last two transmembrane segments flanking a pore loop (S5-P-S6) as the pore (P) domain. The P domain for all K channels contains the sequence T/SxxTxGxG which is termed the K selectivity sequence. Within each of the K_V subfamilies, homomeric and heteromeric channels may form with a range of functional properties (González et al. 2012; Johnston et al. 2010).

K_V channel modulators may inhibit channel activity either by occluding the channel permeation pathway, as in the case of pore-blocking toxins and inner pore blockers, or through interacting with the voltage sensor to stabilise the closed state of the channel, as in the case of gating modifier toxins. Some small molecules act by binding to the gating machinery as gating modifiers, or by interacting with the interface between the α- and β-subunits to alter channel activity (Wulff et al. 2009). Mutations of K_V channel genes may cause neurological diseases such as episodic ataxia and epilepsies, heart diseases and deafness (Lehmann-Horn and Jurkat-Rott 1999; Kullmann and Hanna 2002; Abriel and Zaklyazminskaya 2013; Villa and Combi 2016).

1.2 2TM Potassium Channels

The 2TM domain family of K channels is also known as the inward-rectifier K channel family (K_{ir}). Seven structurally distinct subfamilies of the K_{ir} family have been identified in mammals. This family includes the strong inward-rectifier K channels ($K_{ir}2.x$) that are constitutively active, the G-protein-activated inward-rectifier K channels ($K_{ir}3.x$) and the ATP-sensitive K channels ($K_{ir}6.x$, which combines with sulphonylurea receptors to form the functional channel complex). The pore-forming α subunits form tetramers, and heteromeric channels may be formed within subfamilies (e.g. $K_{ir}3.2$ with $K_{ir}3.3$). K_{IR} channels play central roles in control of cellular excitability and K^+ ion homeostasis. K_{IR} channels possess only

two membrane-spanning helices and have evolved distinct voltage-independent mechanisms for opening and closing, including gating by G proteins, pH and ATP.

Mutations in K_{ir} channels cause a range of diseases including Bartter syndrome (Simon et al. 1996), Andersen-Tawil syndrome leading to ventricular arrhythmias (Plaster et al. 2001), epilepsy (Buono et al. 2004; Ferraro et al. 2004), vasospastic angina (Miki et al. 2002) and neonatal diabetes (Gloyn et al. 2004). Loss of $K_{ir}4.1$ expression abolishes endo-cochlear membrane potential and causes deafness in Pendred syndrome (Wangemann et al. 2010).

1.3 4TM Potassium Channels

The 4TM family of K channels (K_{2P}) underlies background currents, which are expressed throughout the body. They are open across the physiological voltage-range and are regulated by many neurotransmitters and physiological mediators. The pore-forming α-subunit contains two pore loop (P) domains and so two subunits assemble as dimers to form one pore lined by four P domains in the functional channel. Some of the K_{2P} subunits can form heterodimers across subfamilies (e.g. $K_{2P}3.1$ with $K_{2P}9.1$).

K_{2P} channel subtypes have emerging roles in a multitude of physiological responses and pathological conditions (Mathie et al. 2021a), including action potential propagation in myelinated axons (Brohawn et al. 2019; Kanda et al. 2019), microglial surveillance (Madry et al. 2018), inflammation (Bittner et al. 2009), cancer (Pei et al. 2003; Mu et al. 2003; Sun et al. 2016), cardiac arrhythmias (Decher et al. 2017) and pain (Alloui et al. 2006; Devilliers et al. 2013; Vivier et al. 2017).

2 Classical Pharmacology of Potassium Channels

At least some of us will have taken, or will take in the future, drugs that produce their effects through an action on potassium channels. These include certain oral hypoglycaemic agents, such as gliclazide which block ATP-sensitive K channels ($K_{ir}6.x$) in pancreatic beta cells; openers of the same ATP-sensitive K channels such as nicorandil which hyperpolarise vascular smooth muscle cells leading to vasodilation and are used in the treatment of angina; and blockers of $K_V11.1$ (hERG) channels such as amiodarone which delay the repolarisation phase of cardiac action potentials and are used in the treatment of atrial and ventricular fibrillation. All of the above compounds are listed in the WHO model list of essential medicines: (https://www.who.int/groups/expert-committee-on-selection-and-use-of-essential-medicines/essential-medicines-lists).

Each of these drugs has been in clinical use for decades but, perhaps surprisingly, there are no recent additions to this list of essential drugs which activate or block potassium channels as their primary mechanism. However, a small number of new drugs which target potassium channels, such as fampridine, a formulation of

4-aminopyridine (4-AP, see below) used in multiple sclerosis, have been introduced. These older drugs are not particularly potent, nor are they particularly selective for their primary target. As a result, the doses used can be high and can be associated with undesirable side effects.

It is difficult to obtain precise numbers regarding the relative importance of different proteins as current drug targets, not least because it is often unclear what the primary target of an existing therapeutic agent actually is. Nevertheless, relatively recent estimates suggest that existing drugs target just a few hundred proteins (667 human plus 189 pathogen proteins, or protein families, Overington et al. 2006; Santos et al. 2017 but see Imming et al. 2006). Nineteen per cent of these (or around 160 proteins) are ion channels. To consider this from the perspective of existing drugs, there are around 1,600 distinct approved drugs on the market today of which 18% (or around 300 drugs) target ion channels, making them the second largest gene family targeted by existing drugs, behind G protein coupled receptors (GPCRs).

Early identified pharmacological agents that act on K channels are the "classical" K channel blockers, tetraethylammonium ions (TEA) and 4-AP, which block a range of K_V channels to different degrees but are largely ineffective against K_{ir} or K_{2P} channels. There are also a number of naturally occurring toxins which bind potently and selectively to particular potassium channels, such as charybdotoxin, iberiotoxin and apamin (certain K_{Ca} potassium channels), ShK toxin (from the sea anemone, *Stichodactyla helianthus*; $K_V1.3$ channels) and guangxitoxin ($K_V2.1$ and $K_V2.2$ channels) (Matsumura et al. 2021). These toxins have provided considerable insight, both into how channels function and into potential sites of action for the development of novel therapeutics. Furthermore, naturally occurring compounds such as hydroxy-α-sanshool (from Szechuan peppers), which blocks certain two-pore domain potassium channels, including TRESK channels (Bautista et al. 2008; see also Mathie 2010) have revealed the potential importance of these channels as therapeutic targets in the treatment of pain.

The pharmaceutical industry has not yet exploited potassium channels fully as a drug target despite the introduction of novel, faster screening techniques for compounds acting on potassium channels, although there are several compounds currently in different stages of clinical trials (https://www.ionchannellibrary.com/drugs-in-clinical-trials/). The amino acid sequence homology among potassium channel subfamily members can be quite high (>70%), and this can often make it challenging to both identify and develop subtype-selective compounds. Nevertheless, there is ample opportunity to discover and develop novel "first-in class" molecules targeting potassium channels, as considered later in this review.

3 Identifying K Channel Pharmacological Targets

Embarking on a drug discovery programme focussing on a novel ion channel target is an expensive and time-consuming operation. It is not something that is entered into lightly or without the potential of a profitable (drug-to-market) outcome. A pharmaceutical company would expect to see a portfolio of evidence from a range of

different scientific and technical approaches in order to be persuaded that a particular potassium channel is a viable therapeutic target.

At a very fundamental level, knowledge of the exact subunit composition of potassium channels in different cell types and tissues is incomplete and this can be complicated further when channel subunit expression levels are altered in disease. As such the precise subunit composition of potassium channels and what auxiliary subunits may (or may not) be associated with them is often unknown for particular disease states.

Even if one is confident of the potassium channel subtype underlying a particular disease phenotype, there is a paucity of existing pharmacological tools to aid the development of useful assays. Furthermore, despite the resolution of some potassium channel structures and the increased understanding of their function that has resulted from this, there is a lack of resolved structures of potassium channels in complex with channel activators or inhibitors (but see Dong et al. 2015; Lolicato et al. 2017) which restricts identification of pharmacophores for rational drug design (McGivern and Ding 2020). When selective pharmacological tools or structural insights are not available, additional information can be obtained from human genetic data, which show correlations between loss or gain of function mutations in ion channel subunits and disease phenotypes, when selecting which targets to pursue. However, genetic association of a mutation with a disease does not prove causation of the disease (McGivern and Ding 2020).

In the sections below, we outline the key information that might be needed for identifying a disease-associated ion channel target. In most cases, the availability of this information is fragmented or incomplete and one has to make a judgement call as to whether the channel is indeed a viable target. To illustrate this, we pick two examples of different K channels and outline the existing evidence (and evidence gaps) that suggest they may be viable therapeutic targets.

3.1 Physiological and Pathophysiological Role of the Channel of Interest

A detailed knowledge of the physiological and pathophysiological roles of the potassium channel of interest is critical but this is often lacking detail. Fundamental questions such as "do we want to enhance or inhibit the current?" rely on an understanding of the physiological roles of particular ion channels and how this might change in pathophysiology. For example, there is good evidence that $K_V2.1$ channels are important in regulating the neuronal firing in motoneurons where they can either maintain or suppress repetitive firing depending on the existing activity of the neurons (Romer et al. 2019; see also Liu and Bean 2014). Loss of function of these channels can lead to decreased firing and neuro-developmental epileptic disorders (de Kovel et al. 2017). Some mutations of $K_V2.1$ channels cause a loss of ion selectivity for K^+ ions over Na^+ ions which will have complex effects on firing, depending on the degree of synaptic activity. Furthermore, gain of function mutations can, paradoxically, still lead to epilepsy phenotypes (Niday and

Tzingounis 2018). So, depending on both the underlying pathophysiology and the level of tonic firing activity (Romer et al. 2019) opposing therapeutic approaches might be desirable.

Perhaps an alternative way to consider this is to seek to restore optimum/normal levels of activity of the channel target. For example, there is a strong genetic linkage between loss of function mutations of TASK-1 K_{2P} channels and the development of pulmonary hypertension (Ma et al. 2013; Olschewski et al. 2017; Cunningham et al. 2019). More recent studies suggest that reduced TASK-1 channel activity is a more general condition seen not just in genetic pulmonary hypertension but also in the idiopathic condition (Antigny et al. 2016). As such, restoration of "normal" channel function may be a more widely applicable therapeutic intervention in this and other situations.

3.2 Knowledge of Distribution Channel mRNA and Protein Expression in the Appropriate Places in the Body

A fundamental component of the evidence base for suggesting a particular ion channel target is the knowledge that the channels are actually expressed (and functional) in the cells or organs of the body that are relevant. This is exemplified in the case studies below for TREK-2 and $K_V1.3$ channels as therapeutic targets, where there is good evidence that these channels are both expressed and functional in the cells that matter and, indeed, that expression and/or function is then altered in disease states.

3.3 Are There Species Differences? This May Be Important for Extrapolation from Preclinical Physiology and/or Pharmacology Studies

Since much preclinical research occurs on rodent models, it is extremely important to consider species differences between channel protein structures and whether this alters responsiveness to drugs. One example of this is where the respiratory stimulant, doxapram, was found to be a more selective inhibitor of TASK-1 than TASK-3 K2P channels in rodents (Cotten et al. 2006), but this channel specificity is lost for human TASK-1 and TASK-3 channels where doxapram is an equipotent inhibitor of both channel types (Cunningham et al. 2020).

3.4 Evidence from Diseases States of Channel Up- or Down-Regulation

Channelopathies provide important information about the roles of ion channels and have contributed to identification of novel ion channel targets in disease. Mutations in different ion channel genes can give rise to disease states such as episodic ataxias,

epilepsy, diabetes, cardiac arrhythmias, Birk Barel mental retardation syndrome and cystic fibrosis (see Ashcroft 2006; Catterall et al. 2008, Veale et al. 2014b). Many of these channelopathies result from mutations in the coding regions, leading to a gain or loss of channel function or from mutations in the promoter regions leading to over- or under-expression of ion channels. Similarly, genetic ablation studies with knockout animals have confirmed the potential importance of ion channels in disease states but have also provided novel and surprising insights into new roles for specific ion channels in physiological processes, which might be targeted in the future (see Mathie 2010).

3.5 Case Study 1: TREK-2 Channel Activators for Pain

Over 1.5 billion people worldwide suffer from chronic pain. Existing first-line drugs for treatment of chronic pain, including cyclooxygenase inhibitors (non-steroidal anti-inflammatory agents, NSAIDs) and opioid receptor modulators, do not alleviate pain completely and, in certain situations including neuropathic pain, do not work well except at high doses (Mathie and Veale 2015). Furthermore, tolerance and addiction to opioids, particularly if used chronically, is a major current health issue.

Chronic pain conditions are often characterised by persistent over-excitability of peripheral nociceptors brought about by changes in ion channel organisation and/or activity. Increasing evidence points to an important contribution from a number of different potassium channels (see Du and Gamper 2013; Tsantoulas and McMahon 2014; Waxman and Zamponi 2014), including K_{2P} channels (Alloui et al. 2006; Woolf and Ma 2007; Noel et al. 2009; Mathie et al. 2010; Gada and Plant 2018), in pain processing. Among K_{2P} channels, the strongest body of evidence from expression and functional studies highlights the importance of TREK-1, TREK-2 (and also TRESK) channels (see Mathie and Veale 2015).

TREK channels are expressed in both human (mRNA) and rodent (mRNA/protein) small/medium sized dorsal root ganglion (DRG) neurons which relay painful stimuli to the CNS. In these neurons TREKs are often co-localised with excitatory TRPV1 channels (Maingret et al. 2000; Medhurst et al. 2001; Talley et al. 2001; Alloui et al. 2006; Dedman et al. 2008; Loucif et al. 2018). Recent immunohistochemistry studies, using selective commercially available antibodies, also show the expression of all three TREK channels (TREK-1, TREK-2 and TRAAK) in small sized rat DRG neurons (Viatchenko-Karpinski et al. 2018, Acosta et al. 2014). Given the high expression levels of these channels in nociceptive neurons, activation of these channels would be predicted to reduce nociceptor firing, thereby reducing pain.

Despite its broad CNS expression, studies of TREK-2 KO mice did not observe any major behavioural abnormalities (Mirkovic et al. 2012). In humans, initial TaqMan RT-PCR assays showed that there are very low levels of expression of TREK-2 (or TREK-1) channel mRNA in the heart (Medhurst et al. 2001), suggesting low risk of cardiac side effects in humans.

It has been suggested that TREK-2 contributes 69% (Kang and Kim 2006) and 59% (Acosta et al. 2014) of the resting potassium current in neonatal rat small DRGs and rat small $IB4^+$ DRG neurons, respectively. In contrast, TREK-1 and TRESK channels contributed just 12% and 16%, respectively (Kang and Kim 2006). TREK-2 channel activity therefore has a significant impact on the resting membrane potential – and so excitability – of these cells.

TREK-2 channels have been implicated in regulating somatosensory nociceptive neuron excitability. After CFA-induced inflammation, spontaneous pain, measured as spontaneous foot lifting, was increased by siRNA-induced TREK-2 knockdown in vivo (Acosta et al. 2014); furthermore, TREK-2 KO mice were more sensitive to von Frey filaments (Pereira et al. 2014).

There is a wealth of structural information from a variety of different potassium channel subunits, which has formed the basis of our understanding of how all ion channels function. Following the original publications of crystal structures of TWIK-1 and TRAAK (Brohawn et al. 2012; Miller and Long 2012), more recent work has identified a number of different structural forms of TRAAK and TREK-2, which purport to correspond to different open and closed conformations of the TREK family of K_{2P} channels (Brohawn et al. 2014; Lolicato et al. 2014; Dong et al. 2015).

It has been shown that the TREK family of channels are subject to a process known as alternative translation initiation (ATI). For TREK-2 there are three potential translational (ATG or methionine) sites in its N-terminus, thus allowing for the possible generation of three different TREK-2 proteins (Staudacher et al. 2011; Simkin et al. 2008). Furthermore, it has been shown that different isoforms of TREK channels generated by ATI can cause an alteration in drug sensitivity (Eckert et al. 2011; Kisselbach et al. 2014; Veale et al. 2014a).

As well as ATI, the TREK family has also been shown to be subject to alternative exon splicing. For TREK-2, the three isoforms identified do not differ hugely, in terms of their functional properties; however, they are differentially expressed in various tissues. TREK-2a is strongly expressed in the brain, pancreas and kidney, whilst TREK-2b is expressed in the proximal tubule of the kidney and in the pancreas and TREK-2c is expressed mainly in the brain (Gu et al. 2002). It is not known which subtype predominates in DRG neurons.

At least in expression systems, there is accumulating evidence to suggest that all three TREK channels (TREK-1, TREK-2 and TRAAK) can form functional heterodimeric channels with each other (Blin et al. 2016; Lengyel et al. 2016; Levitz et al. 2016) resulting in unique functional properties. Functional recordings of currents with properties suggestive of the formation of TREK-1/TREK-2 heteromeric channels were also demonstrated in native DRG neurons indicating that hetero-dimerisation of TREK channels may occur in native cells to provide greater diversity of leak potassium conductances (Lengyel et al. 2016). A recent study provides convincing evidence for TREK-2 and TRESK heterodimer channels in native trigeminal ganglion neurons (Lengyel et al. 2020).

There are, however, several gaps in the evidence in favour of TREK-2 channels as a therapeutic target for pain. Firstly, there is no direct genetic linkage at this stage

between TREK-2 channels and pain. Therefore, it is not possible to select patients on the basis of genotype. It is also not practically possible to select patients based upon TREK-2 expression levels in neurons. Nevertheless, this is not a unique problem as, in general, the pain therapeutic field suffers from a lack of predictive/efficacy and patient stratification biomarkers.

Secondly, there has not been satisfactory pharmacological validation of TREK-2 activators in vivo due to the non-selectivity of tool compounds. Therefore, it is not clear whether TREK-2 activation alone will have sufficient analgesic effects or co-activation of TREK-1/TRAAK will also be required. Thirdly, the side effects of TREK-2 channel activation are not known and therefore, it is not clear whether selectivity for TREK-2 over TREK-1 will avoid drug related side effects.

3.6 Case Study 2: K_V1.3 Channel Blockers in Autoimmune Disorders

The voltage-gated potassium channel, K_V1.3 regulates the membrane potential of lymphocytes (DeCoursey et al. 1984; Wulff and Zhorov 2008; Chiang et al. 2017) which is critical for the activation of these immune cells (Veytia-Bucheli et al. 2018).

Several studies have confirmed that K_V1.3 is highly expressed in macrophages, microglia and T cells, suggesting that K_V1.3 plays a crucial role in immune and inflammatory responses to human diseases such as multiple sclerosis (MS), rheumatoid arthritis, type 1 diabetes mellitus and asthma (Toldi et al. 2010; Huang et al. 2017; Tanner et al. 2017; Zhou et al. 2018). In these conditions, the expression levels of K_V1.3 channels are significantly elevated (Rangaraju et al. 2009).

The pathogenesis of autoimmune diseases involves activation and proliferation of effector memory T cells (TEM cells) and persistence of autoantigens (Devarajan and Chen 2013). During the activation of TEM cells, the expression of the K_V1.3 channel was up-regulated significantly (Cahalan and Chandy 2009). Accumulated data for K_V1.3 showed higher expression levels in myelin-reactive T cells from the peripheral blood (PB) of MS patients compared to healthy controls (Wulff et al. 2003). Also, in an animal model of experimental autoimmune encephalomyelitis (EAE), it has been confirmed that expression of K_V1.3 is significantly elevated (Rus et al. 2005).

Taken together, these data suggest that K_V1.3 channels are an attractive therapeutic target for immunomodulation (Beeton et al. 2006; Wulff and Zhorov 2008). In patients with multiple sclerosis (MS), type 1 diabetes mellitus, rheumatoid arthritis, psoriasis, or chronic asthma, disease-associated T cells are K_V1.3-dependent TEM cells. Selective K_V1.3 inhibitors suppress the proliferation and cytokine production of these cells (Cahalan and Chandy 2009). There is also a growing body of evidence suggesting that K_V1.3 channel blockers have beneficial therapeutic effect on rheumatoid arthritis, autoimmune encephalitis and other autoimmune diseases (see Chang et al. 2018, Wang et al. 2020).

K_V1.3 channel blockers have been found to alleviate disease symptoms in animal autoimmune diseases, chronic inflammatory diseases and metabolic disease models

without obvious side effects (Perez-Verdaguer et al. 2016). Importantly, positive results have been shown in preclinical trials (see below, also Prentis et al. 2018). Therefore, $K_V1.3$ channel blockers have the potential to be developed as effective drugs for the treatment of MS and EAE (Rangaraju et al. 2009).

Toxin blockers of $K_V1.3$ and other potassium channels have been found in the venom of numerous animals, including the venom of sea anemone (Wulff et al. 2019). In 1995, an effective blocker was extracted from the Caribbean sea anemone (*Stichodactyla helianthus*) by Castaneda et al. (1995) subsequently named ShK toxin (Wulff and Zhorov 2008). ShK and subsequent derivatives of it (see below and Lanigan et al. 2001) reduce the inflammatory response of autoimmune diseases by maintaining the integrity of the blood brain barrier (BBB) (Huang et al. 2017), reducing activation of TEM cells (Beeton et al. 2006) and eliminating respiratory bursts in activated microglia and subsequent secondary damage of neurons by microglia (Fordyce et al. 2005).

The structures of several ShK and ShK-like toxins have been determined, all of which have been reported to modulate ion channels (Chhabra et al. 2014). Like many K channel blocking toxins, they are polypeptides of between 34 and 75 amino acids, characterised by six cysteine motifs (forming 3 disulphide bonds) and a functional lysine residue which enters and blocks the pore of the channels at the selectivity filter. The novel analog ShK-186 (dalazatide) has a 100-fold improvement of selectivity for $K_V1.3$ over $K_V1.1$ and 1,000-fold over $K_V1.4$ as well as $K_V1.6$ (Pennington et al. 2009) compared to the natural ShK toxin. ShK-186 and its analogs had strong therapeutic actions in animal models of human autoimmune diseases including MS and rheumatoid arthritis (Beeton et al. 2001). Unexpectedly, ShK-186 was found to have a long half-life if given by sub-cutaneous injection leading to a sustained concentration, at pM levels, in plasma. This, in turn, gave a prolonged therapeutic efficacy (Tarcha et al. 2012). ShK-186 completed Phase 1a and 1b trials in healthy volunteers in 2016 and was believed to enter phase 2 trials in 2018/2019 (Wang et al. 2020).

Despite these encouraging data, there are still challenges for ShK to be used to the treatment of neuroinflammatory diseases. The first problem underlying the application of these peptides is that they cannot be taken orally, mainly because they have difficulty penetrating the intestinal mucosa. Furthermore, due to the polypeptide's molecular size, hydrophobicity and polarity, the cell membrane penetration of ShK is poor. The second obstacle is that ShK cannot cross the BBB in most neuroinflammatory diseases where this barrier is not compromised by the condition (Li et al. 2015). Thus, there is scope for the development of novel, pharmacologically active agents, either polypeptides or small molecules, to selectively target and block $K_V1.3$ channels in humans.

4 Techniques to Study the Pharmacology of K Channels

Ion channel drug discovery traditionally involves the use of model assay systems, often utilising a recombinant channel expressed in cell lines (see Mathie et al. 2021b). These assays support the identification of active compounds and the characterisation and optimisation of these lead series. Promising lead candidate molecules can then be tested in models of disease before entering clinical trials (see McGivern and Ding 2020; Walsh 2020). The preferred ion channel assay is often a cell line that transiently or stably expresses the appropriate combination of channel α- and auxiliary subunits (if known) and that yields relevant functional and pharmacological responses on (semi) high throughput assay platforms. There is an underlying assumption that the channel of interest expressed in an assay cell line should perform consistently over time, when tested using different methodological approaches and when used in different laboratories (see CiPA study below).

The gold standard approach for studying ion channels remains the manual patch-clamp method. This is, however, an extremely low-throughput technique where single cells are studied one at a time but, with appropriate expertise, this technique allows the collection of both high-quality data and potential mechanistic insight, unsurpassed by other methods. Used in isolation, this technique is unlikely to generate sufficient novel drug candidates to satisfy the demands of either the pharmaceutical industry or therapeutic need (McGivern and Ding 2020).

Accordingly, a range of different assay technologies have been developed and refined to address, primarily, the issue of high throughput. An excellent review of the range of available technologies, from the perspective of K_{ir} channels has recently been published by Walsh (2020, see also McGivern and Ding 2020) and has helped shape our thinking in this section of this review. Walsh (2020) analyses the pros and cons of a number of assay technologies that have been used for screening K_{ir} channels, including membrane potential-based fluorescent dye assays, using either fast-response fluorescence resonance energy transfer (FRET) probes or slow-response probes, thallium-sensitive fluorescent assays, radiometric and nonradiometric rubidium (Rb^+) flux assays, automated patch-clamp assays, a cell-free ion flux assay that uses K_{ir} channel-containing liposomes, and a K^+-dependent yeast growth assay.

In the sections below, we describe some of the key technological approaches that can be utilised to advance discovery of new therapeutic agents targeting K channels. Although these technologies have been used successfully for screening compound libraries against different K channels, each technology has its own limitations in parameters such as sensitivity, specificity, cost and physiological relevance (Walsh 2020). Therefore, there is a need for continued development of ion channel screening technologies.

4.1 Improved Structural Information for Ion Channels

High resolution elucidation of the structure of ion channels using X-ray crystallography was led by the outstanding work on bacterial, and later mammalian, potassium channels by MacKinnon and colleagues (e.g. Doyle et al. 1998; Jiang et al. 2002, 2003). These structures have led to rapid advances in our understanding of ion channel function and the identification of regions of channels that are important for determining channel function and drug binding. Knowing regions of the channel important for drug binding, or pharmacophores, it is then possible to use these pharmacophores as templates for the design of novel therapeutic agents (Mathie et al. 2021a).

The fact that ion channels are present in lipid membranes coupled with the dynamic nature of ion channel proteins has made resolution of structures difficult. Consequently still relatively few ion channels have been resolved using crystallography-based techniques. However, recent advancements in the field of Cryo-EM have led to significant improvements in the observed resolution and a substantial increase in the number of published structures. The recent development of direct electron detector cameras alongside improvements in computational processing has allowed greater resolution of structures, down to the atomic level (see Nakane et al. 2020). For example, many tens of Cryo-EM structures for the transient receptor potential (TRP) channel family have been published in the last few years (Renaud et al. 2018). The recently published human β3 $GABA_A$ receptor structure (Nakane et al. 2020) is (at 1.7 Å), the highest resolution membrane-spanning protein structure obtained to date from cryo-EM studies, which is significantly better than the accepted resolution of at least 2.5 Å required to model drug–protein interactions (Renaud et al. 2018). Improved structural images from cryo-EM techniques will undoubtedly lead to significant advances in the development of selective ion channel blockers and activators and, hopefully, an increase in useful drugs targeting ion channels. Readers are also directed to (Jiang 2021) for discussion of recent progress in structural biology of K^+ channels.

4.2 Flux Assays

Ion channel high throughput screening programs have mainly used either radiometric Rb^+ assays (e.g. Loucif et al. 2018) or fluorescence-based assays of ion flux in stable cell lines (using, for example, Tl^+, Ca^{2+} or Na^+ dyes) to measure permeation of the channel.

4.2.1 Thallium (Tl^+) Flux Assay

Perhaps the most common of these flux-assay approaches, at present, is the thallium (Tl^+) flux assay which takes advantage of the high permeability of K channels to T^{l+} ions in order to measure Tl^+ flux as a surrogate of ion channel activity. The Tl^+-sensitive, fluorescent-based assay for multi-well plate screening of K channels was introduced by Weaver et al. (2004). In this high throughput assay, cells are loaded

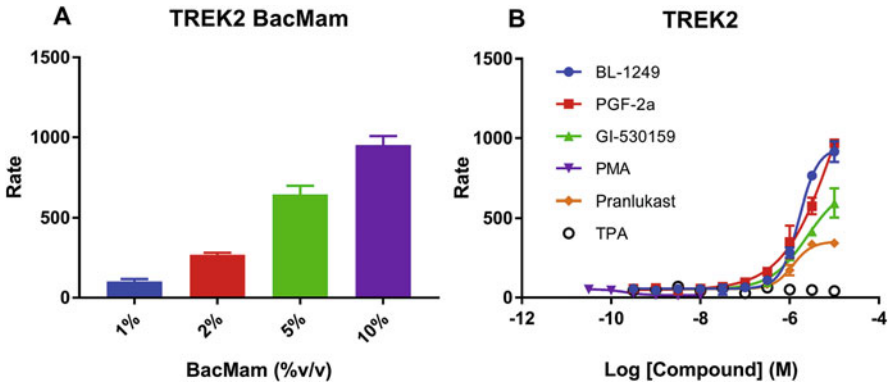

Fig. 2 Thallium flux assays of TREK-2 channel activators using "BacMam" baculovirus transfection. (**a**) Titration of BacMam. As BacMam levels increase (%v/v present in media), there is an increase in the rate of fluorescence change between 13 and 19 s after the addition of thallium, error bars denote S.E.M. This is proportional to increased TREK-2 activity in the assay. (**b**) Pharmacology of TREK-2 activators. Exemplar data showing activity of BL-1249, 11-deoxy prostaglandin F2a (PGF-2a), GI-530159, PMA, Pranlukast and TPA (adapted from Wright PD, McCoull D, Walsh Y, Large JM, Hadrys BW, Gauritcikaite E et al. Pranlukast Is a novel small molecule activator of the two-pore domain potassium channel TREK2. Biochem Biophys Res Commun. 2019; 520: 35–40 with permission)

with a Tl^+-sensitive, membrane-permeant reporter dye. K channel modulators can be rapidly screened by monitoring changes in the Tl^+-induced fluorescent signal.

In our own research, in collaboration with colleagues at LifeArc (Wright et al. 2017, 2019), cells transiently expressing K channels of interest were generated by using U-2 OS cells and a BacMam baculovirus transfection method. Generation of cell lines stably expressing ion channels can be challenging for a number of reasons including inherent toxicity. Moreover, the ability to identify channel activators can be compromised by systems in which the target is over-expressed at high levels due, for example, to saturation of the dye. Conversely, ion channel inhibitors can be hard to detect when expression, and therefore signal, is low. To minimise these issues BacMam can be used to deliver ion channels into mammalian cells. BacMam confers a number of advantages, including safety and reduced time compared to generating stable cell lines but, primarily, it allows the precise titratable expression of the gene of interest (Wright et al. 2013, 2017, 2019, see Fig. 2). In our work with K_{2P} channels this enabled us to generate cell systems in which we were able to intricately and robustly select a level of K channel expression, functionally optimised for the identification of channel activators. Another advantage of the BacMam system is that it allows screening against parental cells in the absence of the heterologous expressed ion channel. The use of a high throughput, fluorescence-based assay also allows the possibility of a "target class" approach to drug discovery (Barnash et al. 2017; McCoull et al. 2021). This approach allows the simultaneous prosecution of multiple targets from a target family. Knowledge accumulated from one program can be leveraged across multiple potential therapeutic targets (McCoull et al. 2021).

One major limitation to the Tl^+ assay is that some cells contain endogenous Tl^+ transport pathways that can interfere with the K channel efflux under study and cause a higher than acceptable rate of "false-positive" hits. Furthermore, ion flux fluorescent assays often require expensive instrumentation such as FLIPR or FDSS workstations that combine sophisticated liquid handling microfluidics with fluorescent imaging. In addition, biosafety and disposal of a toxic heavy metal such as Tl^+ is an issue.

A further technical drawback of plate-based, fluorescence assays is that the activating or inhibiting agent, once added, cannot be removed easily, so it is difficult to study the reversibility of the compound's effects on channel activity. Furthermore, potencies of ion channel modulators from fluorescence assays do not always correlate with manual or automated patch-clamp electrophysiological measurements.

4.2.2 Liposome Flux Assay

The liposome flux assay (LFA) described by Su et al. (2016) is a cell-free assay which provides a putative alternative high throughput screening system for K channel drug discovery. In this procedure, K channels are purified and reconstituted into lipid vesicles. A concentration gradient for K^+ efflux is established by adding the vesicles into a NaCl solution. K^+ efflux is initiated by the addition of a proton ionophore, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), which allows the influx of protons to balance the efflux of K^+ (Walsh 2020). The efflux of K^+ is quantified indirectly by monitoring the proton-dependent quenching of a fluorescent dye. In the original description of LFA, a library of 100,000 compounds was screened to identify both inhibitors and activators of GIRK2 ($K_{ir}3.2$) channels, hERG channels, TRAAK K_{2P} channels and K_{Ca} channels (Su et al. 2016). LFA potentially provides an efficient and low-cost method for K channel screening. However, the technique is not yet widely used and drug potencies obtained using LFA will need to be compared with values obtained using more established ion flux and automated patch-clamp systems before LFA becomes a widely used K channel high throughput method (Walsh 2020).

4.3 Electrophysiological Approaches

Until quite recently, the study and development of new drugs that act on ion channels has been restricted by the lack of high throughput screens that measure current directly passing through the ion channels using electrophysiological approaches. Manual patch-clamp recording techniques (Hamill et al. 1981) allow exquisite resolution of electrical activity but are restricted by the number of recordings that can be made in a given time and the level of technical expertise required to achieve these recordings. They do, however, remain critical for exploring the complex biophysical properties of ion channels and, in detail, the mechanisms of how lead compounds might work. For example, information arising from structural studies can be harnessed to explore the site and mechanism of drug action through site-directed mutagenesis of the ion channel under investigation.

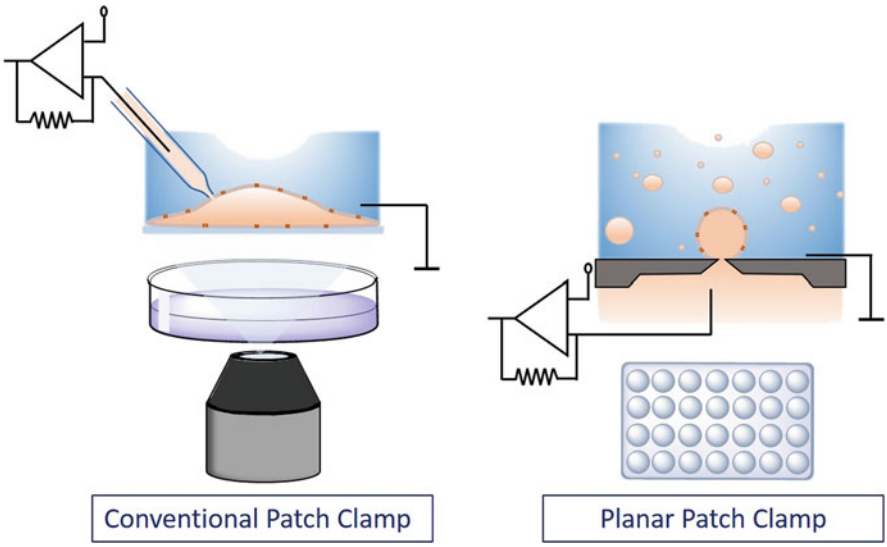

Fig. 3 Schematic representation of conventional patch clamp recording versus high throughput automated patch recording. Adapted and redrawn from an original schematic by Nanion Technologies: https://www.nanion.de/en/technology/technology-explained.html

Now, however, in large part driven by the need for pharmaceutical companies to screen compounds against hERG channels (see below), higher throughput screening technologies have been introduced and continue to be refined and optimised. Automated patch-clamp increases throughput and ease of use compared to the conventional patch-clamp technique making it accessible to a wider audience. The automation of the patch-clamp method was made possible with the development of chip-based planar patch-clamp technology (Fig. 3). Multi-well planar arrays give higher throughput screens by allowing multiple recordings in parallel. Most automated patch-clamp (APC) systems utilise a planar array which contains micron-sized apertures in silicon-based chips. Cells are added in suspension to a multi-well recording plate and, in a similar fashion to forming a seal using a microelectrode in manual parch-clamp recordings, negative pressure is applied to attract cells to the apertures. Further application of negative pressure causes the patch of membrane immediately beneath the aperture to rupture, thus establishing the whole-cell configuration. Alternatively, electrical access to the cell can be obtained using a pore-forming antibiotic such as nystatin or amphotericin B.

A range of different systems now exist, and an excellent analyses of the advantages and disadvantages of the evolving versions of each system have been provided by Dunlop et al. (2008) and, more recently, by Bell and Dallas (2018). Each system allows significant up-scaling of activity when compared with conventional patch-clamp electrophysiological methods or two-electrode voltage clamp from oocytes, increasing output from single digit to hundreds or even thousands of drug data points per day (Bell and Dallas 2018).

In the last few years, and in particular following the development of high-quality miniaturised amplifiers, automated, 384-well plate-based electrophysiology platforms have emerged, ranging from the perforated-patch-based IonWorks Barracuda (Molecular Devices, Sunnyvale, CA) to the latest generation, giga-seal-based instruments such as Qube (Sophion Bioscience), SyncroPatch (Nanion Technologies) and Ionflux (Fluxion). Coupled with this, storage and analysis of data have vastly improved, enabling large amounts of data to be recorded and analysed in a comparatively short space of time (Obergrussberger et al. 2020). These recent whole-cell patch-clamp platforms offer much higher throughput than earlier versions. They incorporate microfluidic networks and temperature control and are capable of recording currents from populations of individual cells, which increases the likelihood of obtaining useful data from each well of an assay plate. This latter feature also allows K^+ current averaging to compensate for cell-to-cell variations in current amplitudes and kinetics. They have been used in screening campaigns for libraries approaching 200,000 compounds.

Automated electrophysiology now provides high-quality data-rich information for driving structure–activity relationship (SAR) and an ability to explore mechanism of action early in screening. Initially developed and used for recombinant ion channels, either stably or transiently transfected into cell lines such as CHO cells and HEK293 cells, the use of these techniques is now being expanded into mammalian cells such as neurons or smooth muscle cells, either freshly isolated or maintained in primary culture (Milligan et al. 2009). In the near future, there is interest in applying high throughput, automated patch-clamp to native cells and human induced pluripotent stem cells (see below). The development of dynamic clamp on an APC instrument, where "currents" of choice may be introduced electronically (Goversen et al. 2018), may prove to be a major breakthrough in the use of this technology to study more physiologically relevant channel properties and regulation (Obergrussberger et al. 2020).

There are still areas for consideration. For example, for the most part, stably transfected cell lines work best in these systems and there can be issues with expression levels and cost associated with this. Furthermore, the sheer volume of data generated requires consideration when deciding how best to analyse and display this. While many APC systems now provide high-quality voltage clamp data that approach traditional whole-cell patch-clamp currents, some instruments have sacrificed data quality in exchange for higher screening throughput (Obergrussberger et al. 2018). In addition, as in the case with fluorescent plate readers (above), the high initial start-up price and consumable costs involved in using APC systems often limit their application to large academic and pharmaceutical research facilities. As such, there remains considerable value in the continued development of lower-throughput, higher fidelity automated systems.

Impressively, the major commercial suppliers of these devices (Sophion, Nanion, Fluxion) have developed advanced programmes to reach out to academic and pharmaceutical researchers in order to develop and expand the range of devices and resources available to make them appropriate to as many needs as possible. The

CiPA initiative described below exemplifies the potential value of such open and constructive collaboration.

5 Quantification and Standardisation of Drug Action on K Channels

For pharmacologists, there is a desire to quantify the effects of agents which regulate receptor or ion channel activity in order to fully define their selectivity and their usefulness as tool compounds or therapeutic agents (Mathie et al. 2021a). It is also critical that measurements can be obtained that are as consistent as possible between different platforms and different research groups. In this regard, there are considerations around quantification and characterisation of pharmacologically active compounds on potassium channels which might enhance our understanding of how these channels function and are regulated and enable us to obtain more consistent and reliable measurements across studies.

Practically, for all of the techniques described above, there are issues around resting K channel activity. The activity and regulation of K channels can vary depending on the recording method used (flux assay versus patch-clamp electrophysiology; mammalian cell versus oocyte expression system). It may also vary between different channel isoforms (generated through splice variants or alternative translation initiation) or between wild type and mutated channels or channels from different species (see above, also Mathie et al. 2021a). It is important, therefore, that these variables are documented carefully between studies and varied as little as possible from one study to another.

When it comes to quantification of effect, again as much standardisation as possible is desirable. For example, for K channel activating compounds, should efficacy (measured as the percentage increase or absolute maximum increase in current evoked) or potency (measured as a calculated EC_{50} value) or both, be used as a quantitative measure of drug activity? If different measurements are used by different groups, comparisons are difficult. One possible solution, regardless of how the measurement is obtained or quantified, is to compare the effects of novel compounds with a known, well-characterised "standard". For example, in our studies of TREK-2 channel activators (see above), we routinely use BL-1249 as a standard activator of these channels and express the effects of other compounds both in absolute terms and in comparison with the effects of a defined concentration of BL-1249. However, this may not be so appropriate for compounds with a different site of action where the degree of tonic channel activity may influence the activity of a compound acting at one site more than a compound acting at a different site on the channel.

The need for consistent and robust quantification of the effects of drugs on ion channels has, perhaps paradoxically, been addressed best by the need to minimise the risks caused by drugs modifying the activity of ion channels in the heart.

5.1 hERG Channels and the CiPA Initiative

Inhibition of hERG ($K_V11.1$) potassium channels by drugs can lead to a concentration-dependent prolongation of the QT interval, a condition described as long QT syndrome and associated with an increased risk of cardiac proarrhythmia (Sanguinetti and Tristani-Firouzi 2006). A number of drugs have been withdrawn from use or had their indications severely limited in many countries because of their propensity to inhibit hERG channels. These include astemizole (an antihistamine), terfenadine (also an antihistamine) and cisapride (which stimulates gastrointestinal motility) (Mathie 2010). hERG channels are also inhibited by a wide range of commonly used drugs such as chlorpromazine, imipramine and amitriptyline (Redfern et al. 2003). As such, pharmaceutical companies have been compelled to introduce preclinical testing of all new chemical entities for hERG-channel blocking activity during preclinical trials (Redfern et al. 2003, but see Kramer et al. 2013). This includes the incorporation of higher throughput screens for ion channels.

More recently, based on a more comprehensive understanding of cardiac electrophysiology and cellular mechanisms underlying the drug-induced abnormal heartbeat, torsade de pointes (TdP), the Comprehensive in vitro Proarrhythmia Assay (CiPA) initiative was established with the aim of integrating multi-ion channel pharmacology measured in vitro into in silico models to assess TdP risk (Sager et al. 2014; Brinkwirth et al. 2020; Kramer et al. 2020; Ridder et al. 2020). If successful, this programme may facilitate the use of nonclinical data as part of an integrated risk assessment strategy to inform clinical decision making, improve accuracy and reduce costs in predicting cardiac liability of new drug candidates. The programme has three primary strands. The first is to characterise and quantify, in vitro, the effects of drugs on multiple cardiac ion channels, the second to accurately reconstruct the cardiac action potential in silico. The third strand is to confirm predictions made using the in silico model using human stem cell derived cardiac myocytes (Su et al. 2021).

As a result, this initiative has brought together a consortium of commercial and academic laboratories, including several CROs, to extend and integrate the use of electrophysiology-based cardiac ion channel screening on six primary cardiac ion channels, including four potassium channels: hERG ($K_V11.1$), KCNQ1 ($K_V7.1$) + KCNE1, $K_{ir}2.1$ and $K_V4.3$ (see Fig. 4).

An important component of this initiative was an attempt to quantify cross-site and cross-platform variability in the study of hERG ($K_V11.1$) channels (Kramer et al. 2020). It is clear that despite best attempts to minimise variability, differences in experimental protocols, instruments and procedures introduce variability and affect IC_{50} values characterising the effects of drugs. The study utilised 12 centrally-supplied, blinded drugs and tested them against hERG and two other non-K channels involved in the cardiac action potential at multiple (17) sites using five APC platforms but using, as far as possible, a standard operating protocol. Whilst many of the results were similar across sites and platforms, there were some notable differences. For example, there was a 10.4-fold variance in IC_{50} for dofetilide block of hERG channels (12–103 nM) across 14 sites, four platforms. This is a

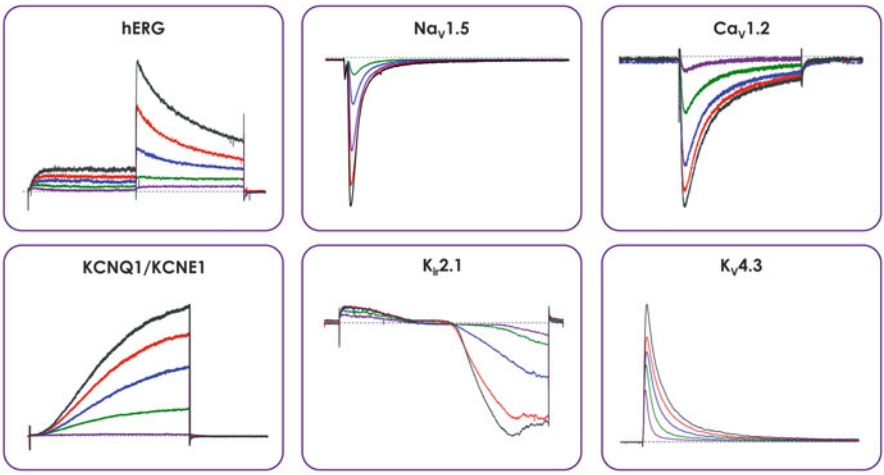

Fig. 4 Ion channel currents investigated in the CiPA protocol. Four different potassium channel currents are studied as part of this protocol: hERG ($K_V11.1$), KCNQ1 ($K_V7.1$) co-expressed with KCNE1, $K_{ir}2.1$ and $K_V4.3$. Other channels that form part of the study are the voltage-gated sodium channel, $Na_V1.5$ and the voltage-gated calcium channel, $Ca_V1.2$. Figure kindly provided by Dr. John Ridley, Dr. Andrew Southan, Dr. Robert Kirby, Dr. Marc Rogers and their colleagues at Metrion Biosciences

potential issue because the whole tenet of the CiPA programme is to develop in silico modelling based on reliable and reproducible IC_{50} values from APCs.

There are a number of potential explanations for variability in the data. The kinetics of block are often important when considering hERG blockers. This depends on the protocols chosen which may have shown subtle variability across platforms, despite the fact that certain quality control parameters (Rm, leak current, baseline current) were applied across all platforms and sites and a standard fitting process used for all concentration response relationships. Other suggested sources of inconsistency include equilibration times, variability in drug forms and concentrations, expressed versus background currents, intracellular buffer and temperature. Temperature, in particular, may be a wider problem for the CiPA programme as experiments are primarily done at room temperature. For example, Lo and Kuo (2019) have described the temperature dependence of amiodarone block of hERG channels and found it to be much more voltage sensitive at 37°C compared to 22°C. This may be an important consideration when extrapolating the CiPA in vitro data for drugs on ion channels collected at room temperature compared to the situation in patients at body temperature.

Despite these sources of variability, the CiPA project represents by far the best attempt to date to obtain reliable and consistent quantitative data of the effects of drugs on ion channels. The project consistently measures and collates these differences and makes strenuous efforts to eliminate or minimise them or at least take them into consideration when evaluating drug action. As such, in our view, the CiPA project, despite having the primary aim of minimising the adverse effects of

drugs, sets an outstanding example for future research models aimed at developing novel therapeutic drugs acting on ion channels.

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