


## WHITE PAPER

# Potassium channels in the heart: structure, function and regulation

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**Abstract** This paper is the outcome of the fourth UC Davis Systems Approach to Understanding Cardiac Excitation–Contraction Coupling and Arrhythmias Symposium, a biannual event that aims to bring together leading experts in subfields of cardiovascular biomedicine to focus on topics of importance to the field. The theme of the 2016 symposium was ‘K<sup>+</sup> Channels and Regulation’. Experts in the field contributed their experimental and mathematical modelling perspectives and discussed emerging questions, controversies and challenges on the topic of cardiac K<sup>+</sup> channels. This paper summarizes the topics of formal presentations and informal discussions from the symposium on the structural basis of voltage-gated K<sup>+</sup> channel function, as well as the mechanisms involved in regulation of K<sup>+</sup> channel gating, expression and membrane localization. Given the critical role for K<sup>+</sup> channels in determining the rate of cardiac repolarization, it is hardly surprising that essentially every aspect of K<sup>+</sup> channel function is exquisitely regulated in cardiac myocytes. This regulation is complex and highly interrelated to other aspects of myocardial function. K<sup>+</sup> channel regulatory mechanisms alter, and are altered by, physiological challenges, pathophysiological conditions, and pharmacological agents. An accompanying paper focuses on the integrative role of K<sup>+</sup> channels in cardiac electrophysiology, i.e. how K<sup>+</sup> currents shape the cardiac action potential, and how their dysfunction can lead to arrhythmias, and discusses K<sup>+</sup> channel-based therapeutics. A fundamental understanding of K<sup>+</sup> channel regulatory mechanisms and disease processes is fundamental to reveal new targets for human therapy.

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**Abbreviations** AF, atrial fibrillation; AP, action potential; APD, action potential duration; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; COP, coatamer associated protein; cryo-EM, cryo-electron microscopy; ER, endoplasmic reticulum; HF, heart failure; hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte; IPC, ischaemic preconditioning; K<sub>v</sub>, voltage-gated K<sup>+</sup>; LQTS, long QT syndrome; MD, molecular dynamics; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; REFER, rate-equilibrium free energy; RMP, resting membrane potential; SR, sarcoplasmic reticulum; SUR, sulfonylurea receptor; VSD, voltage sensor domain.

## Voltage-gated K<sup>+</sup> channel structure and function

*In this session Toby Allen, Francisco Bezanilla, Jianmin Cui, Peter Larsson, Daniel Minor, Sergei Noskov, Jon Sack, Matthew Trudeau, Jamie Vandenberg and Vladimir Yarov-Yarovoy participated as speakers, discussion leaders, or panelists.*

Voltage-gated K<sup>+</sup> (K<sub>V</sub>) channels are gated pores in the membranes of excitable cells that open and close in response to changes in transmembrane voltage to allow the selective passage of K<sup>+</sup> ions across the cell membrane (Bezanilla, 2000). The existence of K<sub>V</sub> channels was first inferred over 60 years ago through a combination of functional and theoretical studies in the squid giant axon (Hodgkin & Huxley, 1952). Many of the principles underlying how these channels sense change in membrane voltage and then transduce that into the opening and closing of the voltage-dependent gates are now well established, but there is still a way to go before we will understand voltage sensing and voltage sensor to pore coupling at the same atomic level resolution that we understand the basis of ion selectivity (Zhou *et al.* 2001; Medovoy *et al.* 2016).

Our current understanding of the molecular basis of cardiac K<sup>+</sup> channel function, including selective ion permeation, voltage sensing and gating, is derived from a combination of experimental observations and modelling. Heterologous expression of cloned channels has greatly facilitated quantitative biophysical and structural studies of K<sup>+</sup> channels. Static structures of prokaryotic K<sup>+</sup> channels (e.g. KcsA, MthK, K<sub>V</sub>AP, K<sub>ir</sub>Bac1.1, K<sub>ir</sub>3.1/K<sub>ir</sub>Bac1.3 chimera) and eukaryotic K<sup>+</sup> channels (e.g. K<sub>ir</sub>2.2, K<sub>V</sub>1.2) determined by X-ray crystallography and more recently by cryo-electron microscopy (cryo-EM) for Slo2.2 (Hite *et al.* 2015) have provided crucial information for constructing homology models of cardiac K<sup>+</sup> channels whose structures have yet to be solved. These structures have also enabled molecular dynamics (MD) simulations that provide insights into the mechanisms of ion selectivity and permeation, channel gating and ligand binding at an atomic level of detail. Structures have not yet been determined for most of the cardiac K<sup>+</sup> channels. However, structures of highly related mammalian K<sup>+</sup> channels (i.e. K<sub>V</sub>1.2, K<sub>ir</sub>3.2 and Slo2.2) as well as the cytoplasmic domains of K<sub>ir</sub>2.1, K<sub>ir</sub>3.1 and K<sub>V</sub>11.1 are available and provide useful templates to construct homology models.

### Questions, controversies, challenges:

- What do we know about the mechanisms of voltage sensing?

In a seminal series of papers published in 1952, Hodgkin and Huxley identified many of the critical features of

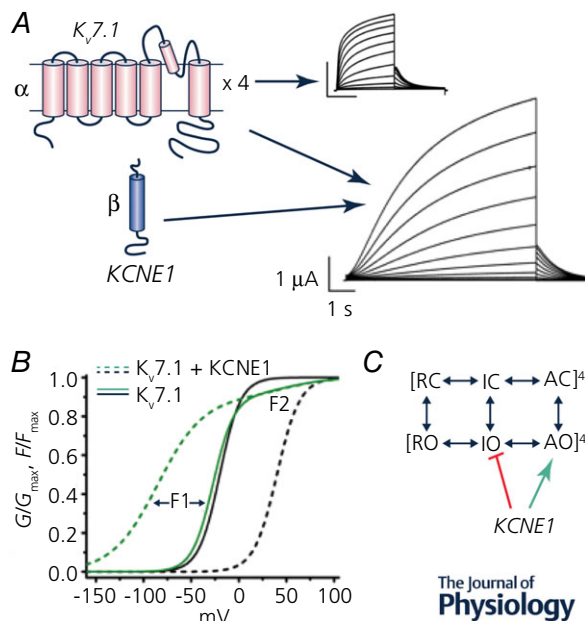
voltage-gated channels; however, we are still trying to work out the precise details of the structural basis of channel function. Perhaps the most prescient prediction made by Hodgkin and Huxley, was that ‘changes in ionic permeability depend on the movement of some component of the membrane which behaves as though it had a large charge or dipole moment’ (Hodgkin & Huxley, 1952). Twenty years passed before the small transient current associated with the movement of this charge, the gating current, was first recorded (Armstrong & Bezanilla, 1973). Measuring gating currents remains to this day at the centre of ongoing efforts to understand how voltage sensors work (see e.g. Lacroix *et al.* 2014). It was another decade before cloning of the first members of the voltage-gated ion channel family led to the identification of the S4 transmembrane domain, with its multiple arginine residues, as the principal component of the voltage-sensing domain in these channels (Noda *et al.* 1984; Papazian *et al.* 1987). In the last 20 years, the development of methods to attach chemical probes ranging from H<sup>+</sup> (Starace & Bezanilla, 2001) to fluorophores (Mannuzzu *et al.* 1996) to electron paramagnetic resonance probes (Cuello *et al.* 2004) has allowed indirect monitoring of the motion of the voltage-sensor domain. These spectroscopic methods have complemented gating current measurements in our quest to understand the extraordinary molecular gymnastics that underlie the function of these channels. Recent studies utilizing these techniques have revealed the gating mechanisms for several cardiac K<sub>V</sub> channels, including K<sub>V</sub>11.1 (ERG1) (Es-Salah-Lamoureaux *et al.* 2010), K<sub>V</sub>1.5 (Vaid *et al.* 2009), and I<sub>Ks</sub> channels formed by co-assembly of K<sub>V</sub>7.1 with KCNE1 (minK) accessory subunits (Barro-Soria *et al.* 2014; Zaydman *et al.* 2014).

In most K<sub>V</sub> channels that have been investigated, the voltage dependence of the voltage sensor domain (VSD) movement occurs over a more negative voltage range than that required for channel activation. Channel opening only occurs after all the VSDs have been activated, and involves a final highly cooperative state transition. In a process called electromechanical coupling, S4–S5 linkers act as mechanical levers that couple outward movement of the S4 segments to opening of the pore domain (Long *et al.* 2005b; Jensen *et al.* 2012). Recent studies have revealed that gating of K<sub>V</sub>7.1 and K<sub>V</sub>11.1 channels differs from this canonical model. Quite unexpectedly, channel gating is relatively normal when K<sub>V</sub>11.1 subunits are split at the S4/S5 linker into separate VSD and pore domains and heterologously expressed in oocytes (Lorinczi *et al.* 2015). Split K<sub>V</sub>1 or K<sub>V</sub>7.1 channels are non-functional. Thus, a rigid covalent linkage between the S4 and S5 segments is not required for normal gating of all K<sub>V</sub> channels. Given the widely accepted canonical model of K<sub>V</sub> channel gating, recent insights into the unusual gating mode adopted by

$K_{V7.1}$  channels and its modulation by KCNE1 subunits (described below) were equally unexpected.

Compared to current conducted by  $K_{V7.1}$  homotetrameric channels,  $K_{V7.1}$ -KCNE1 heteromeric channel currents (i.e. cardiac  $I_{Ks}$ ) are much larger, activate more slowly and at more positive potentials, do not exhibit inactivation and have altered pharmacology (Fig. 1A). A plethora of site-directed mutagenesis and cross-linking studies have guided structural modelling that strongly suggests that the single transmembrane domain of KCNE1 subunits nestles between the VSDs of adjacent  $K_{V7.1}$  subunits. This arrangement would permit a 4:4  $K_{V7.1}$ -KCNE1 stoichiometry. The naturally occurring stoichiometry and the mechanisms by which KCNE1 alters the gating of  $K_{V7.1}$  have been hotly debated. In heterologous expression systems,  $K_{V7.1}$ -KCNE1 stoichiometry has been shown to be either fixed at 4:2 (Plant *et al.* 2014), or variable from 4:1 to 4:4 (Nakajo *et al.* 2010; Murray *et al.* 2016), although the predominant  $K_{V7.1}$ -KCNE1 stoichiometry in native myocytes remains unclear. Ion channel gating can be quantified by measuring both gating and ionic

currents; however, it is not possible to quantitatively measure both signals at the same time. As summarized at the UC Davis meeting by Jianmin Cui, voltage clamp fluorometry of heterologously expressed channels that permits the simultaneous monitoring of ionic currents and VSD movement has recently revealed new and unexpected insights into the molecular mechanisms underlying KCNE1 modulation of  $K_{V7.1}$  channel gating. For these studies, a Cys residue was introduced into the S3-S4 linker of the cloned channel and labelled with a thiol-reactive fluorophore. As the VSD moves across the membrane in response to a stepped change in voltage, the local environment surrounding the tethered fluorophore is altered. The resulting change in the fluorescence emission of the fluorophore can be monitored with a photodiode at the same time ionic current is measured. Unlike a typical  $K_V$  channel, the voltage dependence of VSD movement (fluorescence-voltage or  $F-V$  relationship) and ionic currents (conductance-voltage, or  $G-V$  relationship) of  $K_{V7.1}$  channels essentially overlap (Fig. 1B). In striking contrast to  $K_V1$ , movement of individual VSDs can lead to channel opening of  $K_{V7.1}$ . The  $F-V$  relationship of KCNQ1 splits into two clearly separable components, F1 and F2. The large and fast F1 component represents movement of the VSDs at quite negative potentials and a smaller and slower F2 component can be measured at more positive voltages. The  $G-V$  relationship of  $K_{V7.1}$  nearly superimposes with the F1 component of  $F-V$  (Fig. 1B). KCNE1  $\beta$ -subunits drastically alter the F1 component of VSD movement in  $K_{V7.1}$ , but not the F2 component, and change the  $G-V$  relationship from being superimposed with F1 to overlapping with F2 (Fig. 1B; Osteen *et al.* 2010; Barro-Soria *et al.* 2014; Zaydman *et al.* 2014). A gating model for  $K_{V7.1}$ -KCNE1 channels that includes independent movements of all four VSDs (giving rise to the F1 component) and a final concerted conformational change in the S4 segments that corresponds to channel opening and the F2 component from the voltage clamp fluorometry experiments (Barro-Soria *et al.* 2014) can account for most experimental findings. The model is an oversimplification because it does not account for the many subconductance states of  $K_{V7.1}$ -KCNE1 channels that may correspond to partial openings in response to single VSD movements (Werry *et al.* 2013). This is in contrast to the Zaydman model that is simplified to transitions between a closed, intermediate-open and activated-open states ( $C \leftrightarrow IO \leftrightarrow AO$ ) and where F1 represents the C to IO transition and F2 represents the IO to AO transition (Fig. 1C). In this model, KCNE1 suppresses channel opening when VSDs are in the IO state such that  $K_{V7.1}$ -KCNE1 channels are only open in the AO state. Although the two gating models differ in details, both attribute KCNE1 effects to altered coupling between the VSDs and the pore domain. Recent findings have also shown that VSD-pore domain coupling in  $K_{V7.1}$



**Figure 1.** KCNE1  $\beta$ -subunits alter the gating of  $K_{V7.1}$  channels

**A**,  $K_{V7.1}$  channels are formed by co-assembly of four identical  $\alpha$ -subunits, each composed of six transmembrane segments. Co-assembly of  $K_{V7.1}$  channels with KCNE1  $\beta$ -subunits (single transmembrane domain) form slowly activating channels that conduct cardiac  $I_{Ks}$  in cardiac myocytes. **B**, KCNE1 subunits alter the voltage dependence of voltage sensor movement. The  $F-V$  relationship is shifted to more negative potentials, whereas the  $G-V$  relationship is shifted to more positive potentials. **C**, simplified kinetic model of  $K_{V7.1}$  and  $I_{Ks}$  channel gating. Fourth power notation symbolizes four VSDs per channel. KCNE1 subunits suppresses channel opening when the VSDs are in the intermediate-open (IO) state and stabilizes the activated-open (AO) state. Modified from Zaydman *et al.* (2014).

is dependent on phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) and ATP. PIP<sub>2</sub> binds to basic residues located between the VSD and pore domain (Zaydman *et al.* 2013), whereas ATP binds to basic residues located in the cytoplasmic C linker close to the S6 gate (Li *et al.* 2013).

### Questions and challenges:

- What remains to be elucidated about the mechanisms of voltage sensing?
- Closed state structures of K<sub>V</sub> channels remain elusive
- Can a combination of new approaches and MD simulations provide an atomic level view of voltage-dependent VSD movement?

Using spectroscopic methods it has been established that the VSD of K<sub>V1</sub> channels moves outwards in response to a depolarization and that this outward movement is likely to involve at least a 6.5 Å outward displacement combined with rotation of the S4 helix, which facilitates charge–charge pairing of successive arginine side-chains with relatively fixed counter charges in the S1–S3 segments of the VSD (Bezanilla, 2000). However, the flexibility of attached spectroscopic tags means that it is unlikely that these methods are going to enable us to get a more accurate measure of the extent of this motion. In theory we could obtain more accurate estimates if we had atomic resolution structures of both the up and down states of the VSD in intact K<sub>V</sub> channels. We have crystal structures of voltage gated K<sup>+</sup> channels in the activated (likely to be the open and inactivated) state (Long *et al.* 2005a, 2007). However, given the high entropy of the closed state, which can be inferred from the very shallow voltage dependence of gating current measurements at negative voltages, obtaining a crystal structure of a closed state voltage sensor conformation will be extremely difficult. We suggest that it will be slightly less challenging to get insights into the structure and location of the voltage sensor in the closed state using cryo-EM. First, the use of cryo-EM eliminates the need to crystallize the channel, which is one of the most challenging aspects of the structural analysis of membrane proteins using X-ray crystallography (Liao *et al.* 2013). Second, provided one can capture a sufficient number of images of single particles in the cryo-electron microscope then in theory it should be possible to capture channels with voltage sensors in the open (likely to be > 99.9%) and closed (< 0.1%) states at 0 mV (Kowal *et al.* 2014). Introduction of mutations that stabilize the down state conformation of the VSD would make it more feasible to capture a sufficient number of particles in the closed state.

Ultimately, even if we were to obtain static structures of the stable end states of VSDs we ideally would want to know the pathway by which these domains transit between the closed and open channel state conformations.

In particular, how do the positively charged arginines move through the ‘hydrophobic’ core to transfer charges from the intracellular to extracellular sides? One promising candidate to study the motion of arginine residues is the use of the sulfhydryl-reactive fluorophore monobromo-trimethylammonio-bimane (qBBr). qBBr fluorescence is quenched by tryptophan and, to a lesser extent, tyrosine – so one can monitor movement of qBBr relative to Trp/Tyr residues by monitoring changes in fluorescence (Semenova *et al.* 2009). Another possibility is applying the technique of rate-equilibrium free energy (REFER) analysis where one can infer the motion of a two-state gating transition by studying how mutations perturb the energetics of the transition state (proportional to the logarithm of perturbation to the kinetics of the reaction) relative to the energetics of the equilibrium distribution of the stable end states. This technique has been applied to study the gating of acetylcholine receptor channels (Auerbach, 2010) as well as inactivation gating in K<sub>V11.1</sub> channels (Wang *et al.* 2011), but never before applied to the motion of voltage sensors. In theory this technique can achieve residue level resolution. A more likely scenario, however, is that REFER analysis studies will be used for obtaining constraints for MD simulations.

### Challenges:

- Few cardiac K<sup>+</sup> channels structures have been solved
- MD simulations of channel function rarely include interactions with membrane lipids or other regulatory molecules

Recent progress in MD simulation, particularly with the introduction of the ANTON chips (Dror *et al.* 2012, Jensen *et al.* 2012) and structural modelling of ion channels using Rosetta (Yarov-Yarovoy *et al.* 2006, 2012; Pathak *et al.* 2007), suggests that we will soon be able to simulate the motion of the voltage sensor at atomic resolution. The movement of charged species through variable membrane electrical fields is however a more complex phenomenon than the movement of ions in aqueous solution and through the selectivity filter. Thus, it is possible that we will also need improvements in incorporation of the membrane potential into molecular simulations (Raval *et al.* 2016) before we will be able to achieve accurate simulations of the motion of VSDs in response to a changing membrane electric field. With the availability of two end-points in the VSD (or any other) gating pathway, one can construct and relax the transition pathway using one of several developed techniques – meta-dynamics, string or multi-dimensional umbrella sampling simulations to name a few (Lindahl & Sansom, 2008). The Roux and Perozo groups (Ostmeyer *et al.* 2013) used free energy sampling to investigate molecular mechanisms responsible for slow recovery from

inactivation in the bacterial channel KcsA. This technique allows detailed mapping of the equilibrium free energy surfaces underlying complex conformational transitions. The equilibrium surfaces extracted from free energy simulations can be used for various kinetic modelling schemes to model voltage- or concentration-dependent effects in dynamics of  $K^+$  channels, hence bringing modelling and experiments together (Perissinotti *et al.* 2015).

Several challenges remain before we can connect molecular modelling of cardiac  $K^+$  channels to experimental data and use this information for developing better therapeutic interventions. The most obvious challenge relates to the lack of molecular structures for most relevant cardiac channels. A viable strategy to refine 3D structures of cardiac  $K^+$  channel targets is to combine *de novo* structural modelling driven by experimental constraints from toxin binding, electrophysiology, cryo-EM and other techniques with MD simulations enabled by the ANTON platform. For example, the structural modelling of peptide toxin interaction with  $K_V$  channel VSDs allows the refinement of toxin-channel complex in the activated state of the voltage sensor (Gupta *et al.* 2015). Using the Rosetta molecular modelling suite and MD simulations, an integrated approach for modelling of the  $K_V11.1$  channel based on homologous  $K_V$  channel templates was developed (Lees-Miller *et al.* 2009; Subbotina *et al.* 2010).

Several recent studies have emphasized an important role for lipid-specific modulation in the normal function of several cardiac channels. The functional implications of  $PIP_2$  regulation of  $I_{KATP}$  were firmly established by Hilgemann and Ball 20 years ago (Hilgemann & Ball, 1996), and specific binding pockets for lipids were recently established (Lee *et al.* 2013). Other examples of lipids modulating ion channel function include cholesterol regulation of currents conducted by  $K_{ir}2.1$  (Romanenko *et al.* 2004; Rosenhouse-Dantsker *et al.* 2013) and polyunsaturated fatty acids in the cardiac membrane that may exert anti-arrhythmic activity by attenuating  $K_V7.1$  channel function (Liin *et al.* 2015). However, most (if not all) MD simulations of various cardiac  $K^+$  channels are performed in model one-component lipid bilayers. To start to address the role of lipid-channel interactions using molecular modelling, a model membrane with a more realistic lipid composition is desperately needed. The most recent developments in MD techniques and force-field parameters have yielded simulations of complex membranes with up to 65 different lipid types, including asymmetric component distribution in leaflets. These models provide a high-resolution view of the lipid organization of plasma membranes at an unprecedented level of complexity (Ingolfsson *et al.* 2014). The membrane simulations revealed a

complex and dynamic interplay of lipid components with emergence of transient domains varying in size of composition enriched in cholesterol, gangliosides or phosphatidylinositol mono-, bis- and trisphosphate panel (Ingolfsson *et al.* 2016). The next logical step for the field would be to investigate cardiac ion channel dynamics in the mixed lipid bilayers under a wide variety of conditions, including electrical fields and binding of peripheral membrane proteins. Taken together, simulations of cardiac ion channels with soluble domains in a complex bilayer will open an avenue to understanding specific lipid regulation of cardiac function at the supra-molecular level.

Can we build a complete model of an arbitrary  $K^+$  channel, including its regulation, in a cardiac myocyte? The answer is conceptually 'yes', but we are not there yet. A rapidly emerging area for future research is the development of better approaches for integration of multi-scale processes. For example, lipid peroxidation or residue ionization in ion channels in response to changes in intracellular pH can result in significantly altered functional dynamics, but MD simulation is limited in its ability to model bond-formation and bond-breaking events. The incorporation of various cellular signals such as phosphorylation, glycosylation and binding of short regulatory peptides to  $K_V$  channels into simulations of conformational dynamics and corresponding responses to perturbation remains challenging. More generally, understanding of complex interactions between  $K_V$  channels and various peripheral membrane or soluble proteins requires the development of supra-molecular (non-atomistic) models of protein-protein interactions. The coupling between the cytoskeleton and lipid-modulated function of various  $K^+$  channels (e.g.  $K_{ATP}$ ) while established experimentally, has remained underexplored and the mechanisms responsible for large macro-molecular assembly organization and conformational dynamics are largely unknown. A strategy that incorporates future experimental, structural and modelling studies will be a fundamental test of our understanding of the physical and chemical basis of molecular interactions between  $K_V$  channel modulators, lipid environment and channel at the atomic level. Importantly, this research will enable targeting of any  $K_V$  channel subtype for treatment of cardiac disorders and for monitoring the activity of specific  $K_V$  channel types.

### Cardiac $K^+$ channel regulation

*In the sessions related to  $K^+$  channel regulation, Tamas Banyasz, Nipavan Chiamvimonvat, Laszlo Csernoch, Isabelle Deschenes, Dobromir Dobrev, Eleonora Grandi, Jordi Heijman, Leighton Izu, Robert Kass, Jonathan Lederer, Yu-Fung Lin, Jon Makielski, Manuel F. Navedo, Jeanne Nerbonne, Colin Nichols, Brian O'Rourke, Catherine Proenza, Zhilin Qu, Randall Rasmusson, Ursula Ravens,*

*Crystal Ripplinger, Gail Robertson, Fernando Santana, Daisuke Sato, James Trimmer, Stefan Wagner, Heike Wulff and Antonio Zaza participated as speakers, discussion leaders, or panelists.*

K<sup>+</sup> channels play a critical role in cardiac electrophysiology, and their dysfunction links intracellular signalling, metabolism, remodelling and arrhythmogenesis in many cardiovascular diseases. Below, we discuss our current understanding of cardiac K<sup>+</sup> channel types and mechanisms for their (dys)regulation. The role of K<sup>+</sup> channels in the normal and diseased heart is discussed in more detail in a second white paper (see Chiamvimonvat *et al.* in this issue).

### Types of K<sup>+</sup> channels in heart

Numerous K<sup>+</sup> channel types are expressed in the sarcolemmal membrane of the heart (Table 1), where they exert precise control over action potential (AP) duration (APD) (Fig. 2) (Bartos *et al.* 2015). Each K<sup>+</sup> channel type has distinct kinetics and regulation, allowing control over particular phases of the AP. K<sub>V</sub> channels include the slow, rapid and ultra-rapid delayed rectifiers ( $I_{Ks}$ ,  $I_{Kr}$  and  $I_{Kur}$ ), and the components that rapidly and slowly recover from inactivation of the transient outward current ( $I_{to,f}$  and  $I_{to,s}$ , respectively). Inward-rectifier K<sup>+</sup> current ( $I_{K1}$ ) is indirectly voltage-dependent, a consequence of channel block by intracellular polyamines and Mg<sup>2+</sup>. Total  $I_{to}$  rapidly activates during the AP upstroke and produces a fast and early phase of repolarization. Subsequent activation of  $I_{Kur}$ ,  $I_{Kr}$  and  $I_{Ks}$  controls the AP plateau and APD in large mammals. Activation of  $I_{K1}$  at negative membrane potentials is responsible for the final repolarization and the resting membrane potential (RMP; Fig. 2) (Nerbonne & Kass, 2005, Schmitt *et al.* 2014). Ligand-gated K<sup>+</sup> currents in the heart include the acetylcholine-dependent inward-rectifier K<sup>+</sup> current ( $I_{K,ACh}$ ), the ATP-sensitive K<sup>+</sup> current ( $I_{KATP}$ ), the small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (SK) current ( $I_{SK}$ ), the intracellular Na<sup>+</sup>-activated K<sup>+</sup> current ( $I_{KNa}$ ) and the cAMP-activated pacemaker or 'funny' current ( $I_f$ ), which is carried by both K<sup>+</sup> and Na<sup>+</sup> (Bartos *et al.* 2015, Nerbonne & Kass, 2005, Schmitt *et al.* 2014). Several members of the large family of two-pore-domain K<sup>+</sup> (K<sub>2P</sub>) channels, including TREK-1 (K<sub>2P</sub>2.1) and TASK-1 (K<sub>2P</sub>3.1), are expressed in the heart and contribute to the background plateau current  $I_{KP}$  and the steady-state K<sup>+</sup> current ( $I_{SS}$ ) in rodent hearts. K<sub>2P</sub> channels are regulated by many factors, including temperature, pH, oxygen and membrane stretch (Schmitt *et al.* 2014). Besides these sarcolemmal K<sup>+</sup> channels, several ion channels are located on subcellular membranes, including mitochondrial and nuclear K<sub>ATP</sub> channels and sarcoplasmic reticulum (SR) K<sup>+</sup> channels involved in normalizing SR potential during Ca<sup>2+</sup> release (Takeshima *et al.* 2015).

### Challenges:

- Difficulties in isolating individual K<sup>+</sup> current types
- Incomplete knowledge of the molecular basis of K<sup>+</sup> channel types and their dynamic role during the AP
- Detailed *vs.* simplified mathematical models of K<sup>+</sup> channel gating
- Inconsistent and incomplete datasets for modelling

Several challenges complicate the experimental identification of the precise contribution to repolarization of each K<sup>+</sup>-current type, even in isolated cardiomyocytes. During patch-clamp experiments currents are often separated by use of drugs or toxins to block specific currents. However, many of these compounds are not as selective as commonly assumed. Ion channels are also often studied in heterologous expression systems, which facilitates detailed characterization of their biophysical properties independent of the availability of specific blockers; however, incomplete understanding of the macromolecular complexes that produce native currents and the absence of cofactors endogenous to cardiomyocytes make it difficult to recapitulate all regulatory mechanisms in such systems (see below). Experimental protocols for myocyte isolation and culture and the absence of electrical interactions with the surrounding myocardium can alter ion channel expression and function, further complicating the identification of the role of individual K<sup>+</sup> currents in cardiac electrophysiology and arrhythmogenesis *in vivo*. *Ex vivo* tissue preparations, such as the isolated arterially perfused left ventricular wedge and the Langendorff-perfused heart, also suffer from technical limitations (e.g. hypoxia) and are devoid of neurohumoral inputs, so direct extrapolation of some findings to *in vivo* effects is difficult. Further improvements in molecular biological approaches to down-regulate (e.g. siRNA, CRISPR/Cas9) or overexpress (e.g. lentiviral infection) specific proteins in intact cardiomyocytes and living animals may help to clarify the molecular correlates of individual K<sup>+</sup> currents and facilitate the analysis of their contribution to cardiac electrophysiology and arrhythmogenesis independent from pharmacological tools.

Because of the complexity of the cardiac system, mathematical models and simulation are powerful tools to investigate the role of K<sup>+</sup> channels under normal and pathological conditions. The Hodgkin–Huxley formalism is widely used in cellular electrophysiological models for quantitative characterization of K<sup>+</sup> channels. However, gating parameters in this formulation do not explicitly represent channel kinetic states and are sometimes unable to accurately describe certain channel behaviours or channel–drug interactions (Liu & Rasmusson, 1997; Rudy & Silva, 2006; Bett *et al.* 2011). Markov model transitions typically represent specific channel movements that have been characterized experimentally (e.g. subunit

**Table 1. Cardiac K<sup>+</sup> currents and their molecular identities ( $\alpha$ -subunits)**

| Current      | Protein       | Gene                               | Location in humans          | Notes   |
|--------------|---------------|------------------------------------|-----------------------------|---|
| $I_{Kr}$     | Kv11.1        | <i>hERG (KCNH2)</i>                | 7q36.1                      |   |
| $I_{Ks}$     | Kv7.1         | <i>KCNQ1</i>                       | 11p15.5                     |   |
| $I_{to,f}$   | Kv4.2/4.3     | <i>KCND2/3</i>                     | 7q31<br>1p13.3              | Gene and protein names depend on species; significant expression differences among species and regions of the heart |
| $I_{to,s}$   | Kv1.4         | <i>KCNA4</i>                       | 11p14                       |   |
| $I_{Kur}$    | Kv1.5         | <i>KCNA5</i>                       | 12p13                       | Mostly expressed in atrial cells, and in ventricles for some species (e.g. rodents)                                 |
| $I_{K,slow}$ | Kv2.1         | <i>KCNB1</i>                       | 20q13.2                     |   |
| $I_{K1}$     | Kir2.1        | <i>KCNJ2</i>                       | 17q24.3                     |   |
| $I_f$        | HCN4          | <i>HCN4</i>                        | 15q24.1                     | Pacemaker cells   |
| $I_{K,ACh}$  | Kir3.1/3.4    | <i>GIRK1 (KCNJ3)/GIRK4 (KNCJ5)</i> | 2q24.1<br>11q24             | Both homo- and heteromers   |
| $I_{KATP}$   | Kir6.2        | <i>KCNJ11</i>                      | 11p15.1                     |   |
| $I_{K,Ca}$   | SK1, SK2, SK3 | <i>KCNN1, KCNN2, KCNN3</i>         | 19p13.1<br>5q22.3<br>1q21.3 | SK2 predominant isoform   |

cooperativity) and can represent the dependence of a given transition on the occupancy of different states of the channel. Many recent computational studies have highlighted the importance of accurately describing state-dependent drug binding, instead of relying on steady-state concentration–response curves or EC<sub>50</sub> values (Moreno *et al.* 2011; Aguilar & Nattel, 2015; Lee *et al.* 2016; Morotti *et al.* 2016). The choice between Hodgkin–Huxley and Markov models is a balancing act between need for detail and practicality of simplified models, particularly for multi-scale simulations.

Formulations for individual currents and validation of the whole-cell system require many experiments, which are typically performed in different laboratories for purposes other than model building. This situation often leads to incomplete (or non-physiological) datasets, with large variances that reflect differences in experimental conditions (e.g. temperature, solutions, species) rather than true physiological variability (Krogh-Madsen *et al.* 2016). The re-use of model components based on data from other species often masks the link between model parameters and the original data source (Niederer *et al.* 2009). These inconsistencies might be accentuated in disease models, due for example to different severities and/or extents of disease-induced remodelling. Thus, use of consistent, well-defined data sets and dedicated experiments would be ideal to fit model parameters and validate model responses over the full range of physiological conditions. Moreover, commonly used voltage-clamp protocols predominantly provide information about steady-state ion-channel properties (e.g. activation, inactivation), or assess a single dynamic

parameter for a specific condition (e.g. recovery from inactivation at a given membrane potential). Such protocols do not always provide sufficient information to fully characterize channel kinetics during the complex dynamics of the AP. A new approach to build cell-specific cardiac models was recently developed by coupling genetic algorithm parameter fitting with complex electrophysiology protocols, which allow extracting dynamically rich information inherent to arrhythmias (Groenendaal *et al.* 2015).

### Diversity of cardiac K<sup>+</sup> currents

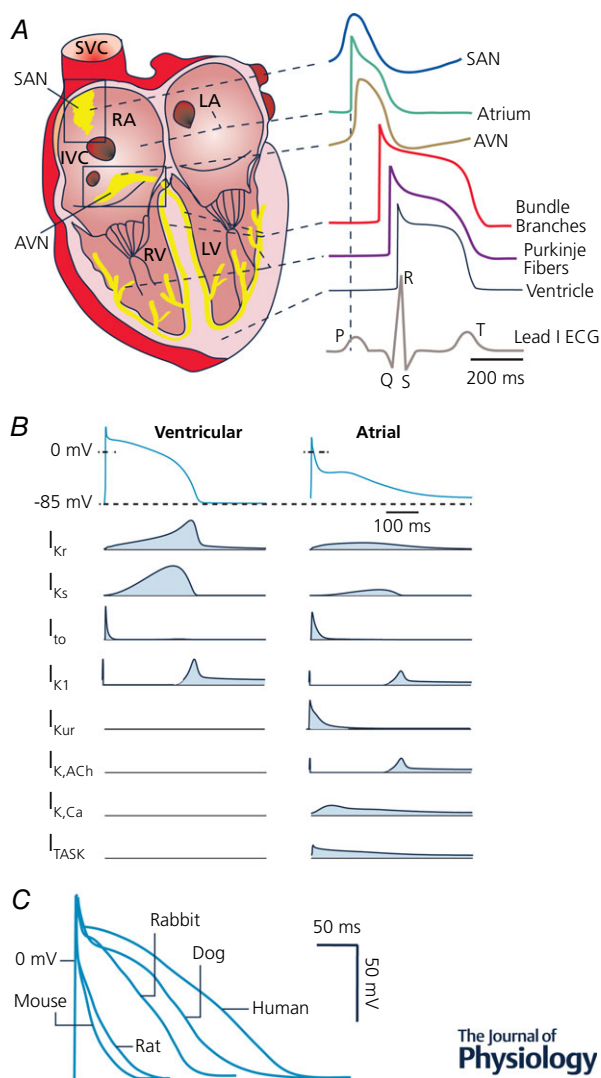
The pronounced heterogeneity in AP morphology and duration among different species and regions of the heart is in large part due to differential expression of multiple K<sup>+</sup> channel types (Fig. 2A and B). APD is much shorter and more triangular in rodents compared to humans (Fig. 2C). However, even large mammals such as the dog, which appear to have reasonably comparable electrophysiological characteristics to humans, respond differently to pharmacological interventions targeting K<sup>+</sup>-channels (O'Hara & Rudy, 2012; Jost *et al.* 2013), indicating a species-dependent contribution of individual currents.

In all species, sinoatrial and atrioventricular cells show spontaneous diastolic depolarization mediated by a complex system, which includes the HCN channel-mediated  $I_f$  in combination with a low  $I_{K1}$  expression. Atrial and ventricular cardiomyocytes have a stable RMP, but distinct AP morphology (Fig. 2A and B). On average, atrial cardiomyocytes have a more depolarized

RMP than ventricular cardiomyocytes due to a smaller  $I_{K1}$ , and a more triangular AP shape due to the atrial-specific expression of  $K_{V1.5}$  ( $I_{Kur}$ ). Further differences have been identified between the left and right sides of the heart, including a larger  $I_{to}$  and  $I_{Ks}$  in the right compared to the left ventricle (Molina *et al.* 2016), and between the different layers of the ventricular wall, with a much larger  $I_{to}$  near the epicardium than near the endocardium (Nerbonne & Guo, 2002).

### Challenges:

- Cell-to-cell variability
- Differential regulation of individual  $K^+$  currents



**Figure 2. Cardiac  $K^+$  channel diversity**

A, AP regional heterogeneity; redrawn from Bartos *et al.* (2015). B,  $K^+$  currents during ventricular and atrial APs; redrawn from Schmitt *et al.* (2014). C, species differences in ventricular AP; redrawn from Kaese & Verheule (2012).

- Limitations of model systems to study  $K^+$  channel-based therapy
- Regulation of  $K^+$  current in other coupled systems

Sequential measurements of  $I_{K1}$ ,  $I_{Ks}$  and  $I_{Kr}$  using AP-clamp in ventricular cardiomyocytes suggest that there is pronounced ( $\sim 10$ -fold) heterogeneity in  $K^+$  current amplitude between different cells (Banyasz *et al.* 2011). Because the action potential is shaped by a balancing act of all ionic currents, larger/smaller outward  $K^+$  currents can be counterbalanced by larger/smaller inward currents to generate normal action potentials. Modelling has predicted that similar electrophysiological behaviour can result from different sets of underlying conductances (Golowasch *et al.* 2002; Sarkar & Sobie, 2010), and experimental work suggests that compensatory changes in current densities maintain relatively stable function in response to changes in activity or overexpression of ion channels (MacLean *et al.* 2003). Variable cell-to-cell channel expression is often not adequately reported or taken into account in traditional cardiac electrophysiological experiments and models, which instead mostly use averaged data and build 'representative' cell models of a specific type. This might be problematic because averages over multiple samples can fail to characterize a system whose behaviour depends on interactions involving a number of highly variable components (Golowasch *et al.* 2002). Thus, range or variance of datasets should be reported, instead of mean values and standard errors. Population-based computational approaches, calibrated by dynamically rich datasets, allow simulating 'good enough solutions' (Weiss *et al.* 2012) and determining those which will adapt better to pathophysiological regulation. Indeed, they have proven valuable for understanding variability in cardiotoxicity (Sarkar & Sobie, 2010) and identifying drug targets with favourable anti-arrhythmic properties (Cummins *et al.* 2014).

The regional and cell-to-cell differences in  $K^+$  channels are dynamic and the contribution of a specific channel type may only become apparent after modulation of other channels. For example,  $I_{Ks}$  is small under basal conditions in ventricular cardiomyocytes of large mammals, with  $I_{Kr}$  providing most of the repolarizing current. However,  $I_{Ks}$  is significantly increased by sympathetic stimulation, reversing the relative dominance of  $I_{Ks}$  vs.  $I_{Kr}$  controlling AP repolarization (Banyasz *et al.* 2014).  $I_{Ks}$  also constitutes an important repolarization reserve that is activated upon loss of other repolarizing currents (Jost *et al.* 2013). Similarly,  $I_f$  does not have an important role in the ventricles under normal conditions. However, during pathological conditions, or after inhibition of  $I_{K1}$ , ventricular cardiomyocytes can show abnormal automaticity, which may be partly mediated by  $I_f$  (Miake *et al.* 2002). Differential regulation of individual  $K^+$



channels makes drug sensitivity dependent on the specific (patho)physiological conditions, and thus is a confounding variable in the study and quantitative description of channel regulation.

Differences in  $K^+$  channel expression have important implications for  $K^+$  channel-based pharmacological therapy in patients. On the one hand, the heterogeneity in  $K^+$  channel expression can be exploited to increase the selectivity of pharmacological interventions. For example, targeting channels predominantly expressed in the atria (e.g.  $K_{2P3.1}$ ,  $K_v1.5$ ,  $K_{ir3.1}$ ,  $K_{ir3.4}$  channels) for rhythm control of atrial fibrillation (AF) may avoid unwanted ventricular proarrhythmic side effects (Heijman *et al.* 2016). On the other hand, these differences raise questions about which model system is the most appropriate to study novel potential  $K^+$  channel-based therapies in patients. Mouse models have the advantages of low cost, ease of genetic manipulation, multi-scale and multi-system analyses, and a homogeneous genetic background for detection of small effects. However, the electrophysiological differences between mice and men (Fig. 2C) make these models less suitable to assess antiarrhythmic efficacy or proarrhythmic risk (Kaese & Verheule, 2012). Large animal models more closely resemble the human  $K^+$  channel distribution and cardiac repolarization time course (Fig. 2C), making them more suitable to evaluate pro- or antiarrhythmic effects. However, their application is restricted by costs, availability of clinically relevant disease models, and limited options for genetic manipulation. Of note are the long QT syndrome (LQTS) type 1 and 2 transgenic rabbits, LQT1 and LQT2 (Brunner *et al.* 2008). Recently, CRISPR/Cas9 was used successfully to genetically modify genomes in various species (Reggio *et al.* 2001; Hai *et al.* 2014; Ni *et al.* 2014), and has the potential to establish large animal models that can more faithfully mimic human cardiac physiology. Human hearts and isolated cardiomyocytes might represent the most clinically relevant samples to study  $K^+$  channel (dys)function. However, the limited amount of available tissue and the highly selected patient population (e.g. explanted hearts from end-stage heart failure (HF) patients, or atrial samples from patients undergoing open heart surgery), as well as the very limited control over confounding clinical variables make their routine application challenging. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a relatively novel option providing potentially unlimited supplies of human cardiomyocytes for any desired pathology. Unfortunately, the phenotype of hiPSC-CMs remains generally immature, including pronounced abnormal automaticity due to the relatively small  $I_{K1}$ . Electronic 'expression' of  $I_{K1}$  using an *in silico* interface and dynamic clamp has proven useful to stabilize the electrical behaviour of hiPSC-CMs, by maintaining a physiological RMP, eliminating

automaticity and reducing AP variability (Bett *et al.* 2013). Such increased stability enables the use of this preparation for quantitative analysis of electrophysiological properties. There are encouraging examples in which application of physical cues (electrical and mechanical, such as oriented growth, stimulation, relevant stiffness matrices, co-culture with fibroblasts and extracellular matrix scaffolds) can push maturation of hiPSC-CMs *in vitro* (Lundy *et al.* 2013; Zhu *et al.* 2014). The unknown genetic variation between different hiPSC-CM lines may also increase electrophysiological variability and limit reproducibility. However, recent advances in gene editing allow the creation of isogenic controls to study the effect of specific genetic variants on  $K^+$  channel regulation and APD (Bellin *et al.* 2013).

Cell types other than myocytes in the heart also express  $K^+$  channels. Cardiac (myo)fibroblasts express  $I_{K1}$ ,  $K_v$  channel currents and several  $Ca^{2+}$ -activated  $K^+$  currents (Yue *et al.* 2011). Their regulation may directly, via electrotonic interactions between fibroblasts and cardiomyocytes, or indirectly, by regulating fibroblast proliferation, fibrosis and paracrine signalling, modulate cardiac electrophysiology and arrhythmogenesis *in vivo*. Several  $K^+$  channels (or their regulatory subunits) are also expressed outside the heart, resulting in a variety of interconnected disease manifestations by their dysfunction. For example,  $K_v$  and  $Ca^{2+}$ -activated  $K^+$  channels in vascular smooth muscle regulate vascular tone and smooth muscle cell migration and proliferation (Ko *et al.* 2008). Similarly, although KCNE2 was first primarily considered a  $K_v$   $\beta$ -subunit in the heart and implicated in LQTS, it is now known to have a wide array of functions in the heart, stomach, thyroid and choroid plexus (Abbott, 2015). These findings highlight the need for a multi-scale systems biology approach to understanding  $K^+$ -channel regulation. A combination of different approaches, from heterologous expression systems to *in vitro* and *in vivo* animal studies, experiments in human samples, and computational models, is likely to be needed to obtain a complete understanding of the regulation of individual  $K^+$  channels and their (patho)physiological relevance.

### Mechanisms for regulation of cardiac $K^+$ channels

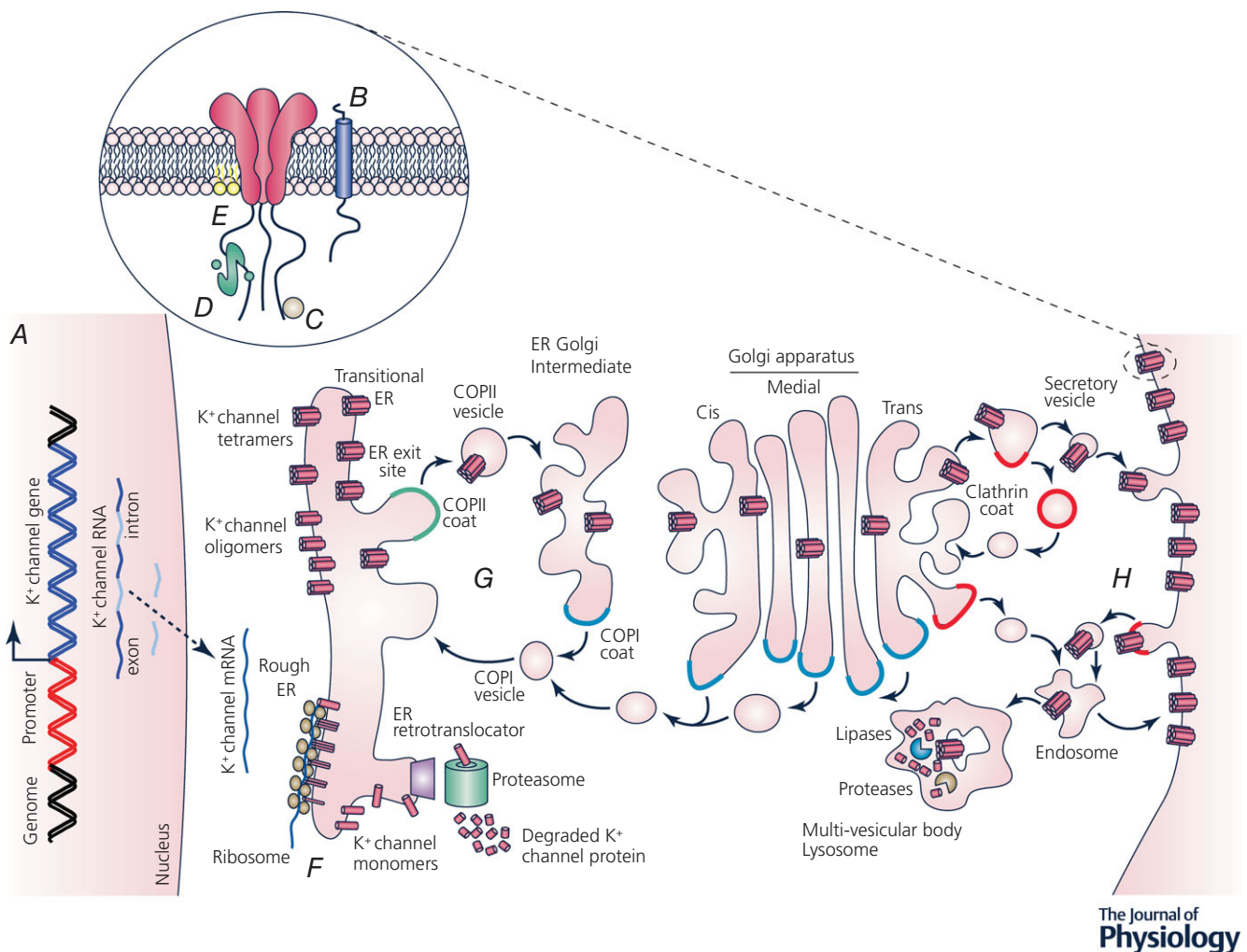
Given the critical role for  $K^+$  channels in determining repolarization rate, it is hardly surprising that  $K^+$  channel function is exquisitely regulated in cardiomyocytes (Fig. 3). Overlapping transcriptional, translational and post-translational mechanisms provide nuanced control over  $K^+$  currents across multiple time scales, ranging from milliseconds to days, or even longer. Regulatory mechanisms modify  $K^+$  currents by controlling channel expression levels, gating sensitivity and kinetics, ligand availability, and the structural states that determine conduction and drug binding. The

complex web of regulation allows for fine control of multiple  $K^+$  currents during different phases of the AP, and within different cardiac regions and cell types. Different (patho)physiological circumstances change the balance of regulatory mechanisms, and hence the predominant currents in a given cell type or region, resulting in highly context-dependent responses to pharmacological agents.

**Transcriptional control.** Transcriptional control of multiple  $K^+$  channels regulates their spatial and temporal profiles to modulate cardiac function during (patho)physiological challenges. For example, the transcription factors Irx5 (Costantini *et al.* 2005) and NFATc3 (Rossow *et al.* 2006) differentially regulate  $K_V4$  expression across the mouse ventricular wall, contributing to the  $I_{to,f}$  transmural gradient. Both Irx5 and NFATc3 have expression and activity profiles opposite that of  $K_V4$ -encoded  $I_{to,f}$ , and their independent deletion in the

heart completely abrogates the  $K_V4$ -encoded  $I_{to,f}$  gradient in some species (Costantini *et al.* 2005; Rossow *et al.* 2006). An expression and activity gradient has also been described for several  $I_{KATP}$  subunits in different cardiac compartments that is established by differential activity of the Forkhead transcription factors FoxO and FoxF2 (Philip-Couderc *et al.* 2008).

Non-coding RNAs (ncRNAs), including long non-coding RNAs and microRNAs, are emerging as important regulators of gene expression in cardiomyocytes (Greco & Condorelli, 2015; Tao *et al.* 2015) and are also associated with  $K^+$  channel remodelling in humans with AF and cardiac hypertrophy (Girmatsion *et al.* 2009; Matkovich *et al.* 2010). Although our understanding of the mechanisms by which ncRNAs regulate cardiac  $K^+$  channel expression and function is in its infancy, these regulatory mechanisms may provide the basis for development of novel therapies.



**Figure 3. Mechanisms for regulation of cardiac  $K^+$  channels**

A, transcriptional regulation; B, interacting proteins; C, post-translational modifications; D, ligands,  $Ca^{2+}$  and calmodulin; E, membrane lipids; F, co-assembly of subunits; G, forward trafficking, including packaging into COPII-coated vesicles, transport to ER-Golgi intermediate compartment and Golgi apparatus, and trafficking to specific membrane destinations into clathrin-coated vesicles; H, internalization.

**Questions:**

- How do physiological and pathophysiological remodelling differ, and how do they interact with K<sup>+</sup> channel regulatory mechanisms?

Aberrant K<sup>+</sup> channel expression and function contributes to electrical remodelling and arrhythmias in several disease states. In HF, AP prolongation is linked to reduced  $I_{to,f}$ ,  $I_{Ks}$ ,  $I_{Kr}$  and  $I_{K1}$  (Yang & Nerbonne, 2016), due in part to transcriptional downregulation of the underlying genes (Nass *et al.* 2008). Similarly, suppressed expression of K<sub>V</sub>4.3 and K<sub>V</sub>1.5 is correlated with reduced  $I_{to,f}$  and  $I_{Kur}$  density during AF (Van Wagoner *et al.* 1997; Yue *et al.* 1999). Downregulation in gene and protein expression levels of K<sub>V</sub>1.5, K<sub>V</sub>2.1 ( $I_{K,slow}$ ) and K<sub>V</sub>4.2/4.3, resulting from Ca<sup>2+</sup>-dependent activation of the calcineurin–NFATc3 signalling pathway (Rossow *et al.* 2009), leads to AP prolongation after myocardial infarction in mice (Rossow *et al.* 2004). A similar decrease in K<sup>+</sup> current density was observed during pathological cardiac hypertrophy caused by pressure overload (Marionneau *et al.* 2008), but was associated with cellular hypertrophy rather than changes in  $I_{to,f}$  and  $I_{K1}$  gene expression.

These observations distinctly contrast with physiological cardiac hypertrophy induced by chronic exercise, where cellular hypertrophy is accompanied by a concomitant increase in the expression of repolarizing ( $I_K$ ) and depolarizing ( $I_{Na}$ ,  $I_{Ca}$ ) currents that normalize the ionic conductances and AP waveform, leading to protection from arrhythmogenic events (Yang *et al.* 2010). These results also highlight the exquisite balance that must exist between K<sup>+</sup>, Na<sup>+</sup> and Ca<sup>2+</sup> conductances to generate a ‘normal’ APD. Evidently, distinct signalling pathways contribute to physiological (insulin-like growth factor 1/phosphatidylinositol-3-kinase  $\alpha$  signalling, independent of protein kinase B (Akt), leading to upregulation of ion channels, including K<sup>+</sup> channels) and pathological ( $G_{\alpha q}$  signalling downstream of G-protein coupled receptors activated by humoral factors) cardiac hypertrophy (Bernardo *et al.* 2010; Yang *et al.* 2012). However, the precise mechanisms underlying differential K<sup>+</sup> channel regulation in physiological *vs.* pathological hypertrophy remain poorly understood. A further understanding of these mechanisms and associated cardio-protective signalling pathways and genes could reveal targets for development of novel therapeutic options.

**Interacting proteins.****Questions, controversies, or challenges:**

- What are the interacting proteins in complex with cardiac K<sup>+</sup> channels, and how do they modulate function?
- Stoichiometry of channel complexes

An emerging concept is that formation of heterogeneous macromolecular channel complexes formed by pore-forming  $\alpha$ -subunit heteromultimers, auxiliary subunits and variable interacting proteins contribute to distinct biophysical properties, differential regulation, and regional- or species-dependent differences in K<sup>+</sup> current diversity.

K<sub>V</sub> $\beta$  subunits are co-assembled with multiple K<sub>V</sub> $\alpha$  subunits to promote trafficking, accelerate inactivation and even act as redox sensors. In ventricular myocytes, K<sub>V</sub> $\beta$ 1 influences the functional expression of K<sub>V</sub>4.3-encoded  $I_{to,f}$  and K<sub>V</sub>2.1-encoded  $I_{K,slow}$  via direct and indirect mechanisms (Aimond *et al.* 2005). Differential expression of KChIP2, which facilitates trafficking and function of K<sub>V</sub>4-encoded channels, is believed to underlie the epi- to endocardial  $I_{to,f}$  transmural gradient in large mammals (Rosati *et al.* 2001). KCNE subunits are single membrane-spanning proteins that associate with several K<sub>V</sub> $\alpha$  subunits to regulate channel activity. For example, co-assembly of KCNE1 and KCNQ1 slows KCNQ1 activation and generates  $I_{Ks}$  (Sanguinetti *et al.* 1996). Mutations in KCNE1 have been associated with LQTS and AF (Crump & Abbott, 2014). Surprisingly, a role for the voltage-gated Na<sup>+</sup> channel accessory subunit, Nav $\beta$ 1, in the functioning of K<sub>V</sub>4.2-encoded channels has also been demonstrated (Deschenes & Tomaselli, 2002).

Scaffolding proteins provide spatiotemporal targeting of regulatory molecules (e.g. kinases and phosphatases) to ion channels with important implications for cardiac function (Langeberg & Scott, 2015). Prototypes are A-kinase anchoring proteins (AKAPs), which dynamically regulate cardiac K<sup>+</sup> channel function via multiple mechanisms. Indeed, the AKAP Yotiao co-assembles with KCNQ1 channels to mediate protein kinase A (PKA)-dependent phosphorylation of KCNQ1 during sympathetic nerve stimulation (Marx *et al.* 2002). Conversely, the anchoring protein AKAP79/150 is required for calcineurin/NFATc3-mediated transcriptional downregulation and functional suppression of K<sub>V</sub> channels following myocardial infarction (Nieves-Cintrón *et al.* 2016). These observations underscore the extensive functional diversity that may coexist within a cell to contribute to a specific phenotype.

The stoichiometry of different channel subunits and splice variants within channel complexes can also profoundly alter the response to regulatory factors. However, the exact composition of subunit isoforms or variants within native channel complexes is difficult to determine experimentally and can vary considerably between different species and cardiac cell types. The mechanisms that determine the stoichiometric balance of different subunits are poorly understood, are often not recapitulated in heterologous expression systems, and may not be specified by amino acid sequence alone.

For example, subunit expression can be regulated by co-translational association with RNA (Liu *et al.* 2016).

**Post-translational modifications.** Several post-translational mechanisms can alter properties or expression levels of cardiac  $K^+$  channels. The most common and best characterized of these mechanisms is phosphorylation by protein kinases such as cAMP-dependent PKA, which phosphorylates  $K^+$  channels as part of the flight-or-flight response, and  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaMKII; Nerbonne, 2011; Mustroph *et al.* 2014), which is overexpressed and hyperactive in HF and persistent AF (Anderson *et al.* 2011). CaMKII acutely regulates  $I_{to}$  by increasing current density and hastening recovery from inactivation (Sergeant *et al.* 2005; Wagner *et al.* 2009; Zhang *et al.* 2015) via a mechanism that may involve direct phosphorylation of  $K_V4.2/K_V4.3$  ( $I_{to,f}$ ; Varga *et al.* 2004) and  $K_V1.4$  ( $I_{to,s}$ ; Roeper *et al.* 1997). CaMKII co-immunoprecipitates with both  $K_V4.2$  and  $K_V4.3$  (Colinas *et al.* 2006), suggesting that it may be a component of native cardiac  $I_{to,f}$  channel complexes. These complexes are composed of several cytosolic (KChIP/ $K_V\beta$ ) and transmembrane (DPP6/10, KCNEs) accessory subunits that determine the time- and voltage-dependent properties and surface expression of native cardiac  $I_{to,f}$  channels (Niwa & Nerbonne, 2010). Each of the protein components might also be a target for CaMKII-mediated regulation. In fact, there are multiple putative CaMKII phosphorylation sites on both KChIP2 and  $K_V\beta2$  (Nerbonne, 2011). Furthermore,  $K_V4.2/K_V4.3$  channels form complexes with SAP97 and CaMKII (El-Haou *et al.* 2009), and in neurons CaMKII-dependent phosphorylation of SAP97 enhances  $K_V4.2$  trafficking (Gardoni *et al.* 2007). Intriguingly, regional heterogeneity in  $I_{to,f}$  expression and function might be associated with heterogeneous regulation of  $I_{to,f}$  complexes. In contrast, chronic CaMKII overexpression that leads to HF development results in a reduction of  $I_{to}$  (Wagner *et al.* 2009), but this appears to be a secondary effect.

CaMKII may also regulate cardiac  $I_{K1}$  channels. CaMKII enhances  $I_{K1}$  acutely, but in failing ventricular myocytes overexpressing CaMKII $\delta$   $I_{K1}$  is attenuated and expression of *KCNJ2* (which encodes  $K_{ir}2.1$ ) is markedly reduced (Wagner *et al.* 2009). What are the mechanisms involved in the acute and chronic CaMKII-mediated regulation of native cardiac  $I_{K1}$  channels? Experiments focused on characterizing endogenous  $I_{K1}$  channel complexes and identifying CaMKII phosphorylation sites on  $K_{ir}2.1$ , as well as of other contributors to  $I_{K1}$ , are needed.

**Calcium.**  $Ca^{2+}$ -activated  $K^+$  channels provide a direct link between intracellular  $Ca^{2+}$  and membrane voltage with every heartbeat. Whether or not  $Ca^{2+}$ -activated  $K^+$  (or  $Na^+$ -activated Slo2.1) channels are important for normal cardiac electrophysiology has long been debated. Recent

evidence suggests that only SK channels contribute to cardiac repolarization. SK  $\alpha$ -subunits are constitutively bound to calmodulin and activate upon intracellular  $Ca^{2+}$  binding to the calmodulin EF hand ( $K_d \sim 500$  nM), which also regulates membrane expression (Zhang *et al.* 2015). While initial experiments revealed an atrial dominant expression pattern for SK channels in normal or non-diseased cardiomyocytes (with the specific blocker apamin prolonging atrial but not ventricular APD), subsequent studies also described SK currents in nodal, pulmonary vein and ventricular myocytes. Furthermore, in ventricular myocytes from diseased animal models or failing human myocytes, SK channel expression is upregulated, suggesting a role for these channels in pathological ventricular repolarization (Chua *et al.* 2011; Chang *et al.* 2013).

SK channel interaction with cytoskeletal proteins such as  $\alpha$ -actinin2 and filamin A is critical for cellular localization, which indirectly links SK channels to their intracellular  $Ca^{2+}$  source (Lu *et al.* 2007; Rafizadeh *et al.* 2014). Specifically, SK2 channels colocalize with  $\alpha$ -actinin2 and L-type Cav channels (Cav1.2/Cav1.3) along the Z-line in atrial myocytes (Lu *et al.* 2009), suggesting that SK channels may be controlled by subsarcolemmal  $[Ca^{2+}]$  via activation of L-type Cav channels (Marrion & Tavalin, 1998; Zhang *et al.* 2015). Interestingly, SR  $Ca^{2+}$  has been suggested as a source for activation of overexpressed SK channels (Terentyev *et al.* 2014), although overexpression can change subcellular localization, and thus could alter regulation compared to native SK channels.

Intracellular  $Ca^{2+}$  also indirectly regulates many other  $K^+$  channels in cardiomyocytes. For example, native and expressed  $K_V7.1/KCNE1$  ( $I_{Ks}$ ) channels are activated by intracellular  $Ca^{2+}$  in a CaM-dependent manner (Tohse, 1990; Ghosh *et al.* 2006). Many findings over the years have improved our understanding of how  $Ca^{2+}$  regulates  $K^+$  channels in the heart. Yet determining the precise mechanisms of  $Ca^{2+}$  sensitivity of  $K^+$  channels in cardiomyocytes remains technically challenging, in part because intracellular  $[Ca^{2+}]$  greater than 500 nM can cause unwanted cellular contraction during recordings.

**Membrane lipids.** Lipids may interact directly and dynamically with ion channels to alter properties such as channel conformation or gating, sarcolemma surface charge, and molecular affinity. The regulation of ion channels by the acidic phospholipid  $PIP_2$  is one of the best understood.

In the heart,  $PIP_2$  is necessary for proper function of numerous  $K^+$  channels via distinct mechanisms. Several  $K_{ir}$  channels are highly  $PIP_2$  dependent, only conducting current when  $PIP_2$  is bound. Studies of  $K_{ATP}$  channels in guinea pig ventricular myocytes were among the first to show rapid current rundown upon  $PIP_2$  depletion

and reactivation when PIP<sub>2</sub> is replenished (Hilgemann & Ball, 1996; Fan & Makielski, 1997). K<sub>ir</sub>2.1 affinity for PIP<sub>2</sub> is important for normal ventricular repolarization in humans. Many *KCNJ2* mutations associated with Andersen–Tawil syndrome reduce K<sub>ir</sub>2.1's affinity for PIP<sub>2</sub> and decrease *I*<sub>K1</sub> (Donaldson *et al.* 2003). Other cardiac K<sub>V</sub> channels such as K<sub>V</sub>11.1 (hERG/*I*<sub>Kr</sub>) and K<sub>V</sub>7.1 (KCNQ1/*I*<sub>Ks</sub>) also rely on PIP<sub>2</sub> for proper function. Activation of voltage-sensitive phosphatases or G<sub>q/11</sub>-coupled receptors causes PIP<sub>2</sub> depletion at the membrane, thus reducing PIP<sub>2</sub> association with K<sub>V</sub>7.1, and decreasing *I*<sub>Ks</sub> (Zaydman *et al.* 2013).

**Ligands.** Ligand-gated K<sup>+</sup> cardiac channels that conduct *I*<sub>KATP</sub>, *I*<sub>K,ACh</sub> and *I*<sub>f</sub> are also subject to the regulatory mechanisms described above, but their regulation is largely via control of ligand availability in response to physiological and pathophysiological conditions, such as metabolic status, autonomic nervous system activity and ischaemia. Cardiac K<sub>ATP</sub> channels are of particular translational interest, because they contribute to ischaemic preconditioning (IPC), in which short bouts of ischaemia protect the myocardium against subsequent more severe insults. *I*<sub>KATP</sub> activation shortens and may even prevent APs and Ca<sup>2+</sup> transients in myocytes that are ATP depleted, allowing energetically compromised cells to conserve energy (Lederer *et al.* 1989). They might synergize with gap junction channels, which are closed by acidosis and elevated diastolic [Ca<sup>2+</sup>]<sub>i</sub> (Spray *et al.* 1982). K<sub>ATP</sub> channels and their regulators have long been investigated as targets for development of new treatments for ischaemic heart disease.

K<sub>ATP</sub> channels are composed of four pore-forming K<sub>ir</sub>6 α-subunits plus four regulatory sulfonylurea receptor (SUR) subunits. K<sub>ATP</sub> channels are inhibited by ATP, which binds to K<sub>ir</sub>6 subunits, and activated by Mg<sup>2+</sup>-ADP, which binds to the SUR subunits. Channels are closed at physiological ATP concentrations, but open as the ATP/ADP ratio decreases during hypoxia or ischaemia. This regulation of channel activity is thought to be critical for IPC, which is prevented by K<sub>ATP</sub> blockers and mimicked by K<sub>ATP</sub> channel openers. However, different K<sub>ATP</sub> channels with distinct properties are expressed in the sarcolemma (sarc-K<sub>ATP</sub>) and the mitochondrial inner membrane (mito-K<sub>ATP</sub>) of cardiomyocytes. The location of K<sub>ATP</sub> channels that mediate IPC remains controversial (Garlid & Halestrap, 2012).

Sarc-K<sub>ATP</sub> channels clearly incorporate K<sub>ir</sub>6.2 and SUR2A subunits, and K<sub>ir</sub>6.1 and SUR1 may also contribute (Zhang *et al.* 2010). Sarc-K<sub>ATP</sub> channels are highly expressed in cardiomyocytes and have a large single channel conductance (~75 pS). Hence opening of even a small percentage of the channels on the sarcolemma is predicted to shorten APD. However, this simple model is belied by the observation of fairly normal ECGs with

no QT shortening in patients with Cantu syndrome, a multi-organ syndrome associated with gain-of-function mutations in SUR2 or K<sub>ir</sub>6.1 (Cooper *et al.* 2015; Levin *et al.* 2015). The lack of pronounced effects of sarc-K<sub>ATP</sub> activation on the ECG appears to be associated with increased L-type Ca<sup>2+</sup> current via a complex compensatory remodelling process, a situation that underscores how co-regulation of different types of ion channels poses challenges to the description of regulatory effects.

Mito-K<sub>ATP</sub> channels were first identified in giant fused mitoplasts (Inoue *et al.* 1991) and their primary function appears to be regulation of mitochondrial matrix volume. Identification of subunits that comprise mito-K<sub>ATP</sub> channels has been challenging owing to the difficulty of isolating pure mitochondrial membrane fractions and the lack of specific probes (i.e. drugs or antibodies). However, recent evidence suggests that ROMK2 subunits form the pore and SUR2 serve as the regulatory subunits in mito-K<sub>ATP</sub> channels (Foster *et al.* 2012). Mitochondrial large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK) channels have also been identified in the heart, where they may improve cardiac energetics (Singh *et al.* 2012).

## Trafficking and sequestration of K<sup>+</sup> channels

*Presenters, discussion leaders and panelists for this session included Geoffrey Abbott, Fadi Akar, Ravi Balijepalli, Nipavan Chiamvimonvat, Henry Colecraft, Brian Delisle, Isabelle Deschenes and Aldrin Gomes.*

### Questions:

- Are we using appropriate cell types (heterologous expression systems, cardiomyocytes, or hiPSC-CMs) to characterize channel trafficking?
- What possible roles do regulatory proteins (e.g. chaperones) play in the trafficking of wild-type or mutant K<sup>+</sup> channels?
- What is the impact of unfolded protein accumulation in the endoplasmic reticulum and how does this affect cellular physiology?
- How do we quantify the relative physiological importance of multiple isoforms encoded by the same gene?
- What are the roles that promiscuous interacting proteins have in regulating K<sup>+</sup> channel trafficking and channel stability?

As noted in the above sections, K<sup>+</sup> channel structure, function, diversity and regulation have been studied extensively. Much less is known about protein folding and trafficking events. A generalized trafficking scheme for the biogenesis and targeting of K<sup>+</sup> channels to the cell surface membrane is shown in Fig. 3. K<sup>+</sup> channel α-subunit helices are inserted in the endoplasmic reticulum (ER) membrane

during translation. The K<sup>+</sup> channel  $\alpha$ -subunit proteins co-assemble, and they are recruited into ER exit sites located in the smooth or transitional ER compartment. K<sup>+</sup> channels traffic out of the ER in coat protein (COP) II vesicles; undergo processing as they transit through the Golgi apparatus; and are then inserted into the cell surface membrane. The turnover of K<sup>+</sup> channels from the cell surface membrane is likely to involve clathrin- or caveolin-mediated endocytosis and degradation by proteases in the lysosomes.

Defects in K<sup>+</sup> channel folding can negatively affect co-assembly, trafficking and the membrane stability of K<sup>+</sup> channels. The meeting presentations centred on the mechanisms by which different LQTS-linked mutations in the genes encoding K<sub>V</sub>11.1 (*KCNH2*) and K<sub>V</sub>7.1 (*KCNQ1*) channels decrease the number of K<sup>+</sup> channels at the cell surface membrane.

An important finding is that the trafficking-deficient phenotype and functional expression for many mutant K<sub>V</sub>11.1 channels is improved by incubating cells with drugs that bind to K<sub>V</sub>11.1 channels (pharmacological correction) (Anderson *et al.* 2014). This finding indicates that some K<sub>V</sub>11.1 mutant channels are promising targets for therapeutic intervention. Heterologous expression studies in mammalian cell lines or hiPSC-CMs show that these trafficking-deficient K<sub>V</sub>11.1 channels are selectively retained in the transitional ER compartment (Smith *et al.* 2011; Smith *et al.* 2013). Although the transitional ER compartment contains ER exit sites from which COPII vesicles bud off for transport to the Golgi apparatus, the mutant K<sub>V</sub>11.1 channels do not undergo ER export because they are excluded from the ER exit sites. Pharmacological correction directly facilitates the trafficking of mutant K<sub>V</sub>11.1 channels sequestered in the transitional ER compartment (Smith *et al.* 2011, 2013). Drug treatment for as little as 30 min is sufficient to improve the functional expression of mutant K<sub>V</sub>11.1 channels at the cell surface membrane for many hours.

Several LQTS-linked K<sub>V</sub>11.1 mutations that do not undergo pharmacological correction disrupt the co-assembly of K<sub>V</sub>11.1  $\alpha$ -subunits (Ficker *et al.* 2002). Heterologous expression of these mutant proteins in cell lines or in hiPSC-CMs show that they exhibit a distinctive immunostaining pattern that consists of several aggregates around the cell nucleus. The K<sub>V</sub>11.1 aggregates do not strongly co-localize with several ER marker proteins, but their immunostaining partially overlaps with ER retrotranslocator channels and cytosolic proteins. These studies suggest mutant K<sub>V</sub>11.1 proteins that disrupt K<sub>V</sub>11.1  $\alpha$ -subunit co-assembly are retrotranslocated into the cytosol and targeted for degradation via the ubiquitin proteasome pathway.

Heterologous expression studies indicate that K<sub>V</sub>7.1  $\alpha$ -subunits expressed alone assemble and traffic well to

the cell surface. On the other hand, whether KCNE1 channel subunits expressed by themselves can target to the plasma membrane appears to be cell type dependent, and may be influenced by the presence of endogenous K<sub>V</sub>7.1 subunits in particular cells used for heterologous expression (Krumer *et al.* 2004; Chandrasekhar *et al.* 2006). In cardiomyocytes, it has been proposed that K<sub>V</sub>7.1 channels and KCNE1 subunits traffic separately and assemble at the surface sarcolemma to form channels that conduct  $I_{Ks}$  (Wang *et al.* 2013). Development of optical tools and approaches to visually monitor K<sub>V</sub>7.1/KCNE1 assembly and trafficking is important to decipher molecular mechanisms of subunit transport and the features that are affected by specific LQT1 mutations. Aromolaran *et al.* (2014) used an optical approach that simultaneously monitors surface (using quantum dot) and total (using yellow fluorescent protein (YFP) fluorescence) K<sub>V</sub>7.1–YFP channels exogenously expressed in rat cardiomyocytes. The surface density of channels remained relatively constant over a fourfold range of K<sub>V</sub>7.1–YFP expression, suggesting the existence of homeostatic mechanisms in cardiomyocytes that maintain K<sub>V</sub>7.1 surface density within tight limits over a wide range of protein expression level. This approach also showed that an LQTS-linked K<sub>V</sub>7.1 mutation in the C-terminus significantly diminishes cell surface channel density in both cardiomyocytes and cells used for heterologous expression (Aromolaran *et al.* 2014).

As noted above, it is well recognized that K<sup>+</sup> channels are heterogeneous macromolecular complexes. Different experimental systems have been utilized to study trafficking mechanisms, each with its own set of advantages and limitations. Heterologous expression systems allow ease of transfection, the ability to precisely determine channel components, and favourable geometry for in-depth analyses. However, they lack the cyto-architecture, intracellular milieu and physiologically important protein–protein interactions present in cardiomyocytes. This could contribute to the phenomenon where there is an inconsistent correlation between severity of biophysical defects in heterologous cells and risk of arrhythmias in patients (Priori *et al.* 1999). Heterologous expression of K<sup>+</sup> channels in primary adult cardiomyocytes offers a more physiologically relevant environment to explore trafficking mechanisms (Aromolaran *et al.* 2014). At the whole-animal level, transgenic rabbits have been useful in providing *in vivo* models of LQTS (Brunner *et al.* 2008). A drawback of transgenic animals is the time and cost required for screening functional consequences of many different K<sup>+</sup> channel mutations. Finally, hiPSC-CMs from LQTS patients provide a model system that is amenable to *in vitro* drug testing to potentially advance personalized therapy (Zhang *et al.* 2014). Overall, an all-of-the-above strategy with respect to the various experimental systems discussed

could be useful in providing a holistic molecular-, cellular- and organ-level view of arrhythmia mechanisms and K<sup>+</sup> channel trafficking.

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## Additional information

### Competing interests

The authors have no conflicts to disclose.

### Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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