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Review

Cardiac voltage-gated ion channels in safety pharmacology: Review of the landscape leading to the CiPA initiative

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ABSTRACT

Voltage gated ion channels are central in defining the fundamental properties of the ventricular cardiac action potential (AP), and are also involved in the development of drug-induced arrhythmias. Many drugs can inhibit cardiac ion currents, including the Na^+ current (I_{Na}), L-type Ca^{2+} current (Ica-L), and K^+ currents (I_{to}, I_{K1}, I_{Ks}, and I_{Kr}), and thereby affect AP properties in a manner that can trigger or sustain cardiac arrhythmias. Since publication of ICH E14 and S7B over a decade ago, there has been a focus on drug effects on QT prolongation clinically, and on the rapidly activating delayed rectifier current (I_{Kr}), nonclinically, for evaluation of proarrhythmic risk. This focus on QT interval prolongation and a single ionic current likely impacted negatively some drugs that lack proarrhythmic liability in humans. To rectify this issue, the Comprehensive in vitro proarrhythmia assay (CiPA) initiative has been proposed to integrate drug effects on multiple cardiac ionic currents with in silico modelling of human ventricular action potentials, and in vitro data obtained from human stem cell-derived ventricular cardiomyocytes to estimate proarrhythmic risk of new drugs with improved accuracy. In this review, we present the physiological functions and the molecular basis of major cardiac ion channels that contribute to the ventricle AP, and discuss the CiPA paradigm in drug development.

1. Introduction

In this article we provide a comprehensive overview of major ion currents contributing to cellular cardiac electrophysiology. Its scope spans fundamental concepts and more advanced considerations. The context is drug-induced proarrhythmia liability. The latest initiative in this regard, CiPA, reflects the recognition that arrhythmia liability is determined by the interaction between the entirety of the cellular electrophysiology matrix, and not simply one current (I_{Kr}).

1.1. Principles of cardiac cellular electrophysiology

Voltage-gated ion channels are complex transmembrane spanning proteins that regulate a broad spectrum of physiological processes throughout the body (Hille, 1978) including excitability of neuronal, skeletal and cardiac muscle cells, as well as modulating contraction and relaxation. In the heart, prominent voltage-gated ion channels include sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) channels, and their inhibition by drugs can to lead to serious adverse effects. KCNH2 gene codes for the Kv11.1 channel, known as the human Ether-à-go-go-Related Gene (hERG), which carries the delayed rectifier potassium current, I_{Kr}, a key component of repolarization during the cardiac action potential (AP). Blockade of I_{Kr} delays cardiac repolarization, prolonging AP duration (APD) and the QT interval on the ECG, and potentially increases the risk for the development of the cardiac arrhythmia, Torsades de Pointes (TdP). Because of the historical association of drug induced I_{Kr} inhibition with TdP, along with the regulatory emphasis on this current, the screening of drugs for effects

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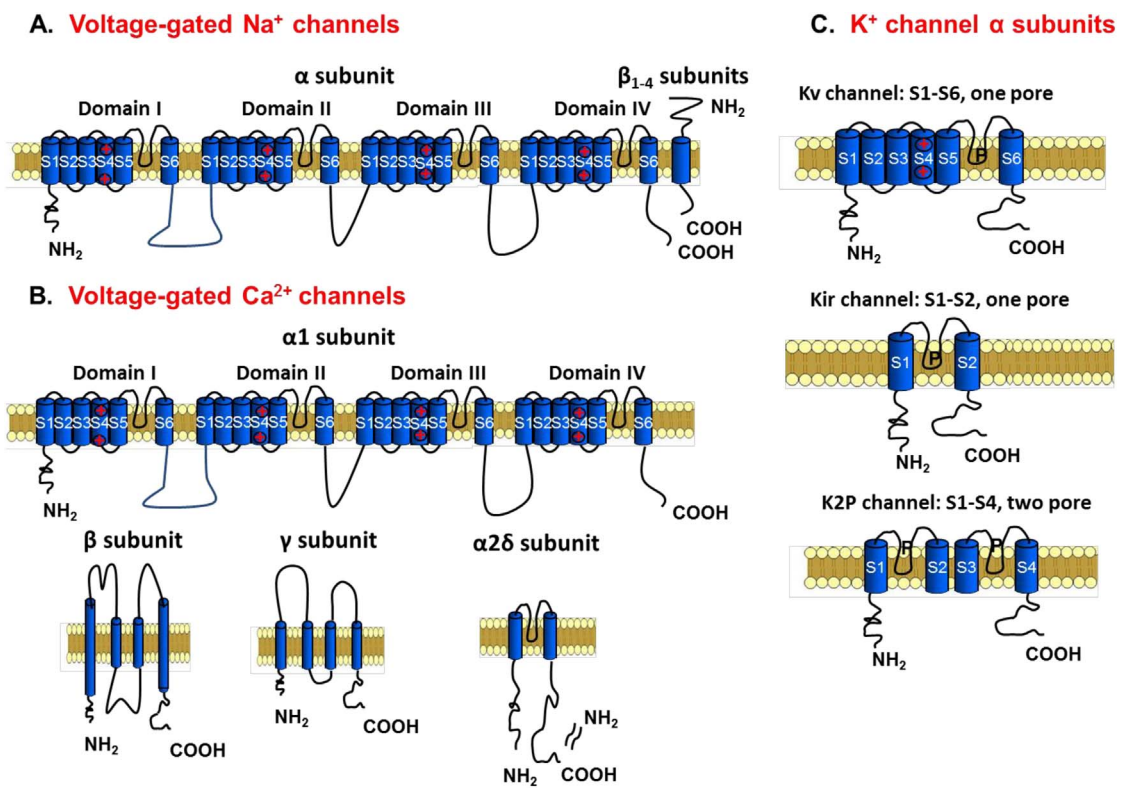

Fig. 1. A schematic view of the structure of voltage-gated Na^+, Ca^{2+} and K^+ channels. A) The Na^+ channel α-subunit is inserted into the lipid bilayer of the cellular membrane and constitutes the channel pore, through which Na^+ ions pass. The subunit consists of four domains (DI-IV). Each domain contains six transmembrane segments. The pore is composed of segments S5–S6, while S4 acts as the voltage sensor. β-Subunits have a single transmembrane segment, a long extracellular N-terminus, and a short intracellular C-terminus. B) The Ca^{2+}-channel α1 subunit demonstrates a similar structural basis to the Na_v channels. The α2δ and β subunits enhance expression and modulate the voltage dependence and gating kinetics of the α1 subunit. C) The K^+-channel α subunit. The top panel shows voltage-dependent K^+-channel (Kv) α subunits that contains six transmembrane segments (S1–S6) with one K^+-conducting pore. The middle panel shows inward-rectifying K^+-channel (Kir) α subunits that contains two transmembrane segments with one pore region. The bottom panel shows the K2P model that contains four transmembrane segments with two pores.

on additional ventricular ionic currents has been largely disregarded in drug safety testing paradigms, despite well characterized roles in the cardiomyocyte AP (Pugsley, Curtis, & Hayes, 2015).

1.2. Structure of voltage-gated ion channels

Voltage-gated Na^+ and Ca^{2+} channels consist of a principal pore-forming α-subunit and various accessory subunits. The α-subunit of the Na^+ channel is approximately 260 kDa, and its function is regulated by one or more auxiliary β-subunits (β1–β4) (Fig. 1A). Voltage-gated Ca^{2+} channels are formed by a group of subunits, including a pore-forming α-subunit (α1) and at least two accessory subunits α2δ and β (Bodi, Mikala, Koch, Akhter, & Schwartz, 2005). The voltage-gated Ca^{2+} channel α-subunit (Fig. 1B) is structurally similar to that of the Na^+ channel (Catterall & Swanson, 2015). All α-subunits of the Na^+ and Ca^{2+} channels comprise four homologous domains (DI-DIV), each of which contains six transmembrane segments (S1–S6). Domains DI-DIV are connected by cytoplasmic linkers. Segments S5 and S6 in each domain are connected via a hairpin-like P (pore) loop that lines the outer pore of channels (Gellens et al., 1992; Terlau & Stuhmer, 1998). In the pore-region of the Na^+ channel α submit, an Asp-Glu-Lys-Ala (DEKA) motif determines Na^+ selectivity. In the Ca^{2+} channel α subunit four glutamate residues, known as the EEEE motif, are responsible for Ca^{2+} selectivity. The S4 segment in each domain contains positively charged amino acid residues and acts as a voltage-gated sensor by moving across the membrane to trigger channel activation, in response to membrane depolarization (Jiang, Ruta, Chen, Lee, & MacKinnon, 2003; Yang, George, & Horn, 1996). The short cytoplasmic intracellular loop connecting domains III and IV in Voltage-gated Na^+ channel is thought to be the inactivation gate, which bends back into the channel and blocks the pore from the inside during sustained depolarization of the membrane (Patton, West, Catterall, & Goldin, 1992; Stuhmer et al., 1989). The inactivation gate is located in the center of a DIII–IV linker with a three-amino-acid stretch, consisting of isoleucine, phenylalanine, and methionine (IFM) (West et al., 1992). Residues of the S6 segment in each domain often provide the binding site for various drugs (e.g., local anesthetics), and contribute to the formation of the internal vestibule in the P-region. The amino (N)- and carboxy (C)-termini of the α-subunits are located on the intracellular side of the Na^+ and Ca^{2+} channels, so as to act as the loops that connect the repeats of the core motif (Ragsdale, McPhee, Scheuer, & Catterall, 1994).

K^+ channels can be classified into three main groups depending on their pore-forming channel structures (Fig. 1C). The first group includes the voltage-dependent K currents (K_v) derived from six families of channel proteins based upon *D. melanogaster* nomenclature. K currents included in this group include the transient outward K^+ channel, Ca^{2+}-activated K^+ channels and KCNQ channels, all of which form tetrameric structures comprised of subunits with six transmembrane segments (S1-S6) and cytoplasmic N- and C-termini. The S1-S4 segments move in response to voltage changes, functioning as the voltage sensor for the channel. The glycine-tyrosine-glycine (GYG) motif in the pore region is the critical sequence responsible for K^+ selectivity (Snyders, 1999). The second group of K^+ channels consists of two transmembrane segments (S1–S2), conducting three inward K^+ currents: the inward-rectifying K^+ (K_{ir}) channels, ATP-sensitive K^+ (K_{ATP}) channels and G protein-coupled inwardly-rectifying K^+ (GIRK) channels. The S1 and S2 segments are connected by a pore-forming P-loop. Cytoplasmic N- and C-termini are similar to the S5, S6 segments in the first group of K^+ channels (Loussouarn, Rose, & Nichols, 2002). The third group of

Table 1
Some properties of voltage-gated Na^+ channels.

Channel	Gene	TTX sensitivity*	Tissue location
Nav1.1	*SCN1A*	Sensitive	CNS, PNS, heart
Nav1.2	*SCN2A*	Sensitive	CNS, hear
Nav1.3	*SCN3A*	Sensitive	CNS, heart
Nav1.4	*SCN4A*	Sensitive	Skeletal muscle
Nav1.5	*SCN5A*	Resistant	Heart, CNS
Nav1.6	*SCN8A*	Sensitive	CNS, PNS, heart, glia, nodes of Ranvier
Nav1.7	*SCN9A*	Sensitive	PNS, Schwann cells
Nav1.8	*SCN10A*	Resistant	PNS
Nav1.9	*SCN11A*	Resistant	PNS
Nax	*SCN6A/ SCN7A*	Resistant	CNS, PNS, heart

CNS, central nervous system; PNS, peripheral nervous system; TTX, tetrodotoxin; the isoform of the Na + channel is 'sensitive' if the channel is blocked at nM concentrations and resistant if blocked at μM concentrations.

K^+ channels is the most abundant group with more than 50 members all of which a have two-pore (P1 and P2) structure with four transmembrane segments (S1–S4) (Kuang, Purhonen, & Hebert, 2015; Rasmusson et al., 1998).

1.2.1. Voltage-gated sodium (Na^+) channels

Ten genes encode the distinct Na^+ channel α-subunit isoforms (Nav1.1–Nav1.9 and Nax). Nav1.1–Nav1.9 isoforms are voltage-dependent channels, whereas Nax (encoded by the SCN7A gene), expressed in glial cells within the brain is Na^+ concentration-dependent; however, it is not homologous to other Na channels and does not exhibit voltage-sensitivity (Noda & Hiyama, 2015) (Table 1). The Na^+ channel isoforms are further characterized pharmacologically by their sensitivity to the specific neurotoxin, tetrodotoxin (TTX). The TTX-sensitive (TTX-S) isoforms (e.g. Nav1.1–1.4, Nav1.1.6 and Nav1.7) are blocked by nanomolar concentrations of TTX, whereas on the other hand, the TTX-resistant (TTX-R) isoforms (e.g. Nav1.5, Nav1.8 and Nav1.9) are blocked only by micromolar concentrations of TTX. Homologous residues, C374 in rNav1.5 and C373 in hNav1.5, confer resistance to TTX in the rat and human isoforms, respectively (Heinemann, Terlau, & Imoto, 1992).

Cardiac voltage gated Na^+ current (I_{Na}) properties can be characterized by studying Nav1.5 channels recombinantly expressed in mammalian cells using the whole cell patch-clamp technique. I_{Na} typically activates within 200-300 μs and inactivates completely within 2–5 ms. I_{Na} is an inward current characterized by a half maximum activation voltage ($V_{1/2}$) of approximately −50 mV to −55 mV, and inactivation $V_{1/2}$ of approximately −90 mV to −95 mV. Compounds that inhibit I_{Na} include Class I arrhythmic agents (Milne et al., 1984), various peptide toxins such as TTX (Heinemann et al., 1992) and ranolazine (Zygmunt, Eddlestone, Thomas, Nesterenko, & Antzelevitch, 2001). The "late" component of I_{Na} has received increased attention over the last few years and is emerging as an important contributor to the cardiac APD (Saint, 2009). It is potentially involved in defining rate-dependent adaption of ventricular repolarization and reverses use-dependence of QT prolonging agents (Guo et al., 2011). Late I_{Na} may result from a time-invariant Na^+ "window current" (the overlap of I_{Na} activation and inactivation voltage-dependent curves), steady-state I_{Na} or the slowly inactivated portion of I_{Na} (Moreno & Clancy, 2012; Noble & Noble, 2006; Zaza, Belardinelli, & Shryock, 2008). In the ventricle, the late I_{Na} is small, only accounting for around 0.5% of the peak amplitude of the fast Na^+ current. Its existence has been proposed for decades (Saint, 2008). While its physiological role has yet to be fully defined, it has similar biophysical properties to the fast Na^+ channel including ion channel selectivity and single channel conductance properties, but the population that constitute the late channels fail to inactivate after opening (Saint, 2008). In contrast to fast I_{Na}, a selective and specific blocker of late I_{Na} is still not available. However some studies showed that the late current is pharmacologically distinct and appears sensitive to block by tetrodotoxin (TTX) and lidocaine as well as ranolazine (Ju, Saint, & Gage, 1992; Saint, Ju, & Gage, 1992). The magnitude of the late Na^+ current in the normal heart is small; however, its magnitude is increased in many pathologic conditions including ischemia (Zaza et al., 2008), heart failure (Pourrier, Williams, McAfee, Belardinelli, & Fedida, 2014) and congenital long QT syndrome type 3 (LQT3) (Wang, Yazawa, George, & Bennett, 1996). This late I_{Na} component is thought to maintain the AP plateau (Ju et al., 1992; Saint, 2008; Saint et al., 1992; Zaza et al., 2008). However, when increased, it can prolong the period of ventricular repolarization and drive the Na^+/Ca^{2+} (NCX) exchanger in reverse mode leading to an elevated intracellular Ca^{2+} concentration thereby further increasing the risk of generating cardiac arrhythmias (Belardinelli, Shryock, & Fraser, 2006; Noble & Noble, 2006).

1.2.2. Molecular basis of cardiac (Nav1.5) channels

Nav1.5 is expressed in the heart, particularly in the intercalated discs (Agullo-Pascual et al., 2014; Cohen, 1996). However, Nav1.5 channels are also found within the brain (Donahue et al., 2000; Hartmann, Colom, Sutherland, & Noebels, 1999), the gastrointestinal tract (Ou et al., 2002), and neonatal skeletal muscle (Kallen et al., 1990). Nav1.5 is encoded by the *SCN5A* gene, located on chromosome 3q21–24. The gene consists of 28 exons spanning approximately 80 kb. It is 2016 amino acids in length and has a calculated molecular weight of 227 kDa (Gellens et al., 1992; Rogart, Cribbs, Muglia, Kephart, & Kaiser, 1989). There have been two alternatively spliced gene variants identified, termed Nav1.5c and Nav1.5d. However, Nav1.5c contains 2015 amino acids and no glutamine at position 1077, thus it is also called Q1077del while Nav1.5d consists of 2016 amino acids containing a glutamine (Q) at position 1077, and therefore it is called Q1077. mRNA studies from the human heart showed the presence of both variants, with levels of Q1077del/Nav1.5d being twice those of Q1077/Nav1.5c, in all heart specimens studied (Camacho et al., 2006; Makielski et al., 2003; Tan et al., 2005). Thus, in the human heart, alternative splice variants (Q1077del and Q1077) may interact with other *SCN5A* mutations, and cause variable biophysical phenotypes that could result in development of various heritable arrhythmias (Tan et al., 2005).

Six groups of β-subunits (β1–β4, β1A, β1B) have been identified in cardiac myocytes with different expression levels. β_1 is localized to the intercalated discs and T-tubules in the heart, β3 to the T-tubules, and β_2 and β_4 to the intercalated discs (Dhar Malhotra et al., 2001; Maier et al., 2004). The β1 subunit is non-covalently associated with the α-subunit and regulates Na^+ current amplitude and the kinetic rate of activation and inactivation of the channel; thus, it has a key role in interacting with the α-subunit of the Na^+ channel compared with the multiple other β-subunits. It has been hypothesized that $Na_v1.5$ channels initiate and propagate cardiac APs from one myocyte to the next at intercalated discs, but that β-subunits interact with different Na^+ channel isoforms regulating the properties of Na^+ channels in discretely different ways in the different areas of the heart (Catterall, Goldin, & Waxman, 2003).

1.2.3. Voltage-gated calcium (Ca^{2+}) channels

There are five types of Ca^{2+} channels, L- (long lasting and large conductance), T- (transient-opening and small conductance), N- (neuron pre-synapse), P- (Purkinje cells), and R- (resistant to peptide toxins) types. The T-type and L-type Ca^{2+} channels are involved in key roles in the heart, contributing to automaticity, cardiac conduction, APs and excitation-contraction (EC) coupling. Ca^{2+} channels (Table 2) influence intracellular Ca^{2+} homeostasis and its consequences, in addition to playing a role in electrogenesis and conduction. In diastole, the intracellular Ca^{2+} concentration is low ($\sim 10^{-8}$ M). Once Ca^{2+} enters the cell, it initiates a variety of signaling pathways depending on the cell type, including cardiac muscle contraction, release of hormones

Table 2
Some properties of voltage-gated Ca^{2+} channels.

Channel	αSubunit/	Gene	Accessory subunits	DPH sensitivity*	Tissue location
L	Cav1.1 (α1S)	CACNA1S	α2δ, β, γ	Sensitive	Skeletal muscle
	Cav1.2 (α1C)	CACNA1C	α2, β, γ	Sensitive	Hear (ventricle), CNS, smooth muscle, bone, adrenal gland
	Cav1.3 (α1D)	CACNA1D	α2, β, δ	Sensitive	CNS, pancreas, kidney, cochlea
	Cav1.4 (α1F)	CACNA1F	unknown	Sensitive	Retina
P/Q	Ca_v2.1 ((α1A)	CACNA1A	α2δ, β	Resistant	CNS (cerebellum)
N	Ca_v2.2 (α1B)	CACNA1B	α2δ/β1, β2, β3	Resistant	CNS (brain) and peripheral nerve system
R	Ca_v2.3 (α1E)	CACNA1E	α2δ, β	Resistant	CNS (cerebellum, neurons)
T	Ca_v.3.1 (α1G)	CACNA1G	unknown	Resistant	Heart (SA node), CNS (neurons), bone
	Ca_v.3.2 (α1H)	CACNA1H	unknown	Resistant	Heart (SA node), CNS (neurons), bone
	Ca_v.3.3 (α1I)	CACNA1I	unknown	Resistant	CNS (brain)

CNS, central nervous system; DPH, dihydropyridine; the isoform of the Ca^{2+} channel is 'sensitive' if the channel is blocked at nM concentrations and resistant if blocked at μM concentrations

and neurotransmitters, activation of Ca^{2+}-dependent enzymes, and gene transcription. Ca^{2+} channels are classified into three groups: Cav1, Cav2 and Cav3. The Cav1 group has four members (Cav1.1–-Cav1.4), mediating L-type Ca^{2+} currents and each are specifically blocked by the dihydropyridine (DHP) class of calcium channel blocking (CCB) drugs (Hess, Lansman, Nilius, & Tsien, 1986). The Cav2 group includes three members (Cav2.1–Cav2.3) and gives rise to the P, N and R type currents, respectively. The Cav3 group includes Cav3.1–Cav3.3 and mediates T type Ca^{2+} currents.

T-type Ca^{2+} channels are expressed in atria (Bean, 1985) and Purkinje fibres (Hirano, Fozzard, & January, 1989) as well as in some pacemaker cells (Hagiwara, Irisawa, & Kameyama, 1988). However, functional T-type Ca^{2+} currents have been difficult to demonstrate in human atrial myocytes. L-type Ca^{2+} channels are expressed in all regions of the heart. Ca^{2+} entry into cardiac cells happens mainly through L-type Ca^{2+} channels, leading to Ca^{2+} release from the sarcoplasmic reticulum and contraction. I_{CaL} also plays a significant role in the initiation of cardiac arrhythmias through early afterdepolarization (EAD) related mechanisms (January & Riddle, 1989) and through mediating slow conduction in ischaemic myocardium (Curtis, 1990). Because of its importance, Cav1.2 will be further discussed in the next section.

1.2.4. Cardiac voltage gated Ca^{2+} current ($I_{Ca,L}$)

During the generation of a cardiac AP, $I_{Ca,L}$ is rapidly activated in a voltage- and time-dependent manner, and inactivates slowly. The kinetics of $I_{Ca,L}$ activation are nonetheless slower than those of I_{Na}, taking a few milliseconds to reach peak amplitude. I_{CaL} activates at membrane potentials within the voltage range of an AP plateau, in line with a steady-state activation curve ranging from − 40 mV to + 10 mV, with a $V_{1/2}$ of around − 10 to − 5 mV (Bers & Perez-Reyes, 1999). Steady-state inactivation yields a $V_{1/2}$ of − 40 to − 45 mV. Overlap of activation and inactivation voltage-dependent curves allows "window current" fall in the AP plateau voltage range, and therefore contributes to the mechanism for EAD generation (January & Riddle, 1989). I_{CaL} decreases to almost zero upon repolarization, when the channel responsible for the slow delayed rectifier K^+ current, I_{Ks}, opens and contributes increasingly to membrane repolarization (Barhanin et al., 1996). Therefore, the biophysical properties of I_{CaL} contribute, in part, to maintaining the plateau phase (Phase 2) of ventricular AP along with the Na^+/Ca^{2+} exchanger (NCX), the antiporter membrane protein that removes Ca^{2+} from cells. Importantly, Cav1.2 is the main route of the Ca^{2+} entry that triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via its effects on type 2 ryanodine receptors (RyR2). The intracellular Ca^{2+} concentration ($[Ca]_i$) is consequently rapidly increased through a process of Ca^{2+} induced Ca^{2+} release. The increased $[Ca]_i$ binds to the myofilaments and initiates cardiac contraction (Bers, 2002). When intracellular Ca^{2+} concentrations reach millimolar concentrations (Hirano et al., 1989; Lee, Marban, & Tsien, 1985), a feedback mechanism leads to rapid inactivation of Cav1.2 and results in an acceleration of channel recovery from inactivation (Bers & Perez-Reyes, 1999). A prolonged plateau provides enough time for I_{CaL} to recover from voltage- and Ca^{2+}-dependent inactivation, resulting in reactivation and increased inward currents that can further depolarize the cardiac membrane potential (January & Riddle, 1989; Ming, Nordin, & Aronson, 1994). In addition, extra Ca^{2+} will be moved into the SR by Ca^{2+} pumps through sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) pathway or extruded out of cells through the forward mode of the NCX (Bers, 2002; Lederer et al., 1990). The NCX current (I_{NCX}) can generate an inward current at the end of repolarization and therefore may contribute to the APD. I_{NCX} can be involved in EADs and delayed afterdepolarizations (DADs) (Nattel, 2002; Sipido, Bito, Antoons, Volders, & Vos, 2007).

1.2.5. Molecular characteristics of cardiac L-type Ca^{2+} channels

There are four subfamilies of voltage-dependent Ca^{2+} channel pore-forming α1 subunits, Cav1–Cav4; each subfamily has several family members or splice variants (Lacinova, 2005). Cav1.2 (or α1C) is the pore-forming α1 subunit of the cardiac L-type Ca^{2+} channel, and is encoded by CACNA1C. Cavβ2 is the most frequently expressed Cav β subunit in the cardiac L-type Ca^{2+} channel (Perez-Reyes et al., 1992). Cavβ2 is a cytosolic protein that binds to α1C via the DI and DII linker, increasing α1C expression and I_{CaL} amplitude and modifying I_{CaL} kinetics (Stotz, Jarvis, & Zamponi, 2004). Functional Cav1.2 channel complexes also need another accessory subunit, Cavα2δ (Arikkath & Campbell, 2003). Co-expression of Cavα2δ with α1C gives rise to an increased I_{CaL} amplitude and accelerated activation and inactivation (Gurnett, De Waard, & Campbell, 1996). The α1 isoform is the ligand-recognition site that determines the effects of drugs on each of the Ca^{2+} channels in the heart. The L-type Ca^{2+} channels possess high affinity, stereoselective-binding domains for channel blocking drugs, and it is the blockade of these Ca^{2+} channels in the heart that mediates the effects of L-type calcium antagonists on heart rhythm.

1.2.6. Voltage-gated potassium (K^+) channels

Cardiac voltage-gated K^+ channels play an essential role in AP repolarization and render the atrial and ventricular cell equivalent to a 'potassium battery' during diastole owing to the K^+ transmembrane concentration gradient and the fact that the main current active during diastole is I_{K1}. The contribution of individual K^+ channels to repolarization can vary depending on a number of factors including channel density in different cardiac regions (atrial, ventricular, epicardium, endocardium, mid-myocardium, etc.). This section will focus on four K^+ currents, namely I_{to}, I_{Kr}, I_{Ks}, and I_{K1}.

1.2.7. Transient outward K^+ channel (I_{to})

I_{to} is composed of two (fast and slow) currents, termed $I_{to,fast}$ and $I_{to,slow}$. $I_{to,fast}$ displays faster gating properties, and contributes to Phase 1 repolarization in APs. In patch clamp studies $I_{to,fast}$ current activates and inactivates rapidly when membrane potentials are depolarized to

relatively positive potentials above approximately −30 mV. The activation and inactivation gating kinetics are time- and voltage-dependent (Greenstein, Wu, Po, Tomaselli, & Winslow, 2000). The time constant of activation of $I_{to,fast}$ is in the order of milliseconds, whereas the inactivation can be described by tens to hundreds of milliseconds (Patel & Campbell, 2005). The activation $V_{1/2}$ is ~+10 mV, while the inactivation $V_{1/2}$ is around −35 mV in the ventricle (Han, Wang, & Nattel, 2000; Nabauer, Beuckelmann, & Erdmann, 1993). $I_{to,fast}$ is a Ca^{2+}-independent current. $I_{to,slow}$ also shows similarly rapid activation and inactivation kinetics, but it recovers slowly from inactivation. Kinetics of recovery from inactivation show that the time constants are of ~30–100 ms for $I_{to,fast}$ and ~100–1000 ms for $I_{to,slow}$ (Giles & Imaizumi, 1988; Patel & Campbell, 2005). $I_{to,slow}$ is the small Ca^{2+}-activated component of I_{to}. $I_{to,fast}$ can be distinguished from $I_{to,slow}$ by differential sensitivity to the K^+ channel toxins Heteropoda toxins (HPTXs) and 4-aminopyridine (4-AP). HPTXs at nanomolar concentrations block $I_{to,fast}$ but not $I_{to,slow}$ (Sanguinetti et al., 1997). 4-AP blocks $I_{to,fast}$ in the closed state, but also blocks the open state of $I_{to,slow}$ in a use-dependent manner (Campbell, Rasmusson, Qu, & Strauss, 1993). Three α-subunit isoforms have been identified in the I_{to} family. Kv4.2, encoded by gene KCND2, is the major α-subunit contributing to $I_{to,fast}$ in rodents; however Kv4.3, encoded by KCND3, is the primary α-subunit for $I_{to,fast}$ in larger mammals such as dogs and humans (Nerbonne, 2000). Kv1.4 (encoded by KCNA4) displays channel gating kinetics and pharmacological properties similar to $I_{to,slow}$ (London, Wang, Hill, & Bennett, 1998; Patel & Campbell, 2005). In addition to the selective blocker 4-AP, $I_{to,fast}$ is inhibited by many other cardiac drugs including the class Ic antiarrhythmic drug flecainide, the class Ia drug quinidine, the class III drug ambasilide, and the class IV drug diltiazem. Quinidine, ambasilide and diltiazem inhibit $I_{to,fast}$ in the open state and in a frequency-dependent manner whereas flecainide, propafenone and the class IV drug nifedipine inhibit $I_{to,fast}$ in the closed state in a frequency-independent manner. Blockade of $I_{to,fast}$ can prolong or shorten ventricular repolarization depending on secondary changes in other currents, and by affecting plateau duration and voltage.

Kv4.2, Kv4.3 and Kv1.4 are pore forming α-subunits underlying cardiac $I_{to,fast}$ that belong to Kv channel Shaker-related subfamilies (Shaker Kv1.x, Shab Kv2.x, Shaw Kv3.x and Shal Kv4.x) (Nerbonne, 2000; Snyders, 1999). $I_{to,fast}$ can also interact with I_{CaL} with the help of the Ca^{2+} binding protein, KChAP, and thereafter affect the overall Ca^{2+} entry, APD and EC coupling. In particular, KChAP promotes Kv4.3 expression and displays rectifier currents. In addition, DPP6 (dipeptidyl aminopeptidase-like protein 6) is another subunit of cardiac Kv4.3 which regulates $I_{to,fast}$ expression (Radicke, Cotella, Graf, Ravens, & Wettwer, 2005). $I_{to,fast}$ density is significantly higher in the epicardium and mid-myocardium than in endocardium, and thus mediates the notch (i.e., classic spike and dome) morphology of APs in epicardial and M cells (Antzelevitch et al., 1999).

1.2.8. Delayed rectifier K^+ currents (I_{Ks} and I_{Kr})

I_{Kr} and I_{Ks} activate slowly because of a single voltage-dependent gate upon depolarization, and because they change the membrane conductance with a delay after a depolarizing voltage step, they are named "delayed rectifier" based on the original description of K^+ channels in axons by Hodgkin and katz (1949). Functional cardiac I_{Ks} derive from the co-expression of pore-forming α subunits and accessory β subunits. The KCNQ1 gene encodes α subunits, whereas KCNE1 encodes β subunits. The pore-forming α-subunit underlying I_{Kr} is Kv11.1, also known as hERG encoded by KCNH2. I_{Kr} is regulated by accessory β subunits encoded by KCNE2 gene. The structure of Kv11.1 is similar to the I_{to} α-subunit structure described in Fig. 1C.

I_{Ks} and I_{Kr} are important outward currents contributing to Phase 3 repolarization of APs in most regions of the heart, but especially within the ventricles. Activation kinetics of I_{Ks} is relatively slower than those of I_{Kr}. The corresponding time constant is in the order of seconds for I_{Ks}, but of tens or hundreds of milliseconds for I_{Kr} (Cheng & Kodama, 2004). In addition, I_{Ks} is activated at more positive membrane potentials than I_{Kr}, giving a steady-state activation $V_{1/2}$ value of approximately +25 to +30 mV for I_{Ks}, and −10 to −20 mV for I_{Kr}. The current-voltage (I–V) relationship of I_{Ks} is linear due to an extremely slow inactivation processes (Mitcheson & Sanguinetti, 1999); whereas I_{Kr} displays an inverted bell-shaped I–V relationship because of rapid inactivation, with current peaking at potentials ranging between 0 and +10 mV. The fast inactivation of I_{Kr} at positive membrane potentials accounts for inward rectifying currents (Smith, Baukrowitz, & Yellen, 1996), which makes I_{Kr} play an additional, albeit small, role in the AP plateau (Sanguinetti & Jurkiewicz, 1990). Inactivation is removed as cells repolarize making I_{Kr} a key player in repolarization. I_{Kr} and I_{Ks} are expressed in guinea pig, rabbit, dog and human ventricles, and are prominent repolarizing currents in these species. In adult rat and mouse ventricles, however, their densities are very low or undetectable, and the fast resting heart rate renders these currents non-functional even if the channels, or the gene homologues, are present.

Pharmacological tools can be used to distinguish I_{Kr} from I_{Ks}. For example, when studying I_K in guinea pig myocytes, I_{Kr} can be selectively blocked by some class III antiarrhythmic agents such as E-4031, dofetilide, and D-sotalol (Sanguinetti & Jurkiewicz, 1990; Tamargo, Caballero, Gomez, Valenzuela, & Delpon, 2004), leaving a drug-resistant component that is I_{Ks} (Sanguinetti & Jurkiewicz, 1990). I_{Kr} can also be blocked by the class Ia-c antiarrhythmic agents quinidine, mexiletine and flecainide, respectively (Tamargo et al., 2004). In addition, non-cardiac-targeting drugs can also inhibit I_{Kr} at therapeutic concentrations, leading to acquired QT prolongation described in section 4. I_{Ks} is selectively blocked (compared with actions on other cardiac K currents) by chromanol 293B (Busch et al., 1996), the benzodiazepine L-735,821 (Jurkiewicz, Wang, Fermini, Sanguinetti, & Salata, 1996), the diuretic indapamide (Turgeon et al., 1994) and the chromanol derivative HMR 1556 (Thomas, Gerlach, & Antzelevitch, 2003). Most of the I_{Ks} inhibitors act by blocking the channel in the activated state. The Ca^{2+} channel blocker bepridil blocks the tail current of I_{Ks} more potently with pulses shorter than 600 ms in an envelope protocol, therefore suggesting that the binding affinity to the channel is greater in the closed state (Yumoto et al., 2004), while chromanol 293B and L-735,821 block I_{Ks} by binding to the pore region and S6 of the α subunits. T312 in the pore region and I337, P339, P340, and A344 in the S6 domain are key amino-acid residues contributing to KCNQ1 channel block (Seebohm et al., 2003).

1.2.9. Inward rectifier K^+ current (I_{K1})

I_{K1} contributes to Phase 3 repolarization, and plays a major role in setting the resting membrane potential in the heart. I_{K1} conductance is greatest at potentials negative to the K^+ equilibrium potential ($E_K = -90$ mV), and is maintained because the channel possesses no inactivation gate (and therefore lacks time-dependent inactivation during diastole). I_{K1} shows strong inward rectification, and is voltage-dependent, shifting in conformation to the rested state at positive potential. The rectification properties of I_{K1} result from the fact that outward conductance is unidirectionally blocked by intracellular multivalent ions (especially Mg^{2+}) and polyamines including putrescine, spermidine and spermine (Lopatin & Nichols, 2001; Nichols, Makhina, Pearson, Sha, & Lopatin, 1996). I_{K1} density is greater in the ventricles than in the atria (Melnyk, Zhang, Shrier, & Nattel, 2002; Schram, Pourrier, Melnyk, & Nattel, 2002). The current can be inhibited by extracellular Ba^{2+} and intracellular Cs^+, and relatively selectively by certain drugs that were developed as potential Class III antiarrhythmics such as RP58866 (Rees & Curtis, 1993) Note that unlike the K_V channels, K_{ir} channels lack an intrinsic S4 "voltage sensing element" and a mechanism for rapid N-type inactivation (i.e., inactivation that occurs by the movement of the cytoplasmic N-terminus to the internal vestibule of the opened channel (Hoshi, Zagotta, & Aldrich, 1991) and therefore do not "sense" and respond to changes in membrane potential

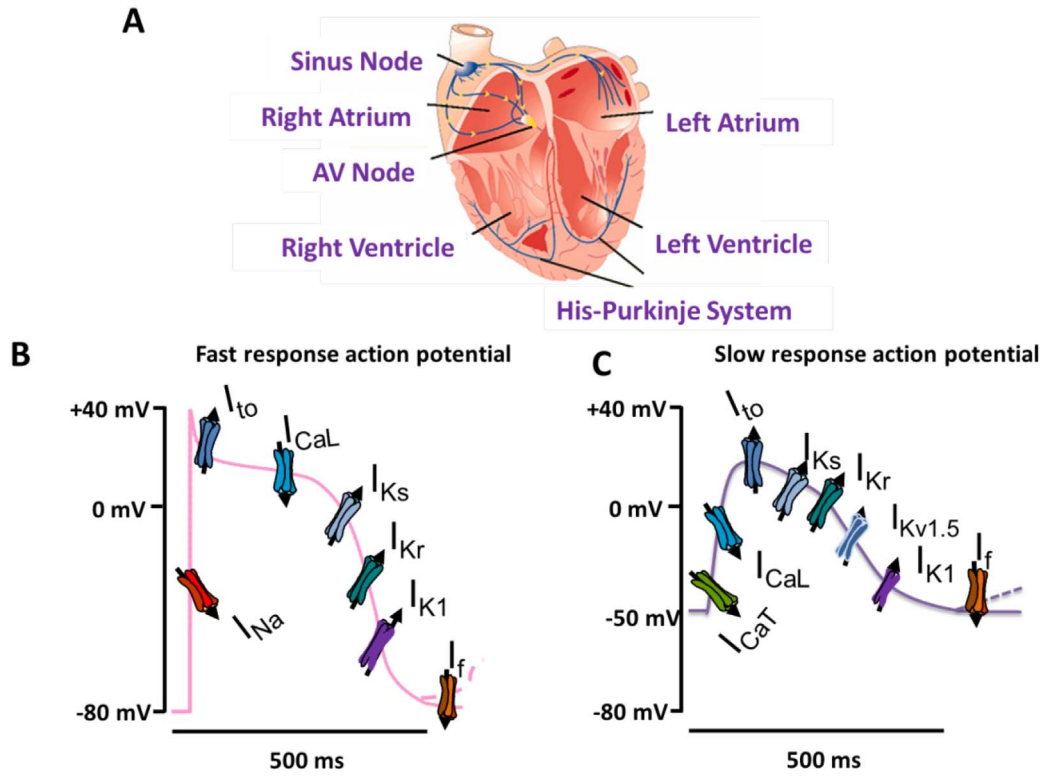

Fig. 2. The cardiac electrical conduction system and genesis of the cardiac action potential in fast-response and slow-response tissues. A) The basic cardiac structure and components involved in conduction of electrical impulses from the sinus node through the His-Purkinje system. B) A schematic of action potentials in fast-response and slow-response tissues. The bottom left panel demonstrates a typical morphology of a cardiac action potential in fast-response tissues (i.e., atrial and ventricular myocardium and Purkinje fibres). Each action potential comprises phase 0 through phase 4. Some channels may conduct in an inward or outward direction, depending on the membrane voltage at any given time point of the electrical cycle. Upright/downright arrows represent depolarizing and repolarizing currents, respectively. The bottom right panel demonstrates a cardiac action potential in slow-response tissue (i.e., the SA node and AV node).

in the manner of other K_V channels, as mentioned above. The K_{ir} channels are nonetheless affected by membrane voltage (Pugsley et al., 2015).

Cardiac I_{K1} has three isoforms, which are encoded by three distinct genes: KCNJ2 (Kir2.1), KCNJ12 (Kir 2.2) and KCNJ4 (Kir2.3). K_{ir} channel pore-forming α-subunits display a S1–S2 structure, as described in Fig. 1C. Kir2.1 is the most important subunit underlying I_{K1}. Mutations in the KCNJ2 gene are linked to LQT7 (Andersen syndrome). KCNJ2 mutant channels have a compromised K_{ir} 2.1 function caused by a dominant-negative effect. Affected patients may have cardiac electrophysiological changes leading to life-threatening arrhythmias. Kir2.1 and Kir2.3 are expressed at higher levels in the ventricle than in the atrium (Melnyk et al., 2002; Schram et al., 2002).

2. The cardiac action potential

Fig. 2B demonstrates the different phases in an archetypal ventricular AP. Phase 4 describes the resting membrane potential (E_m), and has a voltage range of − 80 to − 90 mV. I_{K1} is a main current active in phase 4 in ventricular cells. During phase 4, Na^+ and Ca^{2+} channels are in the rested state meaning that depolarization (triggered in working heart by electrotonic spread of the propagating AP from neighbouring cells) can trigger an AP owing to resultant voltage-dependent shifts to the activated state of these channels (positive feedback) when depolarization reaches the threshold for activation of the Na^+ channels (at about − 60 mV). In the sinoatrial node, the pacemaker current (I_f) which is a non-selective cation current activates as a result of hyperpolarization, and initiates the propagating AP (Robinson & Siegelbaum, 2003). Elsewhere, phase 0 is the AP upstroke caused by Na^+ channels opening. The maximum rate of depolarization during phase 0 (dV/dt_{max}, where dV is the change of membrane potential voltage within the depolarisation interval dt_{max}) determines conduction velocity (Cohen, Datyner, Gintant, Mulrine, & Pennefather, 1985). Phase 1 repolarization is characterized by the formation of a notch in the AP and is caused by fast time-dependent inactivation of I_{Na} and activation of $I_{to,fast}$. Phase 2 is the plateau that results from the opposing influences of inward ($I_{Ca\text{-}L}$) and outward (I_{Kr} and I_{Ks}) currents. Alteration of the amplitude of these currents can increase or decrease the APD and the QT interval. Phase 3 is repolarization, and is driven by I_{Ks}, I_{Kr} and I_{K1} (Matsuura, Ehara, & Imoto, 1987; Sanguinetti & Jurkiewicz, 1990). $I_{Ca\text{-}L}$ is inactivated during phase 3 (Ono & Iijima, 2010). Finally, I_{K1} becomes activated towards the end of phase 3 (Bodi et al., 2005). NCX carries an inward current that is driven by the membrane potential at the terminal AP repolarization.

The morphology of cardiac APs has been shown to vary markedly between cardiac regions. For example, APs recorded from the sinoatrial (SA) and atrioventricular (AV) nodes display lower amplitudes and slow upstroke velocities when compared to ventricular tissue, reflecting the more positive E_m in diastole (phase 4) and the subsequent reduced contribution of Na^+ current and increased contribution of Ca^{2+} to phase 0 in this tissue (Fig. 2C). In the pacemaker cells of nodal tissues phase 4 is characterized by a slow steady spontaneous depolarization, named diastolic depolarization, that leads to a 'threshold' potential (TP), resulting in the all-or-none generation of APs, and pacemaker activity (Bers, 2008). This is due mainly to the presence of the non-selective pacemaker or 'funny' inward current (I_f), and a reduced contribution of I_{K1}. Atrial APs show a shortened plateau phase and repolarize faster than ventricular cells (Fig. 2C). In addition, the ultra-rapid delayed rectifier potassium current (I_{Kur}) generated by the Kv1.5 channel selectively contributes to phase 3 in human atrial APs (Nattel, 2002; Ruan, Liu, & Priori, 2009).

2.1. Mechanisms of cardiac arrhythmias being studied

The surface electrocardiogram (ECG) is the main tool used to diagnose abnormal cardiac activity since each of the different segments and intervals represent the activity of specific ion channels in discrete regions of the heart. In humans, common causes of arrhythmias include myocardial ischemia, infarction, or reperfusion of a previously ischaemic myocardium. Most of these conditions can be readily reproduced in situ and in ex vivo preparations, such as with Langendorff assays. While the pathology of arrhythmias may not appear to be relevant to the safety pharmacologist, our understanding of the underlying ionic mechanisms, based on decades of studies in numerous refined non-clinical models, has contributed to the development of the CiPA paradigm (see below) in its role of assessing the 'proarrhythmia potential' for a new drug. For a very comprehensive guide to the study of cardiac arrhythmias in humans and animals, see Curtis et al. (Curtis et al., 2013).

The underlying causes of cardiac arrhythmias are normally inferred, since it is not possible to distinguish between re-entry, flow of injury current and abnormal automaticity in an intact human, or even an isolated perfused animal heart with any certainty. Indeed, abnormal automaticity is an event in a cell and requires single cell AP recording for verification. Moreover, unless captured during ECG recordings (i.e., after the patient has begun to receive medical assistance), the initiating mechanism underlying arrhythmias can never be determined with certainty. Paradoxically this is most relevant to the most lethal arrhythmias, ventricular fibrillation (VF) and sustained TdP which commonly occur unobserved, and in the case of VF the first episode a patient experiences is usually lethal if first aid is not administered within 2–3 min of its onset. Unfortunately it is not possible to infer mechanisms of *lethal* arrhythmias by the controlled initiation or observation of spontaneously occurring *nonlethal* arrhythmias because the mechanisms, at least in animal models in which they can be studied, are different (Curtis et al., 2013).

2.1.1. Early and delayed afterdepolarization-induced arrhythmias

Early afterdepolarizations (EADs) occur during the repolarization phase of the AP (Binah & Rosen, 1992). They are precipitated by bradycardia or long cardiac pauses, allowing for APD prolongation and for sufficient time for $I_{Ca\text{-}L}$ to recover from inactivation, leading to reopening of calcium channels within the plateau range and the generation of inward current initiating EADs. In the setting of QT prolongation, a small number of Nav1.5 channels have been shown to also remain open within the plateau voltage, carried by the late I_{Na} (Pugsley et al., 2015; Saint, 2009).

Delayed afterdepolarization (DAD) is abnormal depolarization interrupting phase 4 of the cardiac AP (Fig. 3B) during diastole (Fozzard, 1992). DADs are dependent upon the duration of the diastolic interval but, unlike EADs, the amplitudes of DADs increase during tachycardia. DADs may result from an elevated cytosolic Ca^{2+} concentration in circumstances where the cell's capacity to regulate this is impaired. This may then trigger spontaneous Ca^{2+} release from the SR during repolarization. This so-called setting of Ca^{2+} overload may be accelerated by digitalis toxicity, β-adrenoceptor stimulation and low extracellular K^+. Digoxin toxicity can cause diastolic SR Ca^{2+} release, thereby initiating DAD events (Rosati et al., 2001), and this is likely to be exacerbated if there is additional underlying ryanodine receptor dysfunction. Such spontaneous Ca^{2+} release from the SR extrudes Ca^{2+} via the NCX and causes cardiomyocyte depolarization, eventually leading to DAD-induced triggered activity (Pogwizd, Qi, Yuan, Samarel, & Bers, 1999). DAD events also induce ventricular tachycardia and fibrillation, but they are precipitated in the background of rapid heart rates (Priori et al., 1999).

2.1.2. Re-entrant mechanisms of arrhythmias

Although EAD and DAD mechanisms may account for the initiation of some arrhythmias (with the caveats outlined at the start of this section), the major (perhaps exclusive) mechanism that sustains ventricular arrhythmias is re-entry. Re-entry is failure of an impulse to self-terminate owing to the presence of regions of slow conduction that render separate parts of the wave front out of synchronization. The slower part of the wave front may then encounter adjacent tissue that has recovered excitability and may then propagate in this abnormal direction (through the adjacent tissue), re-entering the tissue it excited earlier – the hallmark of re-entrant arrhythmias (Fig. 3C). Abnormalities in cardiac tissue (e.g., ischemia) can generate a tissue substrate for re-entry (Nattel, 2002) by changing conduction velocity or refractory period. Injury-induced conduction block facilitates re-entry by creating a region of 'adjacent' excitable tissue distal to the block, and an island of inexcitable tissue around which a re-entrant circuit may flow. This is exacerbated when the block is unidirectional, which is a feature of ischemia-induced injury in Purkinje fibres. Because the small fibres impinge on larger regions of ventricle there is a 'sink-source mismatch' and although conduction from Purkinje fibre to ventricle may fail locally, conduction in the opposite direction may be permitted as a consequence of the larger current source when an 'adjacent' wave front propagates retrogradely from the large mass of ventricle into the smaller damaged fibre (Antzelevitch, 2001). Re-entry can occur in a single circuit, or multiple unstable re-entrant circuits simultaneously, producing more irregular activity such as multiple monomorphic or polymorphic ventricular tachycardia (VT) or VF (Binah & Rosen, 1992).

3. Heterologous expression systems: in vitro patch clamp electrophysiological studies

Mammalian cells are used as the principal host for cloned human ion-channel expression studies conducted within pharmaceutical drug development. The most common heterologous expression systems include human embryonic kidney 293 (HEK 293) cells and Chinese hamster ovaries (CHO) cells, both of which are used primarily in the conduct of drug safety pharmacology studies. These systems can be made to over-express ion-channels of interest, avoiding contamination by background or endogenous currents. Transient or stable expression of exogenous ion channel proteins in these host system is achieved using recombinant DNA technology. Although these expression systems generally yield consistent results, some inconsistencies have been highlighted between *Xenopus* oocytes and HEK 293 cells (Farrokhi, Hrmova, Burton, & Fincher, 2009; Tan, Bezzina, Smits, Verkerk, & Wilde, 2003), in part because of the high level of protein binding that occurs in the oocytes related to the presence of the yolk leading to reduced apparent potency for some drugs and limiting our ability of determining their TdP liability.

Studies are usually conducted at room temperature or at physiological temperatures (~37 °C). While temperature definitely affects channel kinetics, it does not appear to greatly influence potency, with a few exceptions (Kirsch et al., 2004). Data obtained at either temperature can provide important information about drug actions.

4. Acquired long QT syndrome ($aLQT_S$)

Certain classes of therapeutic drugs have an associated risk to cause acquired long QT syndrome (aLQTs) (Kannankeril, Roden, & Darbar, 2010). This syndrome represents one of the most critical potential side effects for clinical consideration since in certain instances it can lead to the potentially lethal arrhythmia, TdP (Curtis et al., 2013). A wide range of drug classes have been shown to increase the QT interval, and for many, this is statistically associated with an increased risk of TdP including: antiarrhythmics, antihistamines, antibiotics, antimicrobials, antipsychotics, oncology agents and others (Table 3). Because of this, aLQTs has been used as a risk indicator for TdP (Curtis et al., 2013). Not all drugs that prolong the QT will cause TdP, not all patients with aLQTs will develop TdP, but by definition, all drugs that cause TdP have been

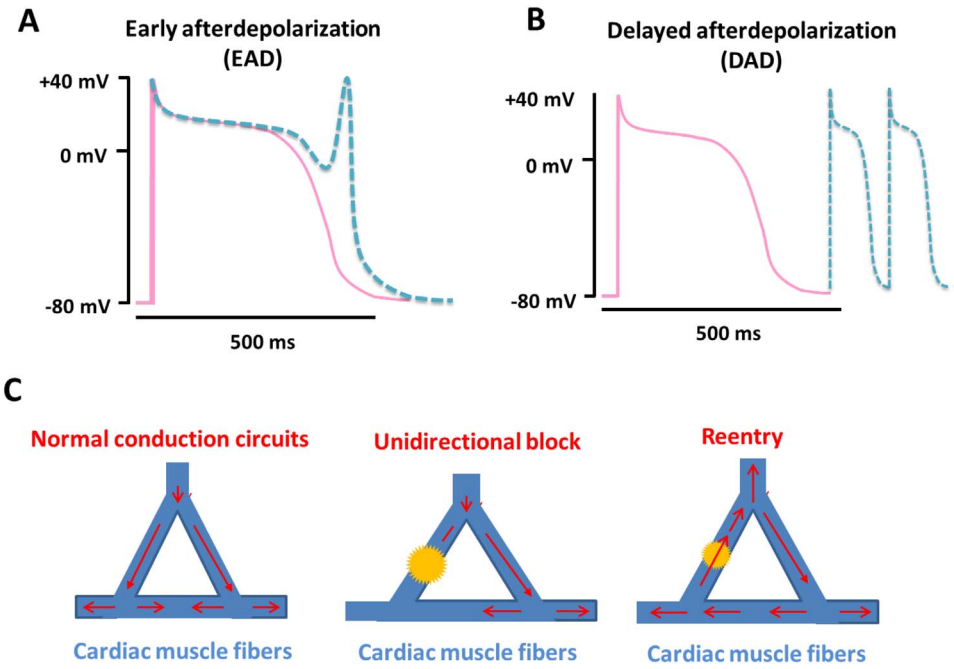

Fig. 3. Mechanisms underlying the development of cardiac arrhythmias. A) An early afterdepolarization (EAD)-induced action potential. B) Delayed afterdepolarizations (DAD)-triggered action potentials. C) Substrate of re-entry for ventricular arrhythmia development. The bottom left panel represents the normal conduction in a bifurcated pathway. The bottom middle panel indicates that electrical conduction is blocked in an area of refractory tissue (i.e., unidirectional block). The bottom right panel shows that if conduction velocity is slower than the tissue refractory period, then reentry can occur. See text for details.

shown to prolong the QT interval (Curtis et al., 2013). Drugs that prolong QT may be approved by FDA, some with a label restriction (e.g., ziprasidone). While most aLQTs is associated with direct inhibition of I_{Kr}, some drugs have been shown to affect hERG channel trafficking at the cell surface (Marzuillo et al., 2014).

4.1. Factors affecting TdP liability

Studies have suggested that pharmacological inhibition of I_{Kr} leading to QT prolongation and TdP liability is typically mediated through binding to the S6 domain of the α subunit of the channel (Mitcheson, Chen, Lin, Culberson, & Sanguinetti, 2000). Drug exposure is a major factor affecting outcome. Like many others, the effects of terfenadine mediated QT prolongation, can be enhanced by the

Table 3
Drugs with an increased propensity for the development of QT prolongation and precipitation of Torsades de Pointes (TdP) arrhythmias.
Source: www.torsades.org; Woosley, RL and Romero, KA, www.Crediblemeds.org, QTdrugs List, [March 8, 2016], AZCERT, Inc. 1822 Innovation Park Dr., Oro Valley, AZ 85755].

Compound category	Compound	Compound category	Compound
Antiarrhythmic drugs (Class IA, Class III and Class IV)	Ajmaline	Anticancer drugs	Prenylamine
	Quinidine		Arsenic trioxide
	Procainamide	Antihistamines	Astemizole
	Disopyramide		Diphenhydramine
	Propafenone		Loratadine
	Almokalant		Terfenadine
	Azimilide	Psychiatric drugs	Amitriptyline
	Dofetilide		Chlorpromazine
	Ibutilide		Chloral hydrate
	Sotalol		Citalopram
	Amiodarone		Doxepin
	Tedisamil		Droperidol
	Verapamil		Haloperidol
	Dronedarone		Imipramine
Antimicrobial drugs	Amantadine		Lithium
	Azithromycin		Maprotiline
	Clarithromycin		Pimozide
	Erythromycin		Sertindole
	Fluconazole		Thioridazine
	Grepafloxacin		Zimelidine
	Itraconazole		Ziprasidone
	Ketoconazole	Vasodilators	Bepridil
	Pentamidine		Lidoflazine
	Sparfloxacin		Vandetanib
	Trimethoprimsulfamethoxazole	Others	Cisapride
Antimalarial drugs	Chloroquine		Domperidone
	Halofantrine		Cesium

inhibition of cytochrome (CYP) P450 3A4 – resulting in higher plasma concentrations (Jurima-Romet, Crawford, Cyr, & Inaba, 1994; Roden, 2004). Similarly, amiodarone (Ohyama et al., 2000) and cisapride (Desta, Soukhova, Mahal, & Flockhart, 2000) were also shown to increase plasma concentrations following CYP3A4 inhibition, resulting in a greater prolongation of the QT interval. Beyond exposure, multiple risk factors may exacerbate aLQTs or TdP risk, such as electrolyte imbalance (e.g., hypokalemia, hypomagnesemia, and hypocalcaemia), stroke, bradycardia, structural cardiac disease and congestive heart failure (Vos, van Opstal, Leunissen, & Verduyn, 2001).

4.2. Drug-induced alterations in ion channel trafficking

Trafficking of ion channels is the process by which newly synthesized proteins are transferred from the endoplasmic reticulum (ER) to the cell membrane, and later removed by the early endosome and degraded. Ribosomes produce nascent polypeptides, which are unfolded chains of amino acids critical for the ion-channel sequence (Curran & Mohler, 2015). Subsequently, these polypeptides are assembled and properly folded in the ER and Golgi apparatus. The fully synthesized ion-channel proteins are then sorted via the endosome and transported to the cell membrane where they are stably expressed. This is the anterograde trafficking pathway. It is balanced by a retrograde trafficking pathway via endosome and lysosome that downregulates channel density (Hwang, 2008; Johannes & Popoff, 2008).

Drugs can alter I_{Kr} expression by affecting different steps within the trafficking pathway (Cubeddu, 2016). Many drugs such as tricyclic antidepressants (Dennis, Nassal, Deschenes, Thomas, & Ficker, 2011), the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Hancox & Mitcheson, 2006), the macrolide antibiotic erythromycin (Duncan et al., 2006) and the azole antifungal ketoconazole (Takemasa et al., 2008) can affect trafficking. These drugs are thought to reduce channel density by inhibiting the transport of the fully mature channel protein from the ER to the membrane surface. Membrane I_{Kr} channel density has been shown to be decreased after long-term exposure to the chemotherapeutic medication arsenic trioxide, which has no direct inhibitory effects when perfused directly on to the channels (Drolet, Simard, & Roden, 2004). Instead, arsenic trioxide was found to downregulate the chaperon complex formation in the trafficking pathway, resulting in the reduced availability of the channel for transport from the ER to the cell membrane (Ficker et al., 2004). The antimicrobial drug pentamidine has also been shown to slow the protein maturation process by directly altering the folding processes of I_{Kr} channels without affecting the chaperon complex (Kuryshev et al., 2005). Probucol, an anti-hyperlipidemic drug, increases I_{Kr} channel degradation in the trafficking pathway (Guo et al., 2007). These observations highlight the numerous mechanisms that are involved in channel trafficking and the limitations of in vitro drug safety screening that may not have scope for assessment of effects on trafficking. Furthermore, many of the drugs that affect trafficking also inhibit I_{Kr}, providing additive effects on AP prolongation.

5. The value of assessing the inhibition of multiple cardiac ion channels

The I_{Kr} assay plays a key role in helping to identify, early in the discovery process, compounds that have the propensity to prolong the QT interval through I_{Kr} inhibition. However, many compounds have inhibitory effects on multiple cardiac ion channels leading to complex alterations in the cardiac AP which may or may not translate into prolongation of the QT interval and/or a TdP liability. For example, the Class Ia antiarrhythmic drug, quinidine, is known to block multiple ion channels including I_{Na}, I_{CaL} and I_{Kr} as well as I_{Ks}, I_{K1}, $I_{K\text{-}ATP}$ and I_{to} (Salata & Wasserstrom, 1988). However; despite this complex pharmacology it is used to restore normal sinus rhythm to patients with symptomatic atrial fibrillation/flutter, and to treat patients with life-threatening sustained ventricular tachycardia. At low therapeutic plasma concentrations, it can prolong the QT interval and potentially induce TdP (effects that may be related to its I_{Kr} blockade); however, at elevated plasma concentrations, multiple ion channel block occurs and the potential QT-prolongation effects are reduced (Ometto, Arfiero, & Vincenzi, 1990; Selzer & Wray, 1964). Moreover, the Class IV antiarrhythmic drug, verapamil, has been shown to produce equipotent inhibitory effects on I_{CaL} and I_{Kr} (Zhang, Zhou, Gong, Makielski, & January,, 1999), but does not cause QT prolongation or increase risk of TdP because the effects on I_{CaL} mitigate I_{Kr} block, leading to limited changes in APD and TdP liability (Fermini & Fossa, 2003; Winters, Schweitzer, Kupersmith, & Gomes, 1985). Even if QT prolongation occurs, a drug does not necessarily have a notable TdP liability. For example, ranolazine (i.e. inhibition I_{Kr} and I_{Nalate}) may cause QTc prolongation but have not been associated with TdP (Jia et al., 2011; Martin et al., 2006). Thus, proarrhythmic effects emerge when the balance between inward and outwards currents is altered significantly. Because of the inherent difficulty in understanding and integrating the potential effects of compounds that affect multiple ion channels at similar concentrations, the use of logistic regression model may likely increase our ability to predict and translate, more efficiently, in vitro ion channel effects to in vivo outcomes, as proposed in the CiPA paradigm (Colatsky et al., 2016; Kramer et al., 2013).

5.1. Stem cells as a tool for proarrhythmia screening

There are two types of human stem cells: embryonic (ESC), which originate from fertilized eggs with the potential to differentiate into different tissues and organs, and adult pluripotent cells of somatic origin, which have limited gene activation potential, but retain the ability to differentiate into only a few types of tissues. Induced pluripotent stem cells (iPSC) offer the potential to assess the risk of drug-induced proarrhythmia using a human derived model. The potential advantage is that it is possible to have an assay in which cells express a full range of ion channels as expressed in human ventricular myocytes. This approach is unique as cells can be differentiated to express not only normal, but also many variant cardiac disease phenotypes (e.g., LQTs (Moretti et al., 2010; Itzhaki et al., 2011; Malan, Friedrichs, Fleischmann, & Sasse, 2011; Egashira et al., 2012; Terrenoire et al., 2013), catecholaminergic polymorphic ventricular tachycardia (CPVT) (Yazawa et al., 2011), overlap syndrome of cardiac Na^+ channelopathy (Davis et al., 2012) and arrhythmogenic right ventricular cardiomyopathy (ARVC) (Ma et al., 2013)). The potential for development of drug-induced arrhythmia may be evaluated by analyzing the parameters for each phase of the cardiac AP, as previously described. IPSC cardiac myocytes (iPSC-CM) are currently being investigated for use in drug safety ion-channel evaluations. IPSC-CMs can be cultured to obtain a large number of cells and can be used with manual or automated patch-clamp technology (Himmel, 2013; Mercola, Colas, & Willems, 2013).

Currently, induced pluripotent stem cell-derived cardiomyocytes (IPSC-CMs) are being evaluated for their physiological similarity to adult human myocytes. At the moment, in terms of cardiac AP parameters, iPSC-CMs exhibit several distinct differences from adult human CMs. IPSC-CMs exhibit a less negative resting membrane potential when compared to adult ventricular myocytes (Davis et al., 2012; Ma et al., 2011). In addition, they show major differences in ion channel expression levels, especially for I_{Kr} and I_f (Khan, Lyon, & Harding, 2013). In general, the IPSC-CM phenotype currently used resembles that of a relatively immature CM and exhibits a variable electrophysiological profile when compared to adult CMs (Himmel, 2013). Individual IPSC-CMs can exhibit either an atrial or ventricular AP morphology and many processes are being developed to enhance ventricular cell differentiation and selection for use in studies. As such, AP waveforms recorded from iPSC clusters presently tend to display heterogeneous phenotypes. Thus, it is imperative to improve selection

of ventricular CMs for study, and further investigation is necessary in order to establish optimal in vitro study conditions for use in proarrhythmia screening.

5.2. CiPA: the next step in proarrhythmia assessment

The CiPA initiative is a consortium composed of a number of collaborators including, FDA, HESI, CSRC, Japan Nation Institute of Health Sciences (NIHS), Health Canada, European Medicines Agency (MEA), Pharmaceutical and Medical Devices Agency (PMDA, Japan), Japan iPS Cardiac Safety Assessment (JiSCA), academics, in silico modellers, and partners from contract research organizations, the pharmaceutical industry and device companies. The new paradigm, has the objective to engineer, early in the drug discovery and development process, assays allowing the evaluation of the proarrhythmic risk of compounds, instead of concentrating on their ability to inhibit the hERG current and to prolong the QT interval (Sager, Gintant, Turner, Pettit, & Stockbridge, 2014). This new paradigm is based on the fundamental mechanistic understanding of the role of ion channels in delayed ventricular repolarization, alterations to which lead to repolarization instability and arrhythmias. It is composed of two distinct nonclinical series of tests: 1) the in vitro study of drug effects on multiple ion channels (not just hERG), and incorporation of these effects in an in silico model of a human ventricular action potential (AP) in an effort to reconstruct the effects on ventricular repolarization and identify potential mechanism-based metrics that can assess proarrhythmia risk, and 2) confirmation of in silico results using human ventricular myocytes, likely derived from human induced pluripotent stem cell (iPSC) cardiomyocytes (Fermini et al., 2016).

The in silico models will be built prospectively, using known torsadogenic drugs of varying risks. The iPSC-CM electrophysiology studies will be useful in confirming predictions based on the in silico model, as will phase I ECG studies in clinical trials should that data be available at the time of conduct of the CiPA assay. Additionally, ion channel inhibition protocols will be standardized, and will likely include multiple pharmacological endpoints. The in silico model will recapitulate the cardiac AP based on drug effects on these multiple ion channels and be aimed at determining potential (yet to be determined) proarrhythmia markers rather than just effects on APD alone. Drugs will be rank ordered for proarrhythmia risk. In vivo ECG analysis in both animals (SP CV studies) and humans (phase I clinical trials) will play important roles in evaluating risk and confirming CiPA outcomes.

6. Conclusion

Proarrhythmia risk assessment for drug-induced TdP is constantly evolving. Over the years, cardiac ion channel testing emerged as an important tool for this. The proarrhythmia screening methodologies have migrated from an ‘hERG centric’ approach to a more integrated strategy including a broader spectrum of in vitro cardiac ion channel data, in silico modelling, in vivo studies and clinical trial data to protect later stage clinical trial participants and ultimately patients. The next decade of research in proarrhythmia risk assessments will require honing of the integrated risk assessment, now named CiPA, and validation of its component parts and the way it is deployed to best effect. This may help avoid throwing ‘the baby out with the bath water’. The evolution of the CiPA methodology is certain to give rise to publications, propelled by increasing knowledge of drug effects on cardiac electrophysiology.

Conflict of interest

None of the authors have any conflicts of interest, other than their employment in commercial pharmaceutical companies, academic institutions or contract research organizations. No information is presented in this paper that advocates for or promotes commercial products from any of our organizations.

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