EXPERT OPINION

1. Introduction
2. Structure, function and pharmacology of cardiac K^+ channels
3. Drug-induced long QT syndrome and induced pluripotent stem cell-derived cardiomyocytes in K^+ channel screening and safety profiling
4. Technologies for cardiac K^+ channel screening
5. Conclusions
6. Expert opinion

Targeting cardiac potassium channels for state-of-the-art drug discovery

Kenneth B Walsh

University of South Carolina, School of Medicine, Department of Pharmacology, Physiology and Neuroscience, Columbia, SC, USA

Introduction: Cardiac K^+ channels play a critical role in maintaining the normal electrical activity of the heart by setting the cell resting membrane potential and by determining the shape and duration of the action potential. Drugs that block the rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed rectifier K^+ current have been widely used as class III antiarrhythmic agents. In addition, drugs that selectively target the ultra-rapid delayed rectifier current (I_{Kur}) and the acetylcholine-gated inward rectifier current (I_{KAch}) have shown efficacy in the treatment of patients with atrial fibrillation. In order to meet the future demand for new antiarrhythmic agents, novel approaches for cardiac K^+ channel drug discovery will need to be developed. Further, K^+ channel screening assays utilizing primary and stem cell-derived cardiomyocytes will be essential for evaluating the cardiotoxicity of potential drug candidates.

Areas covered: In this review, the author provides a brief background on the structure, function and pharmacology of cardiac voltage-gated and inward rectifier K^+ channels. He then focuses on describing and evaluating current technologies, such as ion flux and membrane potential-sensitive dye assays, used for cardiac K^+ channel drug discovery.

Expert opinion: Cardiac K^+ channels will continue to represent significant clinical targets for drug discovery. Although fluorescent high-throughput screening (HTS) assays and automated patch clamp systems will remain the workhorse technologies for identifying lead compounds, innovations in the areas of microfluidics, micropatterning and biosensor fabrication will allow further growth of technologies using primary and stem cell-derived cardiomyocytes.

Keywords: antiarrhythmic agents, cardiac, potassium channels, screening, sensors

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1. Introduction

Cardiac K^+ channels are proteins that span the sarcolemmal membrane and provide a high-conductance pathway for the movement of K^+ down their electrochemical gradient. In the heart, these channels play a critical role in maintaining normal electrical activity by setting the cell resting membrane potential and by determining the shape and duration of the cardiac action potential [1-4]. Cardiac K^+ channels have traditionally been divided into two major categories defined as voltage-gated (Kv) and inward rectifier (Kir) channels [1-4]. The Kv channels include the rapidly activating and inactivating transient outward current (I_{to}), the ultra-rapid delayed rectifier current (I_{Kur}) and the rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed rectifier current. The Kir channels include the cardiac Kir current (I_{K1}), ATP-sensitive (I_{KATP}) and acetylcholine-gated (I_{KAch}) channels. In addition to these classic K^+ channels, Ca^{2+}-activated (I_{KCa}) [5] and two-pore domain (I_{leak}) [6] K^+ channels have recently been identified in the atria of a number of species including humans.

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Article highlights.

- Cardiac K^+ channels play a critical role in maintaining normal electrical activity of the heart.
- Drugs that block voltage-gated K^+ channels are widely used as class III antiarrhythmic agents.
- Given the projected worldwide prevalence of cardiac arrhythmias such as atrial fibrillation, it is essential to develop new K^+ channel screening technologies for discovering effective antiarrhythmic agents. In addition, assays utilizing primary and stem cell-derived cardiomyocytes will be required for evaluating the safety of cardiac and non-cardiac drug candidates.
- Although fluorescent high-throughput screening assays and automated patch clamp systems will continue to be the mainstay for identifying lead compounds, newer technologies such as microelectrode and field effect transistor arrays will allow the greater utilization of native cardiomyocytes in drug screening/safety testing programs.

This box summarizes key points contained in the article.

Cardiac K^+ channel blockers have been widely used as class III antiarrhythmic agents since they prolong the cardiac action potential duration and increase refractoriness of cardiac tissue [3,4]. Atrial fibrillation (Afib) is the most prevalent arrhythmia worldwide affecting more than 30 million adult patients [7,8]. Recently, drugs selective for atrial K^+ currents, such as I_{Kur} and I_{KAch}, have shown efficacy in providing rhythm control in patients with Afib [7,8]. Given that the prevalence of Afib is predicted to double over the next 20 years [9], it is essential to design and develop new technologies for cardiac K^+ channel drug discovery. Assays utilizing primary and stem cell-derived cardiomyocyte will be of particular importance both for K^+ channel screening and for evaluating the cardiotoxicity of potential cardiac and non-cardiac drug candidates. This review provides a brief background on the structure, function and pharmacology of cardiac Kv and Kir channels and then focuses on describing and evaluating current technologies used for cardiac K^+ channel drug discovery.

2. Structure, function and pharmacology of cardiac K^+ channels

The contribution of the various K^+ channels to the cardiac ventricular and atrial action potential waveforms is shown in **Figure 1A**. The initial rapid depolarization of the myocytes (Phase 0) is caused by the fast inward Na^+ current (I_{Na}). The subsequent activation of I_{to} and I_{Kur}, along with the inactivation of I_{Na}, gives rise to early rapid repolarization seen in the action potential (Phase I). During the plateau phase (Phase II), inward current through L-type Ca^{2+} channels (I_{Ca}) and slow inactivating (so-called late) Na^+ channels is balanced by outward current through the delayed rectifier K^+ currents. Repolarization of the action potential (Phase III) occurs via K^+ efflux through I_{Kr}, I_{Ks} and I_{K1}. The cardiac resting potential (Phase IV) of approximately -80 to -90 mV is maintained primarily by I_{K1}. Finally, during periods of metabolic stress (increased intracellular ratio of ADP to ATP) or high parasympathetic (vagal) tone, activation of I_{KATP} and I_{KAch} channels, respectively, can cause a reduction in the action potential duration. As described below, each of the cardiac Kv and Kir channels represents a potential therapeutic target for drug discovery.

Cardiac K^+ channels consist of a pore-forming α subunit that is often associated with an accessory β subunit [1-4]. The structure and function of cardiac Kv and Kir channels is given in **Table 1**. The α subunits are composed of a: i) membrane spanning ion pore that provides a conductive pathway for K^+ movement across the cell membrane; ii) selectivity filter that allows K^+ passage through the channel while excluding other ions; and iii) gating mechanism that controls the movement of the channel from the closed to open states in response to changes in the membrane potential or in the presence of a channel-activating ligand. The pore is also the region where drugs and toxins bind to produce modulatory effects on the gating and conductance properties of the channel. Structurally, Kv channel α subunits contain six transmembrane segments (S1 – S6) with cytoplasmic N- and C-terminal regions and a pore loop between S5 and S6 [1-4]. In general, the β subunits function in regulating the channel-gating kinetics and drug sensitivity. In addition, the β subunits may be required for the successful intracellular trafficking and cell membrane incorporation of the channel. The interaction of the β subunit with the α subunit is of particular importance for the cardiac I_{Ks} and I_{to}. I_{Ks} is composed of the Kv7.1 α subunit (KCNQ1 gene) and the minK β subunit that is encoded by the KCNE1 gene (**Table 1**) [10,11]. When compared to the cardiac I_{Ks}, heterologous expression of Kv7.1 alone produces a small and rapidly activating K^+ current [10,11]. In contrast, co-expression of Kv7.1 along with the minK subunit results in currents that display significantly enhanced amplitudes along with slower activation and deactivation kinetics [10,11]. I_{to} consists of the Kv4.x family of α subunits (Kv4.2 or Kv4.3 in the heart) and KChIP2 β subunit (**Table 1**) [12,13]. The KChIP subunit has been shown to increase the plasma membrane expression of the Kv4.x channels and to confer kinetic properties to the currents that more closely resemble those of the native cardiac I_{to} [12,13].

In contrast to cardiac Kv channels, Kir channels consist of two transmembrane domains connected by a pore region [1,2,14,15]. The inward rectifying I_{KAch} and I_{KATP} channels are of particular relevance for drug discovery because of their unique regulatory properties. I_{KAch} is a member of the superfamily of proteins known as the G-protein-coupled Kir K^+ (GIRK) channels [14,15]. The cardiac I_{KAch} channel is composed of the Kir channel subunits Kir3.1 and Kir3.4 (GIRK1/4) which are arranged in a tetramer (**Table 1**) [14,15]. Binding of muscarinic agents such as acetylcholine or carbachol to the muscarinic receptor causes a dissociation of the $\beta\gamma$ subunits of the G inhibitory protein, which

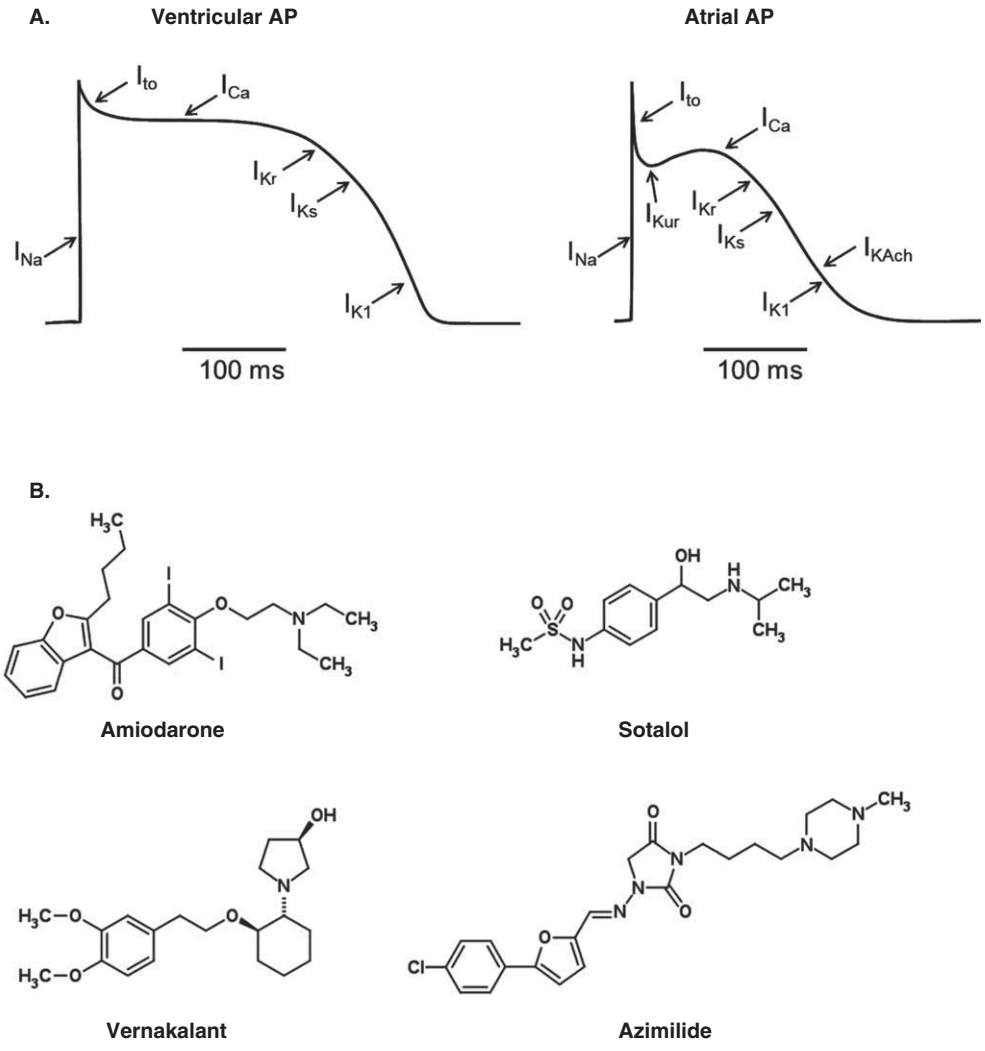

Figure 1. K^+ channel blockers are shown as antiarrhythmic agents. A. Schematic diagram of cardiac ventricular and atrial action potentials and the underlying ion currents responsible for the various phases of the action potential. Note that I_{Kur} and I_{KAch} are selectively expressed in the atrium. **B.** Structures of current and investigational class III antiarrhythmic agents are shown. Whereas amiodarone blocks several ion channels including I_{Na}, I_{Ca}, I_{to}, I_{Kr} and I_{Ks}, sotalol specifically blocks I_{Kr}. Vernakalant (multiple channel blocker) and azimilide (I_{Kr} and I_{Ks} blocker) are currently undergoing clinical trials.

Figure 1A was adapted from [3] with permission of Nature Publishing Group.

AP: Action potential; I_{Ca}: Inward Ca^{2+} current; I_{K1}: Cardiac inward rectifier current; I_{KAch}: Acetylcholine-gated inward rectifier current; I_{Kr}: Rapid components of the delayed rectifier current; I_{Ks}: Slow components of the delayed rectifier current; I_{Kur}: Ultra-rapid delayed rectifier current; I_{Na}: Inward Na^+ current; I_{to}: Inactivating transient outward current.

subsequently binds to and activates I_{KAch} [14,15]. Cardiac I_{KATP} channels consist of four Kir6.2 subunits and the sulfonylurea receptor (SUR) subunit SUR2A (**Table 1**) [1,2,14,16]. The SUR subunit confers sulfonylurea and K^+ channel opener (KCO) sensitivity to I_{KATP} [14,16]. Although I_{KATP} was initially identified in the sarcolemmal membrane of cardiac myocytes [17], it was later demonstrated that KCOs provide cardioprotection against ischemia via a mitochondrial site of action [18,19]. Subsequently, mitochondrial I_{KATP} channels incorporated into lipid bilayers were shown to be modulated by the KCO diazoxide and the I_{KATP} channel blocker 5-hydroxydecanoate [18,19]. Although the molecular nature and pharmacological relevance of the mitochondrial I_{KATP} channel remain controversial, mitochondrial K^+ channels represent new targets for developing anti-ischemic compounds [20].

Cardiac K^+ channels are regulated by protein kinases that phosphorylate serine/threonine and tyrosine residues on the proteins. Studies have demonstrated that the phosphorylation state of K^+ channels can affect the drug sensitivity of the current [21,22] and thus the presence or absence of protein kinase regulation should be considered in drug assay design. I_{Ks} and I_{to} were two of the first cardiac ion currents to be identified as targets of protein kinase-mediated phosphorylation. Catecholamines, β-adrenergic agonists, phosphodiesterase

Table 1. Structure, function and location of cardiac Kv and Kir channels.

Current	α subunit	β subunit	Role in AP	Location	Ref.
$I_{to,f}$	Kv4.2 and Kv4.3	KChIP2	Early rapid repolarization in Phase I	Atrial and ventricular tissue	[12,13,94]
$I_{to,s}$	Kv1.4	Kvβ1.x suggested	Early rapid repolarization in Phase I	Atrial and ventricular tissue	[94,95]
I_{Kur}	Kv1.5	Kvβ1.x and Kvβ2	Early repolarization during Phases I and II	Atrial tissue	[96-98]
I_{Kr}	Kv11.1 (hERG)	KCNE2	Repolarization during Phases II and III	Atrial and ventricular tissue	[99-101]
I_{Ks}	Kv7.1 (KVLQT1)	KCNE1(mink)	Repolarization during Phases II and III	Atrial and ventricular tissue	[10,11]
I_{K1}	Kir2.1 and Kir2.2		Repolarization during Phase III and maintains resting membrane potential Phase IV	Atrial and ventricular tissue	[14,102,103]
I_{KATP}	Kir6.2	SUR2A and SUR1	Opens during Phases III and IV in response to metabolic stress (↑ADP/ATP)	Atrial and ventricular tissue	[14,17,104]
I_{KAch}	Kir3.1/Kir3.4		Opens during Phases III and IV in response to parasympathetic stimulation	Atrial, AV nodal and SA nodal tissue	[15,105,106]

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AV: Atrioventricular; hERG: Human ether-à-go-go; I_{Kur}: Ultra-rapid delayed rectifier current; Kir: Inward rectifier; Kv: Voltage-gated; SA: Sinoatrial; SUR: Sulfonylurea receptor.

inhibitors and other agents that stimulate protein kinase A (PKA), augment the amplitude of I_{Ks} and shorten the action potential duration [23,24]. I_{Ks} is also regulated following α-adrenergic receptor stimulation and in the presence of phorbol ester compounds that activate protein kinase C (PKC) [23,24]. Putative phosphorylation sites on I_{Ks} have been identified at serine27 of the Kv7.1 subunit (PKA site) [25] and serine102 of the minK subunit (PKC site) [26]. In addition, an A-kinase anchoring protein (A-KAP) has been shown to form a macromolecular enzyme complex with the KCNQ1 subunit of I_{Ks} that may be necessary for PKA regulation of the channel [25]. In contrast to I_{Ks}, PKC inhibits I_{to} in cardiac ventricular myocytes isolated from human, rabbit and rat myocardium [27,28]. Phorbol esters also suppress the cloned Kv4.2/4.3 channels when expressed in Xenopus oocytes [29]. Although I_{Kr} is also regulated by PKA and PKC, the nature of this regulatory action is more complex and can vary depending on the species, experimental protocol and stimulating agent used in the studies.

Given the essential role that K^+ channels play in maintaining the cardiac resting membrane potential and shaping the action potential, it is not surprising that these channels represent important targets for drug discovery. Classical K^+ channel blockers used experimentally to characterize cardiac K^+ channels include inorganic cations, quaternary ammonium compounds, aminopyridines and peptide toxins. In general, Kv channels, such as I_{to} and I_{Kur}, are sensitive to 4-aminopyridine and are relatively insensitive to tetraethylammonium salts and Ba^{2+}. In comparison, Kir channels, such I_{K1} and I_{KAch}, are sensitive to Ba^{2+} and are insensitive to 4-AP. Peptides isolated from various species of marine snails, scorpions and spiders have provided a rich source of selective blockers of both Kv and Kir channels. Several peptide toxins that bind to Kv11.1 (human ether-à-go-go [hERG]) channels have been identified [30]. These toxins act mechanistically as either pore-blocking toxins or as channel-gating modifiers. ErgTx1 and BeKm-1, isolated from the scorpions *Centruroides noxius* and *Buthus eupeus*, bind within the pore of the channel, whereas APETx1, a peptide purified from the sea anemone *Anthopleura elegantissima*, acts to modify channel-gating [30]. It is noteworthy that heteropodatoxins and phrixotoxins, from the venom of the spiders *Heteropoda venatoria* and *Phrixotrichus auratus*, represent two of the only groups of compounds that selectively block Kv4.2/4.3 channels and I_{to} when applied at low concentrations [31,32]. Tertiapin, isolated from the venom of the bee *Apis mellifera*, has been widely used as a blocker of some Kir channels (Kir1 and Kir3 families) [33]. In addition, toxin inhibitors to the Kv2.x channels such as stromatoxin-1 and jingzhaotoxin-III have been useful in probing the function of these channels in cellular excitation [34].

In recent years, small-molecule K^+ channel blockers have been introduced as class III antiarrhythmic agents and have been clinically used for the treatment of Afib and ventricular fibrillation (**Figure 1B**). Currently prescribed class III antiarrhythmic drugs are listed in **Table 2** along with some newer K^+ channel modulators that are under clinical investigation. Despite the introduction of catheter ablation procedures to treat Afib and cardioverter-defibrillator implantation for ventricular fibrillation, there remains a critical need for the development of new class III antiarrhythmic agents. It is predicted that by 2015 the global antiarrhythmic drug market will be worth $3.5 billion with newer class III agents such as vernakalant and azimilide (**Figure 1B**) commanding a significant percentage of the market [35]. To meet future demands, novel approaches to K^+ channel screening will need to be developed in order to identify compounds that are both selective for a given K^+ channel subtype and clinically efficacious in the content of native cardiac myocyte electrophysiology.

3. Drug-induced long QT syndrome and induced pluripotent stem cell-derived cardiomyocytes in K^+ channel screening and safety profiling

In addition to providing new class III antiarrhythmic agents, K^+ channel screening assays are essential in evaluating the cardiac risk of potential drug candidates. Long QT syndrome (LQTS) is cardiac disorder that results from a prolonged action potential duration that is manifested as a lengthening of the QT interval measured using a surface electrocardiogram [36]. LQTS is associated with sudden death due to the onset of a severe ventricular arrhythmia known as torsades de pointes [36]. The most common forms of LQTS are caused by mutations in the Kv7.1 (I_{Ks}) and the Kv 11.1 (hERG) (I_{Kr}) α subunits (**Table 1**) [36] that result in the decrease of repolarizing K^+ currents during Phase III of the action potential. In the 1990s, it was reported that several non-cardiac drugs, including cisapride, astemizole and terfenadine, induce torsades-de-pointes by blocking the cardiac I_{Kr} [37]. It is now recognized that a number of different classes of drugs with diverse chemical structures inhibit the hERG channel [37]. Consequently, current regulatory guidelines require both *in vitro* and *in vivo* drug testing to evaluate the risk of cardiac toxicity. The *in vitro* studies are focused on determining drug-induced changes in the action potential duration and specifically on examining drug inhibitory effects on I_{Kr}.

A reliable source of functional human cardiomyocytes would be of great benefit both for hERG channel safety profiling and for K^+ channel drug discovery. However, human cardiomyocytes obtained from adult tissue have a finite lifespan in culture, and attempts to transform these cells in order to establish immortalized human cardiomyocyte cell lines have not been successful. A major advancement toward generating cardiomyocytes occurred with the discovery that adult somatic cells, such as dermal fibroblasts, could be reprogrammed using embryonic transcription factors to create human-induced pluripotent stem cells (iPSCs) [38,39]. Subsequently, several teams of investigators reported the generation and differentiation of human iPSC-derived cardiomyocytes (iPSC-CMs) [40-42]. Human iPSC-CMs display many of the genomic, biochemical, contractile and electrophysiological characteristics of primary cardiomyocytes [43,44]. Of particular relevance, iPSC-CMs express several Kv channels including I_{to}, I_{Kr} and I_{Ks} [44,45]. In addition, iPSC-CMs can be produced in large quantities and in high purity making them potentially suitable for K^+ channel drug screening and toxicological analysis. Finally, disease-specific iPSC-CMs can be generated from patients with a variety of cardiac electrical abnormalities. For example, iPSC-CMs have been produced from patients with LQTS arising from mutations in Kv7.1 (LQT1) and Kv 11.1 (LQT2) channels [46,47]. Thus, the availability of these cells could allow K^+ channel pharmacology to be accessed in more clinically relevant arrhythmic models.

4. Technologies for cardiac K^+ channel screening

Numerous technologies exist for screening cardiac K^+ channels (**Figure 2**). In addition to the usual caveats associated with specific ion channel-screening procedures (see below), there are a number of unique considerations to keep in mind when designing screening assays for new K^+ channel modulators. First and foremost, consideration must be given to the kinetic properties of the K^+ channel under study and the use-dependent characteristics of K^+ channel blockade. Fluorescent-based and ion efflux screening assays are useful for screening ligand-gated (I_{KAch}) and slowly activating/inactivating K^+ currents, but they have limited utility for I_{to} (Kv4.2/Kv4.3) and other rapidly activating/inactivating currents that require high sampling rates (i.e., using the patch clamp technique). Further, a number of K^+ channel modulators, especially the I_{Kr} blockers, display the unwanted property of reverse use-dependence, that is, the compounds are less effective at high heart rates during Afib. Thus, high-throughput fluorescent screening must be supplemented using assay conditions that more closely emulate normal cardiac electrical pacing. Second, many of the current class III antiarrhythmic agents bring about their antiarrhythmic action by blocking multiple types of ion channels (**Table 2**). For example, amiodarone, a first-line agent in the treatment of Afib, blocks I_{Na}, I_{Ca}, I_{to}, I_{Kr} and I_{Ks}. However, current screening strategies are directed at selectively targeting K^+ channels expressed in a particular cardiac tissue. Since both I_{Kur} (Kv1.5) and I_{KAch} (Kir3.1/3.4) are preferentially expressed in the atrium (compared to the ventricle) [2,3], new compounds for Afib such as XEN-D0101 (I_{Kur} blocker) and NTC-801 (I_{KAch} blocker) were developed as selective modulators of these channels (**Table 2**). Therefore, the clinical efficacy of the newer agents must be evaluated alongside the older nonselective drugs. Last, the pros and cons of testing K^+ channel blockers in native cardiac tissue at an early phase of discovery should be carefully considered. As pointed out, replication of native cardiac functional and pharmacological properties may be dependent on the association of the K^+ channel with auxiliary subunits, protein kinases, A-KAPs and other cardiac-specific regulatory factors. Therefore, a clonal cardiac cell line, such as HL-1 cells, or stem cell-derived cardiomyocytes might be preferred for running a medium-throughput assay. Along these same lines, it should be recognized that the expression levels of several cardiac K^+ channels, including I_{to}, I_{Kur} and I_{KAch}, change during Afib due to a process known as electrical remodeling [4,7]. Consequentially, the overall antiarrhythmic effects of selective I_{Kur} and I_{KAch} blockers may differ in normal and remodeled atrial tissues.

4.1 Fluorescent membrane potential-sensitive dye assays

Membrane potential-sensitive dyes (MPSDs) are widely utilized in primary screening assays of cardiac K^+ channels. Membrane potential can be an extremely sensitive indicator

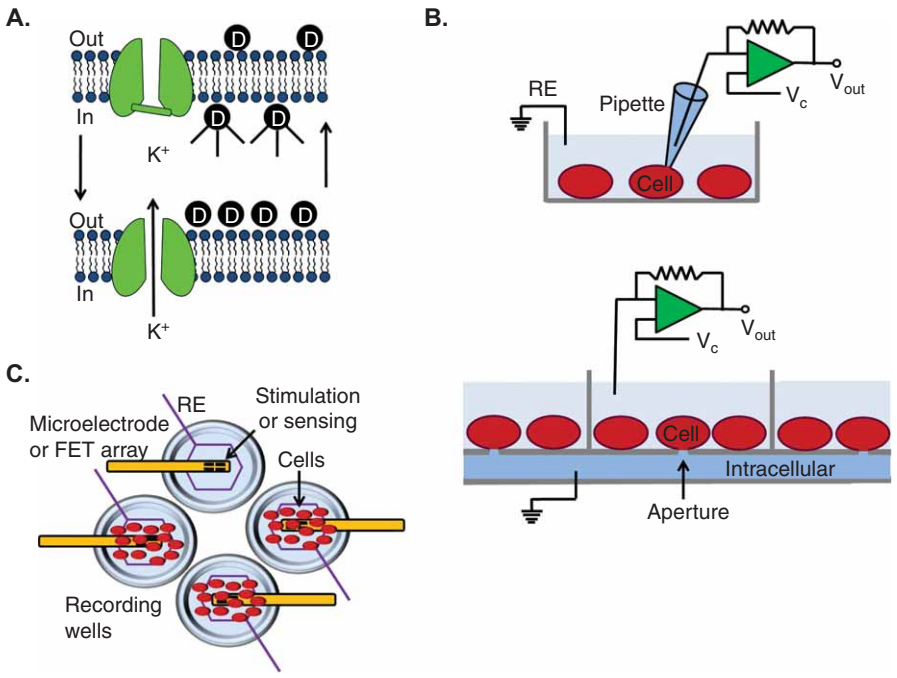

Figure 2. Cardiac K^+ channel screening technologies are shown. (A) Fluorescent membrane potential-sensitive dyes are widely used in K^+ channel screening programs. Changes in the cell membrane potential, resulting from the opening or closing of K^+ channels, alter the distribution of dye molecules **(D)** across the plasma membrane and thus the fluorescent signal. **(B)** Electrophysiological methods include the traditional whole-cell patch clamp technique (upper panel) and newer automated patch clamp systems (bottom panel). **(C)** MEA and FET arrays provide a noninvasive technology for measuring field potentials in cultured cardiomyocytes. A RE is either integrated into the MEA/FET or is included in each recording well (as shown).
FET: Field effect transistor; MEA: Microelectrode array; RE: Reference electrode.

of K^+ channel modulation since relatively small changes in K^+ currents can produce relatively large changes in voltage. In one of the most commonly used MPSD protocols, cells expressing a K^+ channel of interest are incubated with a negatively charged fluorescent oxonol dye, such as bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], which distributes across the plasma membrane (**Figure 2A**). The oxonol molecules inside the cells become strongly fluorescent on binding to intracellular proteins and other cytoplasmic components. Activation of Kv or Kir channels with the subsequent K^+ efflux from the cells causes the membrane potential to become more negative. As a result, the MPSD molecules redistribute to the outside of the cell and the fluorescent signal is decreased. MPSDs have been used in high-density plating formats for screening cardiac Kv and Kir channels including the hERG [48,49], K_{ATP} (Kir 6.2) [50,51] and I_{KAch} (Kir3.1/3.4) [52] channels. Recently, kits containing a MPSD along with a fluorescent quencher have been introduced to reduce well-to-well variations in the fluorescent signal and to allow 'no-wash' dye assays for HTS. Although fluorescent MPSDs provide a convenient and inexpensive approach for measuring changes in membrane potential, they require nonphysiological methods (toxins, high KCl concentrations, etc.) for Kv channel activation and are limited by slow signal responses compared with automated patch clamp (APC) measurements. In addition, oxonol dyes are sensitive to changes in temperature and can be perturbed by test compounds, thus making MPSD assays deposed to high rates of false-positive results.

4.2 Ion flux analysis

Ion flux screening assays using nonradioactive Rb^+ were developed in the 1990s for the analysis of Ca^{2+}-activated K^+ channels [53]. In this procedure, cells are first loaded with Rb^+ and the subsequent efflux of Rb^+ through specific K^+ channels was determined using atomic absorbance spectrometry (AAS). Rb^+ flux assays have been successfully employed in the screening cardiac Kv1.5 [54], Kv11.1 (hERG) [48,55] and Kv7.1/KCNE1 (I_{Ks}) [56] channels. One major advantage of the Rb^+ flux assay is that the signal-to-noise ratio is high when compared to most MPSD assays. However, the assay is limited to screening Kv channels that display slow inactivation kinetics. Consideration must also be given to the time required for cell Rb^+ loading and efflux measurements. In addition, unlike MPSD assays, the technique does not provide real-time experimental analysis since the concentration of the Rb^+ must be determined *post hoc* with AAS.

Weaver *et al.* introduced a thallium (Tl^+)-sensitive, fluorescent-based assay for multiwell plate screening of K^+ channels [57]. In this procedure, cells are first loaded with a Tl^+-

Table 2. Current and investigational class III antiarrhythmic agents.

Drug	Manufacturers	Mechanism	Comments
Amiodarone	Sanofi and Pfizer	Na^+, Ca^{2+} and Kv channel block	Widely used for maintenance of sinus rhythm despite multi-organ toxicity
AVE0118	Sanofi Aventis	I_{Kur}, I_{to} and I_{KAch} block	Cardioversion in animal models, but no clinical studies reported
AZD7009	AstraZeneca	Na^+ and Kv channel block	Entered Phase II trial but was discontinued
Azimilide	Proctor and Gamble	I_{Kr} and I_{Ks} block	Displays modest efficacy in treating Afib
Dofetilide	Pfizer	I_{Kr} block	Maintenance of sinus rhythm with risk of QT prolongation
Dronedarone	Sanofi Aventis	Na^+, Ca^{2+} and Kv channel block	Amiodarone derivative with less toxicity but heart failure contraindications
MK-0448	Merck	I_{Kur} block	First specific I_{Kur} blocker in clinical trials
NIP-141/142	Nissan Chemical	I_{KAch} block	No clinical studies reported
NTC-801	Nissan Chemical and Bristol-Myers Squibb	I_{KAch} block	First specific I_{KAch} blocker in clinical trials
dl-Sotalol	Berlex and Bristol-Myers Squibb	I_{Kr} block and β-adrenergic antagonism	Maintenance of sinus rhythm with risk of QT prolongation
Vernakalant	Cardiome and Merck	I_{Kur}, I_{to} and I_{Na} block	In Phase III trial
XEN-D0101	Xention	I_{Kur} block	Second specific I_{Kur} blocker in clinical trials

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Afib: Atrial fibrillation; I_{KAch}: Acetylcholine-gated inward rectifier current; I_{Kr}: Rapid components of the delayed rectifier current; I_{Ks}: Slow components of the delayed rectifier current; I_{Kur}: Ultra-rapid delayed rectifier current; I_{Na}: Inward Na^+ current; I_{to}: Inactivating transient outward current; Kv: Voltage-gated.

sensitive, membrane permeant reporter dye such as the BTC-AM or FLUXOR. The cells are then incubated in assay buffer containing varying amounts of K^+ and Tl^+. Since many K^+ channels are permeable to Tl^+, K^+ channel modulators can be rapidly screened by monitoring changes in the Tl^+-induced fluorescent signal. The Tl^+ assay has recently been utilized as a primary screen for identifying activators and inhibitors of GIRK channels [58,59], such as the GIRK1/GIRK4 (Kir3.1/3.4) channels that comprise the cardiac I_{KAch}. In addition, the assay has been tested in HEK293 cells expressing hERG channels [60,61]. Although Tl^+ flux measurements displayed assay reliability comparable to that obtained with APC systems, rightward shifts in the dose–response curves for a number of hERG channel blockers were noted when compared with conventional whole-cell patch clamp recordings [60]. The largest reductions in potency were observed with drugs having large hydrophobicity such as amiodarone and astemizole [60], although these differences may result from different conformational states of the hERG channel assayed using the Tl^+ flux and patch clamp technologies [61]. In addition to these pharmacological limitations, biosafety and disposal issues must be taken into account with the use of a toxic heavy metal such as Tl^+.

4.3 Patch clamp electrophysiology

Whereas fluorescent membrane potential dyes and ion flux analysis have been valuable for HTS assays of cardiac K^+ channels, electrophysiological procedures that allow careful control of the cell membrane potential provide a unique advantage over these technologies. The traditional, whole-cell patch clamp technique has been considered the 'gold standard' for investigating the molecular pharmacology of cardiac Kv channels and has been applied in studies of both recombinant and native cardiomyocyte K^+ channels (**Figure 2B**). However, this procedure is labor-intensive, requires a technically skilled staff to carry out the experiments and is extremely low in compound screening throughput. Numerous APC instruments including the Q-Patch, Patchliner, IonFlux, PatchExpress and IonWorks have been introduced over the past 15 years [62,63]. By enabling whole-cell recording to be made from multiple cells in parallel, APC systems have gained popularity in lead optimization and secondary screening of Kv and Kir channels. In general, APC systems utilize a planar array-based format containing a micron-sized aperture in the center of silicon, glass or plastic chips (**Figure 2B**). Cells are added in suspension to the recording chambers and negative pressure is applied to attract single cells to the apertures. Further application of negative pressure causes the patch of membrane immediately beneath the aperture to be ruptured, thus establishing the whole-cell configuration. To date, most APC studies have been carried out with recombinant K^+ channels expressed in Chinese hamster ovary or HEK293 cells that display consistent membrane sealing and stable recording characteristics, as well as large currents. Some systems also provide population recording features that allow data averaging to compensate for cell-to-cell variations in current amplitudes. Some effort has been made in using APC instrumentation to record Kv channels from primary cardiac myocytes and from murine and human stem cell-derived cardiomyocytes [64-66]. In addition to supplying a more appropriate 'cellular environment' for ion channel study, APC recordings with cardiomyocytes can supply data from multiple Kv and Kir channel subtypes; in contrast to just one heterologously expressed channel. Although many APC systems now provide high-quality voltage-clamp data that approaches

traditional whole-cell patch clamp currents, some instruments sacrifice data quality in exchange for higher screening throughput [67,68]. Finally, as in the case with top-line fluorescent plate readers, the high initial startup price and consumable costs involved in using APC systems often limit their application to large academic and pharmaceutical research facilities [68].

4.4 Microelectrode arrays and field effect transistors

Microelectrode arrays (MEAs) and field effect transistors (FETs) represent novel and innovative new technologies for studying cardiac K^+ channels under more physiologically relevant conditions. MEAs are commercially available in various multiwell plate formats with arrays of stimulating and recording electrodes integrated into each well (**Figure 2C**). Primary chick and rodent cardiomyocytes, as well as iPSC-CMs, are plated directly on the MEAs and field potentials are recorded either from spontaneously beating cells or during field stimulation [69-71]. The recorded field potential signal reflects the three major components of the cardiac action potential, that is, a rapid depolarization phase (I_{Na}), a slow plateau phase (I_{Ca}) and a repolarization phase (I_{Kr} and I_{Ks}). In general, results of pharmacological studies using MEAs with primary myocytes and human iPSC-CMs have recapitulated those obtained from cardiac action potential measurements. For example, drugs such as E-4031 that block I_{Kr} prolong both the action potential and the field potential duration [70,72,73]. In contrast verapamil, a drug that blocks both L-type Ca^{2+} and hERG channels, has no effect on the duration of the action potential or the field potential signal since block of the outward I_{Kr} is balanced by the inhibition of the inward I_{Ca} [70,72,73]. Although MEA/human iPSC-CMs systems could eventually be utilized as a first-line technology for evaluating drug I_{Kr} toxicity, there are a number of other considerations. One major limitation of the assay is that currently produced iPSC-CMs display an immature phenotype with structural, electrical and contractile properties that more closely resemble fetal (as opposed to adult) cardiomyocytes [74]. In addition, since field potential signals measured with MEAs represent the summation of currents from multiple cardiac ion channels, data on specific K^+ channels, as recorded with APC systems and other screening assays, are not provided.

FETs are devices that use an applied voltage as a mechanism for electrical switching and amplification [75,76]. FETs are fabricated primarily from silicon and graphene and consist of three terminals called the 'source', 'gate' and 'drain' [75,76]. Ion-sensitive FETs (ISFETs) were developed with the goal of measuring ion concentrations in small fluid volumes [77,78]. With ISFETs, an ion-selective membrane is incorporated into the gate of the FET by applying a polymer containing an ionophore such as valinomycin (K^+) or monensin (Na^+) [77,78]. Permeation of the ion onto the sensing region of the ISFET causes a change in the surface charge of the gate and thus allows for the selective and rapid detection of ions. The small size (1 – 4 mm^2) and biocompatibility properties of the ISFETs represent a marked advantage over large ion-selective electrodes for the measurement of ion fluxes (**Figure 3**). Recently, our laboratory has employed K^+-sensitive ISFETs for measuring K^+ efflux through Ca^{2+}-activated K^+ (KCa) channels. **Figure 3A** displays small conductance KCa currents (I_{KCa}) recorded from cardiac H9c2 cells using the whole-cell patch clamp procedure. The H9c2 cardiac cell line was established from embryonic rat ventricle and has been used as an *in vitro* myocyte model in cardiac biochemical and physiological studies [79]. Application of depolarizing voltage steps to potentials of ≥ -20 mV resulted in the activation of both Kv and time-dependent currents. The time-dependent I_{KCa} were strongly blocked by 100 nM apamin (**Figure 3A**), a blocker of small conductance KCa channels [80]. **Figure 3B** shows an example of K^+ efflux signals recorded from the H9c2 cells using a K^+-sensitive ISFET. H9c2 cells were grown on coverslips and added to a fast-flow chamber containing an ISFET. Application of the Ca^{2+} ionophore A23187, to activate the KCa channels in the H9c2 cells, caused a slow but steady increase in the external K^+ concentration. This efflux was almost completely inhibited by treatment of the cells with apamin. Uncoated FET devices have also been used to measure voltage signals resulting from K^+ efflux through recombinant hERG channels expressed in HEK293 cells [81]. These signals display kinetics and voltage-dependent properties similar to whole-cell K^+ currents that are simultaneously measured using the patch clamp procedure [81].

5. Conclusions

This review has provided a short survey of cardiac K^+ channel screening technologies. Given the projected worldwide prevalence of cardiac arrhythmias such as Afib, it is essential to develop new technologies and design novel strategies for discovering effective antiarrhythmic agents. By prolonging the atrial action potential duration, K^+ channel blockers increase the refractoriness of atrial tissue and demonstrate efficacy in controlling Afib [3,4]. The combination of high-throughput fluorescent imaging (primary screen) and moderate-throughput APC systems (secondary screen) constitutes the current state of the art for identifying cardiac K^+ channel modulators. However, this approach relies on measurements obtained in non-cardiac, heterologous cells overexpressing Kv and Kir channel subunits that do not replicate the regulatory and biophysical properties found in native cardiac cells. In addition, fluorescent and APC assays are designed for the purpose of targeting one specific recombinant ion channel. In contrast, a number of class III antiarrhythmic agents, including amiodarone and vernakalant, control Afib through a mechanism involving blockage of multiple cardiac K^+ channels. Novel cardiomyocyte assay systems, such as MEAs and FETs, while presently at the early stage of validation and optimization, may offer a valuable future technology for phenotypic-based drug discovery.

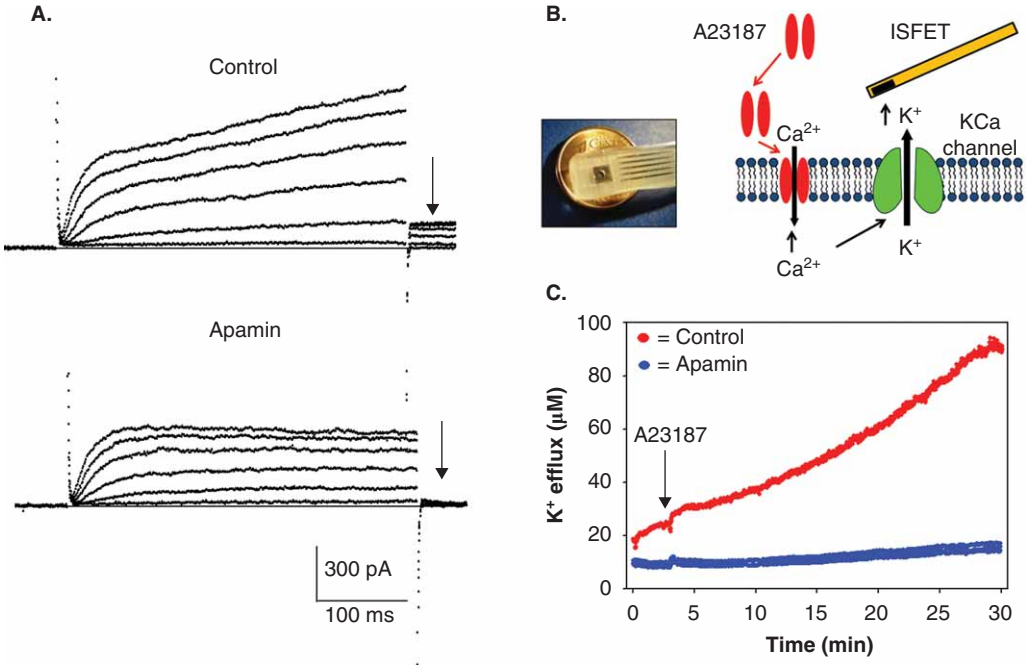

Figure 3. Application of FETs for K^+ channel drug discovery is shown. A. Outward K^+ currents measured from an H9c2 cell during voltage steps applied from a holding potential of -50 mV to -20 through +30 mV in 10 mV increments in the absence and presence of apamin (100 nM) is shown. Apamin blocks the time-dependent Ca^+-activated K^+ currents (I_{KCa}) and the I_{KCa} tail currents (arrows). **B.** K^+-sensitive FET is shown (gold rectangle [size = 2 × 2 mm] D+T Microelectronica, ESP) encapsulated on a printed circuit board (left panel). Increases in intracellular Ca^{2+}, produced by the Ca^{2+} ionophore A23187, activate KCa channels, and the subsequent K^+ efflux is measured using an ISFET (right panel). **C.** K^+ efflux from the H9c2 cells is shown recorded with an ISFET sensor. Buffer containing A23187 is added following the basal efflux measurement (see arrow). Pretreatment with apamin inhibits the A23187-induced K^+ efflux by blocking the KCa channels.

FETs: Field effect transistors; ISFET: Ion-sensitive field effect transistor.

6. Expert opinion

There are a number of factors to weigh when evaluating cardiac K^+ channel screening technologies. Assay throughput, costs, reliability and the relevance of the assay to cardiac electrophysiology are all important issues to consider in the early stages of drug discovery. Fluorescent plate reader technology has proved to be invaluable for the reliable, low cost and expeditious screening of large chemical libraries. The introduction of no-wash protocols using quenchers and new fluorescent dyes has enabled high-density plating formats to be integrated robotically with imaging-based plate readers. MPSD and Tl^+-based fluorescent assays have been validated for screening cardiac hERG, GIRK1/4, K_{ATP} and K_{Ca} channels. However, the use of nonphysiological methods (toxins, high external KCl concentrations, etc.) for K^+ channel activation and the tendency of MPSD systems to produce high rates of false-positives, remain major concerns. Electric field stimulation devices, coupled with fluorescent imaging, have been fabricated for the study of voltage-gated Na^+ channels [82,83] and might provide a more functional approach for Kv channel screening. Alternatively, mutant K^+ channels could be designed that open at more negative membrane potentials and thus require lower KCl concentrations for channel activation in fluorescent studies. For example, a hyperactive KCa channel was recently generated via the introduction of a double mutation in the cytoplasmic domain of the wild-type KCa channel [84]. When expressed in HEK293 cells, the mutant channel displays a negative shift in its voltage-dependence of activation. This shift in the current activation curve allows fluorescent assays to be run at membrane potentials close to the cell resting membrane potential [84].

APC systems not only provide the voltage-control and kinetic resolution of traditional whole-patch clamp setups but also incorporate parallel recording capabilities, microfluidic systems and robotic control which allow for much higher screening throughput. It is likely that, as the cost of APC instruments and associated consumables fall, these systems will play a larger role in primary screening campaigns of cardiac K^+ channels. Indeed, the 386-well plate IonWorks Quattro system has been successfully adapted for screening large compound (> 200,000) libraries against the Kv2.1 [67] and hERG [85] channels. The development of high-throughput APC systems has also allowed hERG channel safety pharmacology to be moved to an earlier phase in drug discovery programs. Human iPSC-CMs represent a particularly appealing

model system for applying APC technology since these cells express several Kv channels including I_{to}, I_{Kr} and I_{Ks} [44,45]. In addition, drugs that induce LQTS have proarrhythmic actions on iPSC-CMs that are comparable to those produced in cardiac Purkinje fibers [86,87]. Preliminary studies suggest that APC methods could be adapted for recording K^+ currents in iPSC-CMs [65,66], although cell fragility, low K^+ channel densities and recording instability will be critical problems to overcome.

MEAs and FETs provide a more physiologically relevant assay system for K^+ channel screening since electrical signals can be noninvasively measured from cardiomyocytes plated on the devices and grown in cell culture. Field potentials recorded with MEAs have been measured from chick and rodent cardiac myocytes, as well as iPSC-CMs [69-71]. Drug-induced changes in the field potential duration have been shown to correlate qualitatively with changes measured in the duration of the action potential [69-71]. However, due to the relatively low throughput and low information content of current systems, the application of MEAs in K^+ channel screening and cardiac safety testing has been limited. Cardiac myocytes have also been directly plated onto FET devices, and extracellular signals, analogous to those recorded with MEAs, are measured from single and multiple cells [88,89]. FETs fabricated from graphene offer several advantages over MEAs including their small size (100 μm^2 to 1 mm^2), high sensitivity and material flexibility [88,89]. Further, the development of K^+-sensitive ISFETs presents the exciting possibility of recording K^+ fluxes from single cardiomyocytes during the activation of specific Kv and Kir channels. However, a number of obstacles, including the compatibility of FET surfaces for cell growth, sensor stability in culture media and graphene degradation over time will need to be addressed before ISFETs can be tested as possible K^+ channel screening devices.

In conclusion, cardiac K^+ channels will continue to represent significant clinical targets for drug discovery. HTS fluorescent assays using MPSDs and Tl^+ flux, along with APC systems, will remain the workhorse technologies for identifying new lead compounds and advancing our knowledge of Kv and Kir channel pharmacology. In the future, refinements in APC materials and methodologies, as well as the greater use of MEAs, FETs and other electronic sensors, will support the introduction of iPSC-CMs and primary cardiomyocytes into drug screening/safety testing programs. Although several challenges must be overcome, advancements in the areas of microfluidics, micropatterning, sensor nanotechnology and iPSC-CM maturation procedures [90-93], will be applied in order to realize the full potential of these new technologies.

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Affiliation

Kenneth B Walsh
University of South Carolina, School of Medicine, Department of Pharmacology, Physiology and Neuroscience, Columbia, SC 29209, USA
Tel: +1 803 216 3519;
Fax: +1 803 216 3538;
E-mail: walsh@uscmed.sc.edu