Allosteric modulation and ligand binding kinetics at the Kv11.1 channel

Yu, Z.

Chapter 2

Kinetic studies of the K_v11.1 (hERG) channel

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Abstract

The K_v11.1 channel, encoded by the human ether-à-go-go-related gene (hERG), mediates a rapid delayed rectifying potassium current (I_{Kr}) underlying repolarization of the cardiac action potential. This channel has been proven to be involved in a diversity of physiologic and pathological processes. “Loss-of-function” mutations in the gene or blockade of the channel by a wide range of prescription medications can prolong the cardiac action potential duration (APD), producing the long QT syndrome (LQTS) that is associated with a markedly increased risk of ventricular arrhythmias and sudden cardiac arrest. On the other hand, “gain-of-function” mutations in the gene shorten the ventricular APD and cause the short QT syndrome (SQTS). Accordingly, the K_v11.1 channel has elicited intense scientific interest in the past several decades. The counter-intuitive gating kinetics of the channel have been suggested to play a specific role in suppressing arrhythmias, and recent studies have pointed out the requirement of incorporating ligand binding kinetics with channel block affinity to refine the K_v11.1 liability of drug candidates. In this review, the biogenesis, structure, function, and pharmacological applications of the K_v11.1 channel are briefly outlined first. Afterwards, the unusual gating kinetics of the channel together with the influences on its gating are summarized. Finally, binding and corresponding kinetics of ligands at the channel as well as assay conditions and techniques for measuring these parameters are discussed.

Introduction

Ion channels are membrane-spanning proteins that conduct ions across the cell and internal organelle membranes. They are involved in a tremendously diverse range of fundamental physiological processes, from rapid responses driven by the neuronal system or fast contractile forces produced by skeletal and smooth muscles to more protracted procedures such as cell growth, differentiation and migration.[1] After G protein-coupled receptors (GPCRs), ion channels constitute the second largest target class for known drugs.[2] Potassium-selective channels, represented by some 70 known loci in the mammalian genome, are the largest and most diverse group of ion channels.[3] This superfamily of potassium channels is mainly divided into three groups: voltage-gated potassium channels (K_v), inward rectifying potassium channels (K_{ir}) and calcium-activated potassium channels (K_{Ca}). Of these, K_v channels are the largest group, and they are encoded by 40 genes and further divided into 12 subfamilies (K_v1-12).[4]

The K_v11 family name, "ether-à-go-go" (EAG) coined by Kaplan and Trout in 1969, was a humorous reference to the similarity between the go-go dancers of the 1960s and the way the legs of a mutant fly shake under ether anesthesia.[5] In this family, the K_v11.1 channel, of which the pore-forming subunit is encoded by the human ether-à-go-go related gene (hERG), has received the greatest scientific scrutiny due to its association with potentially life-threatening cardiac arrhythmias. The K_v11.1 channel is often referred to by several partial synonyms: KCNH2 and hERG to the gene, I_{Kr} to the native current, and K_v11.1 to the fully assembled channel. Missense mutations on human chromosome 7 where KCNH2 is located or pharmacological blockade of the K_v11.1 channel lead to a prolongation of the action potential (AP) duration (APD) that could translate into long QT syndrome (LQTS), Torsades de Pointes (TdP) and sudden death.[5, 6] Electrophysiological studies on these mutants and K_v11.1 blockers have revealed that gating kinetics of the channel play an important role in I_{Kr} suppression.[7-9] Moreover, the assessment of K_v11.1 liability for drug candidates only with IC_{50} values is considered to be unrefined. Therefore, more attention should be paid to the binding kinetics of ligands at the channel in order to achieve a better understanding of the underlying mechanisms of drug actions and subsequently to evaluate more comprehensively their proarrhythmic profiles.[10] In this context, this review will focus on gating kinetics and ligand binding kinetics investigated on the K_v11.1 channel as well as a general introduction of the channel production cycle, its structure, physiological functions, and pharmacological applications during the drug development process.

Production and degradation of the K_v11.1 channel

The production of the K_v11.1 channel begins in the nucleus with transcribing KCNH2 to mRNA, which then acts with the ribosome to synthesize polypeptides. The resulting polypeptide chains are assisted by cytosolic chaperones (heat shock protein 70 [Hsp70] and 90 [Hsp90]) to prevent misfolding or protein degradation, and are further used in the formation of channel subunits in the endoplasmic reticulum.[11] The produced subunits assemble into a tetramer (immature channel) that is exported to the Golgi apparatus, where the channel undergoes a complex glycosylation to obtain a mature and stable conformation before being trafficked to the plasma membrane. The K_v11.1 trafficking is an essential process that contributes to the maturation of the channel ensuring its proper physiological functions.[11] In some cases, the defective trafficking of mutant channels results in a QT interval prolongation, which can be rescued by pharmacological chaperones that stabilize the K_v11.1 channel by binding to specific sites of the channel inner pore.[5] On the other side, degradation of mature channels occurs in lysosomes after being internalized in endocytic vesicles and tagged with ubiquitin. In addition, when the K_v11.1 maturation process is defective, its subunits are also tagged by ubiquitination for degradation and further transferred to the proteasomes or recycled via recycling endosomes. The K_v11.1 channel density at the cell surface is determined by the balance between the transport of mature channels to the cell membrane and their corresponding degradation.

Structural features and physiological functions of the K_v11.1 channel

Structure

Consistent with other K_v channels, the K_v11.1 channel has a tetrameric structure formed by four α-subunits (**Figure 1A**) and auxiliary subunits. As shown in **Figure 1B**, each α-subunit is composed of six transmembrane segments that constitute two major domains with helices S1-S4 contributing to the voltage-sensing domain (VSD) and helices S5-S6 together with the pore loop outlining the pore domain. The VSD undergoes conformational changes upon perturbation of the transmembrane potential through an array of positively charged residues located within S4 helices, whereby the lengthy S5-Pore linker, also known as the "turret", is a peculiar feature of the K_v11.1 channel. The α-subunits co-assemble along the pore axis to form the cavity and selectivity filter (SF) of the channel to allow the central ion conduction. The stability of the SF (**Figure 1C**) is dominated by the

interaction of surrounding pore helices with filter residues.[6] With regard to the auxiliary subunits, a β-subunit, MinK-related peptide 1 (MiRP1), has an important role in accelerating I_{Kr} deactivation and modulating the response of the K_v11.1 channel to drugs.[12] Missense mutations in the KCNE2 gene encoding the MiRP1 have been identified to associate with LQTS and ventricular fibrillations.[13] Notably, the expression levels of MiRP1 in atria and ventricular muscle appear to be low, and thus, exert minor effects on biophysical and pharmacological properties of the channel.[14] However, it is highly expressed in Purkinje fibers of the ventricular conduction system and atrial pacemaker cells.

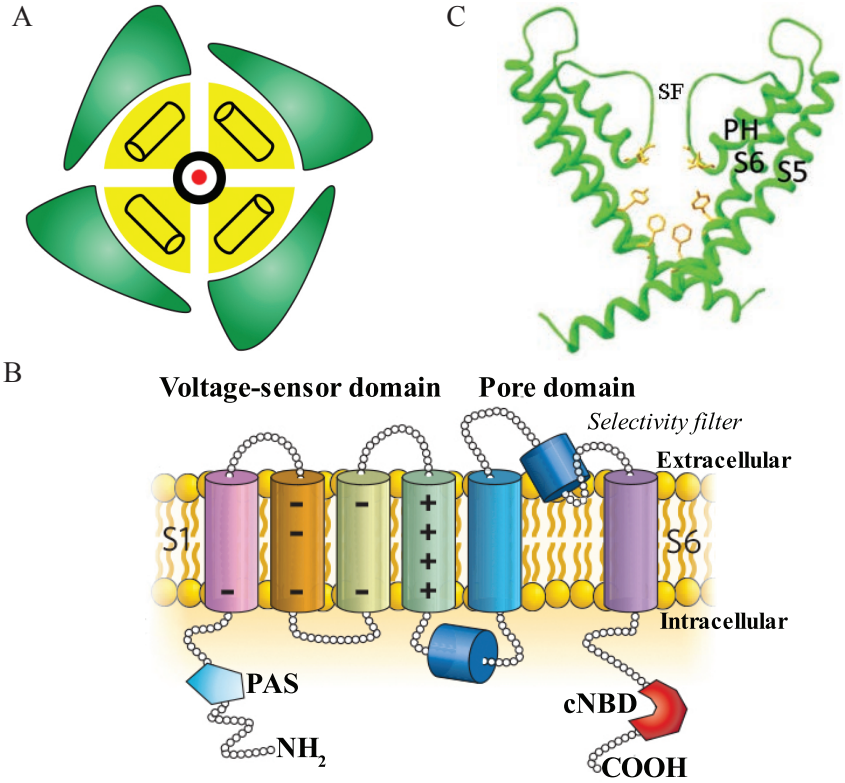

Figure 1. (A) The tetrameric structure of the K_v11.1 channel with the voltage-sensing domain (VSD) colored in green and pore region in yellow, and the red dot representing the potassium ions flow.[5] **(B)** Schematic diagram of a single K_v11.1 subunit containing 6 α-helical transmembrane domains (S1-S6).[6] **(C)** Homology model of the selectivity filter (SF) of the K_v11.1 channel based on the crystal structure of the K_v1.2 channel.[15]

Like all other potassium channels, the structure of the K_v11.1 channel is mainly divided into transmembrane and cytoplasmic regions. The four α-subunits and auxiliary subunits are located in the transmembrane segment, while the NH_2- and COOH-terminals are arranged at the cytoplasmic side. The extracellular loop

between S5 and the pore region contains a glycosylation site that is important for maturation and trafficking of the channel protein.[8] The NH_2-terminal contains a Per-Arnt-Sim (PAS) domain and a stretch of 240 amino acids that are responsible for protein trafficking and definition of the K_v11 family, respectively.[5, 16] On the other hand, the COOH-terminal linked to the S6 helice consists of a cyclic nucleotide binding site (cNBD), contributing to the opening and closure of the permeation pathway for ions. A major difference between the $K_v11.1$ channel and other 6-transmembrane domain potassium channels is derived from the vestibule site. The $K_v11.1$ channel has a larger central cavity due to the absence of a Pro-X-Pro (PXP) motif from the S6 domain that causes the inner helices to bend in other potassium channels.[17] This structural openness is also supported by drug-trapping experiments which indicate that the $K_v11.1$ channel can accommodate a wide variety of structurally diverse drugs and large molecules.[18] These $K_v11.1$ blockers are able to access the binding sites inside the pore, within S6 domains and the end of pore helices, where the important residues for high-affinity blockade of compounds are located.[3]

Physiological functions

The $K_v11.1$ channel is expressed in multiple organs including heart, central nervous system (CNS), gastrointestinal tract (GIT) and endocrine system, and its physiological functions differ according to their distributions in these specific tissues.[6, 19] In the CNS of mammals this channel maintains the membrane potential and development of neurons of the spinal cord and carotid glomus cells, whereas it regulates the gut motility in the GIT. In the endocrine system the $K_v11.1$ channel has been found to be involved in insulin secretion and epinephrine release in chromaffin cells. Apart from these functions, the $K_v11.1$ channel has a dominant presence in normal human cardiomyocytes where its physiological function is best characterized.

As shown in **Figure 2**, the generation of the myocardial AP reflects the sequential activation and inactivation of a series of ion channels, and the AP can be divided into five different phases.[6, 20, 21] In Phase 0, a rapid depolarization occurs when the openings of potassium channels decrease and fast sodium channels open. During Phase 1, there is an early and fast repolarization mainly due to the inactivation of sodium channels and activation of the transient outward potassium channels (I_{to}). Thereafter, the activation of the long opening calcium channels (L-type Ca^{2+}, I_{Ca}) produces a small steady influx of Ca^{2+}, which counteracts the outward K^+ currents induced by an ultra-rapid potassium delayed rectifier. This leads to the formation of a prolonged plateau called Phase 2. The repolarization

in Phase 3 is rapid and robust, and it is initiated by deactivation of sodium and calcium channels and activation of K_v11.1 (I_{Kr}) and K_v7.1 (I_{Ks}) channels. The K_v11.1 channel plays a very important role in Phase 3 repolarization because of its unique gating kinetics: slow activation and fast inactivation coupled to fast recovery from inactivation and slow deactivation. This rapid and delayed rectifier potassium currents (I_{Kr}) terminate the cardiac AP and return the membrane potential to baseline (Phase 4). Since the K_v11.1 channel closes (de-activates) slowly towards the end of the repolarization, there is a constant efflux of potassium ions through the channel. Thus, the resting cell membrane potential is close to the Nernst equilibrium potential for potassium. This also contributes to the refractoriness of cardiac cells in the early diastolic period immediately after repolarization.[5] When a premature beat occurs during the early diastolic period, a large outward current antagonizes the depolarization of the cell and thus propagation of the beat. In this case, functional reduction of I_{Kr} by "loss-of-function" mutations or pharmacological blockade on the K_v11.1 channel translates into a clinical setting in patients prone to arrhythmias determined by premature beats. As shown in **Figure 2**, different waveforms in the electrocardiogram (ECG) reflect distinct phases of the APD in a cardiomyocyte.

Target and antitarget of the K_v11.1 channel in drug development

Roles in cancer

Changes in the expression of the K_v11.1 channel have been associated with dysregulations of cell proliferation in a plethora of tumor types from different cell lineages. The K_v11.1 channel in tumor cells has been postulated to facilitate cell migration in diverse hematopoietic neoplasm through an integrin-related signaling pathway and to enhance the proliferation, invasiveness and lymph node dissemination of cells as well as to reduce cell differentiation.[23, 24] By contrast, the K_v11.1 channel has not been expressed at a significantly high levels in the corresponding non-cancerous cells. Therefore, the K_v11.1 channel, in different tissues, may be used as a biomarker for abnormal cell behaviors and proliferation. Nevertheless, the K_v11.1 channel has also been unraveled to induce cell apoptosis in various cell types by different mechanisms, independent of their capacity to inhibit cell proliferation via cell cycle arrest.[24] Further exploration of this apoptotic pathway together with the channel up-regulation in different tumor types may lead to the development of new anti-tumor therapies in the future. For instance, leukemia is a type of cancer that originates from the bone marrow hematopoietic

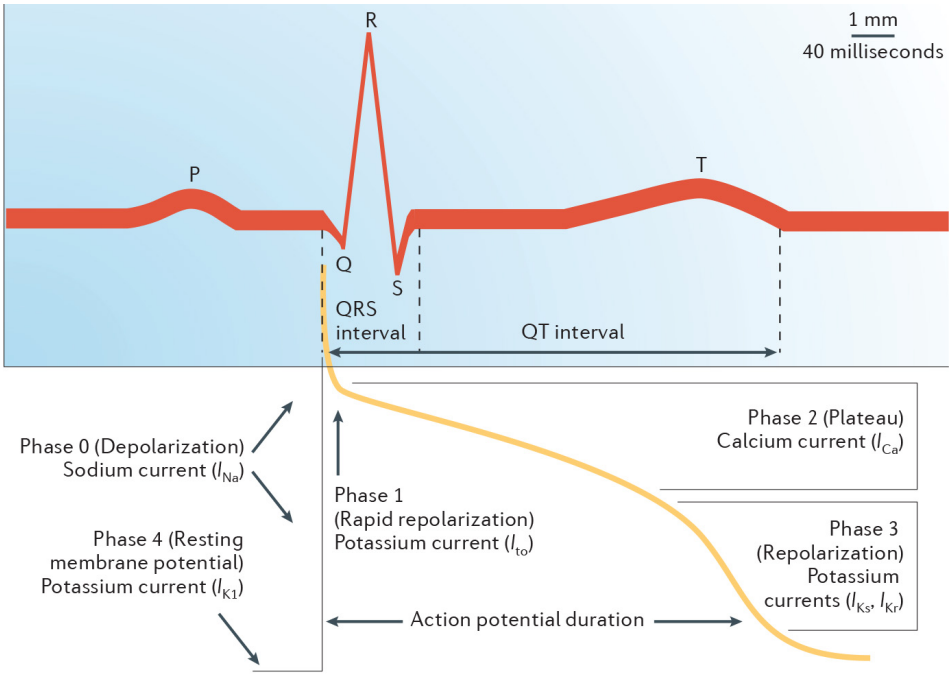

Figure 2. Standard model of the cardiomyocyte QT interval on an electrocardiogram (upper panel) and the corresponding action potential duration (lower panel). The QT interval is a measure of the duration of ventricular depolarization and subsequent repolarization.[22]

stem cells. K_v11.1 channels are expressed in $CD34^+/CD38^-/CD123^{high}$ cells and induce significant proliferation of leukemia cells, whereas they are not detected in normal bone marrow cells.[25] Thus, a specific K_v11.1 blocker could be used to potentially prevent leukemia via reducing abnormal cell proliferation without affecting healthy cells that do not express the K_v11.1 channel. Taken together, the K_v11.1 channel may be used as a novel diagnostic and prognostic marker in human cancers as well as a new target for anti-neoplastic treatments.

Roles in schizophrenia

The contribution of the native K_v11.1 channel to the intrinsic electrical properties of neurons is poorly understood. K_v11.1-3.1 is the primate specific isoform of the K_v11.1 channel expressed in the CNS, and it has been associated with schizophrenia.[26] In this isoform the first 102 amino acids of the most abundant isoform K_v11.1-1a are replaced with 6 amino acids, which results in faster channel deactivation but slower inactivation rates. Although both isomers are comparably expressed at the hippocampus and prefrontal cortex in a control group, the ex-

pression ratio of K_v11.1-3.1 to K_v11.1-1a demonstrates an approximate 2.5-fold increase among patients with schizophrenia. Investigations on the K_v11.1 channel in neural cells may facilitate our understanding of the pathology of schizophrenia, and would provide a new solution for treating this mental disorder in the future.

Roles in smooth muscle related diseases

The K_v11.1 channel is pH sensitive, implying a molecular link for regulating electrical signaling through acidity of the gastrointestinal lumen. Several antibiotics like erythromycin display such adverse reactions as cramps and diarrhea, which might be caused by unintentional blockade of the K_v11.1 channel.[24] Akbarali *et al.* reported that the K_v11.1 channel fine-tuned the resting membrane potential of the circular smooth muscle cells in opossum esophagi, and channel blockers initiated the contraction of muscle segments by depolarization.[27] In human jejunum the K_v11.1 channel was immunohistochemically detected in circular and longitudinal smooth muscle cells, where it was assumed to have a fundamental role in controlling the motility patterns by modulating the electrical behavior of muscle cell.[28] Administration of methanesulfonanilide K_v11.1 blockers like E-4031 and MK-499 impacted the rhythmic contractions of jejunum. Altogether, these findings could help in the search for new targets for the treatment of gastro-esophageal reflux diseases, achalasia as well as intestinal motility disorders.

Human uterine contractions are triggered by an AP possessing a spike followed by prolonged depolarization.[29] Activation of the K_v11.1 channel results in the inactivation of the AP by suppressing the amplitude and duration of contraction before labor. In obese women, enhancement of the K_v11.1 activity by reducing the expression of β-inhibitory proteins shortened the duration of uterine APs and weakened contractions. This finding offers an explanation for the higher incidence of caesarian delivery in obesity, which should be further studied as it offers a new insight into the poor labor outcomes of obese women.

Roles in endocrine dysregulations

In human pancreatic islets, the K_v11.1 channel has been proven to contribute to the regulation of insulin secretion and the firing of β-cells.[30] Blockade of the K_v11.1 channel is sufficient to cause hyper-excitability during application of the insulin releasing drugs. This suggests that alterations in the channel function might be associated with some hyperinsulinemic conditions. The role of the K_v11.1 channel in hyperinsulinemic conditions is awaiting further elucidation that may lead to the discovery of more effective treatment for diabetes.

Roles in arrhythmia

Cardiac arrhythmias, anything from mild palpitations to severe conditions that have the potential to induce cardiac arrest or sudden death, are a major mortality cause in developed countries. The K_v11.1 channel has been suggested to play a particularly pivotal role in the suppression of arrhythmias, yet it has also been found to account for 30-40% of congenital LQTS cases and is the most common cause of drug-induced arrhythmias and cardiac death all over the world.[31, 32]

The K_v11.1 channel as a target in arrhythmology

The short QT syndrome (SQTS) is a cardiac channelopathy associated with atrial fibrillation and sudden cardiac death.[4] The SQTS type 1 variant (SQTS1) involves mutations at KCNH2 that encodes the α-subunit of the K_v11.1 channel.[33, 34] Brugada *et al.* identified two different missense mutations at position 588 in the S5-P loop region (i.e. N588K) of the K_v11.1 channel, which were linked to the hereditary SQTS. These mutations resulted in a dramatic increase in I_{Kr} leading to a heterogeneous shortening of the APD and refractoriness.[5] For this SQTS1, class III antiarrhythmic drugs, also known as K_v11.1 blockers, may be a therapeutic approach for controlling dysregulations of the cardiac rhythm by lengthening the APD and increasing the myocardial refractoriness without significantly altering the conduction velocity.[35] Notably, the sensitivity of SQTS1 to distinct K_v11.1 blockers differs in normalizing the shortening of QT intervals. For example, quinidine restored the shortened QT interval towards a normal range, whereby administration of sotalol and ibutilide in the same condition failed to normalize the abnormal QT interval.[36] This could be explained by the state-dependent inhibition of K_v11.1 blockers to the channel, as the N588K-K_v11.1 channel exhibits altered voltage-dependent inactivation kinetics compared to the wild type channel.

Missense mutations of the KCNH2 gene are also associated with LQTS type 2 variant (LQTS2) and an increased risk for sudden cardiac death. There are four classes of mechanisms determining the LQTS2 abnormalities, acting on K_v11.1 synthesis (class 1), intracellular transport or protein trafficking (class 2), channel gating (class 3) and potassium ion permeation (class 4). Anderson *et al.* performed two comprehensive analyses on 201 LQTS2-linked mutations in four structural domains (NH_2- and COOH-terminus, S1-S4 [VSD], S5-S6 [pore region], extracellular and intracellular linkers), and confirmed that the most common underlying mechanism for LQTS2 was the disturbance of protein trafficking (class 2).[37] Therefore, correction of the K_v11.1 trafficking deficiency could become a useful therapeutic principle in rescuing LQTS2. Most mutations causing trafficking defects can be pharmacologically amended by highly selective K_v11.1 blockers like

E-4031.[38] This compound displayed the highest chance to successfully correct the LQTS2 in channels expressing mutations at the NH_2-terminus, followed by those in the pore domain and then C-linker.[37] Nevertheless, when the wild type K_v11.1 channel was co-expressed, the correction ability of E-4031 changed priorities to mutations in the pore domain followed by the ones in the NH_2-terminus and finally these at the C-linker site. Additionally, K_v11.1 activators, which can augment the I_{Kr}, offer another novel anti-arrhythmic strategy for treatment of LQTS2. The first K_v11.1 activator RPR260243 was reported in 2005,[39] and only a limited number of such compounds have been available until now. Although these activators have shown pronounced antiarrhythmic effects in various *in silico, in vitro* and *in vivo* models, they are found to induce other proarrhythmic side effects such as increasing the APD dispersion[31] and shortening the APD. Overall, activation of the K_v11.1 channel is a new research area, which remains to be exploited in the future.

K_v11.1-related cardiotoxicity

As mentioned previously, mutations of the K_v11.1 channel affect heart conditions characterized by "loss-of-function" or "gain-of-function", which are related to LQTS (**Figure 3A**) and SQTS (**Figure 3B**), respectively. A genetic heterogeneous disorder induced by "gain-of-function" mutations of the K_v11.1 channel is the congenital SQTS, which is identified by a shortened QT interval (< 330 ms) and episodes of syncope, paroxysmal atrial fibrillation or life-threatening arrhythmias.[36] On the other hand, the LQTS represents a cardiac clinical condition involving the K_v11.1 channel, which is characterized by "loss-of-function" of the channel. LQTS can be either congenital or acquired. Congenital LQTS is caused by mutations disrupting the channel functions as described previously. Mutations often lead to deficiencies in protein trafficking, which reduces the amounts of functional K_v11.1 channels located in the cell membrane.[36] Although mutations at other ion channels can also result in LQTS, the reported LQTSs are dominated by the K_v11.1 and K_v7.1 channels. Acquired LQTS refers to drug induced prolongation of the QT interval. This response may be induced by a specifically designed drug that blocks the cardiac repolarizing currents through binding to either the K_v11.1 channel (e.g., dofetilide, sotalol, ibutilide) or to other ion channels involved in cardiac repolarization (e.g., alfuzosin to the sodium channel).[40] However, almost all drugs known to cause acquired LQTS appear to preferentially block the K_v11.1 channel. Furthermore, QT prolongation may arise as a side effect from compounds designed to act on non-cardiac targets. Drugs that belong to different chemical classes and possess various therapeutic indications are able to exert cardiotoxic effects by interfering with the K_v11.1 channel, such as antihistamines

(e.g., terfenadine), gastrointestinal prokinetic agents (e.g., cisapride), psychoactive agents (e.g., chlorpromazine) and antibiotics (e.g., cotrimoxazole).[12] To date, the most common cause of withdrawal from or restrictions of drugs in the market is related to their potential to cause QT prolongations and TdP via blockade of the K_v11.1 channel. This underlines the importance of early evaluation of possible blockade of the K_v11.1 channel for drug candidates, desirably in the early phase of drug development.

Several factors have been identified to account for the promiscuity of the K_v11.1 channel towards drug binding.[6, 12, 41] Firstly, two aromatic residues Tyr652 and Phe656 are highly conserved in the K_v11.1 channel, and they are critical for the binding of structurally diverse drugs. A number of drugs can form a cation-π interaction between their positively charged nitrogen atoms and Tyr652 as well as a hydrophobic attraction of aromatic rings with Phe656. Secondly, the K_v11.1 channel lacks a PXP motif on the S6 helix leading to a large size of the inner cavity, which makes the channel accommodate a range of K_v11.1 blockers encompassing ample molecules. Lastly, multiple distinct binding sites for drugs at the K_v11.1 channel have been discovered, such as an intracellular binding pocket for dofetilide and an extracellular binding region for BeKm-1.[42] This provides another reasonable explanation for the promiscuity of the K_v11.1 channel towards drug binding. Therefore, it is now common practice for pharmaceutical industry to screen for K_v11.1 liability in new chemical entities and conduct thorough clinical QT studies for potential drug candidates. In spite of the seriousness of K_v11.1 related-cardiotoxicty of drugs, the exact molecular and physiological interrelationships behind K_v11.1 blockade, QT prolongation and TdP remain to be explored. As shown in **Figure 2**, depolarization and repolarization of cardiac myocytes are complex processes that result from multiple competing and complementing ionic currents. For example, the potent K_v11.1 blocker verapamil does not display a repolarization liability due to its inhibition of the inward calcium current.[40, 43] In this context, a Comprehensive *in vitro* Proarrhythmia Assay (CiPA) has been proposed as a novel safety screening proposal intended to replace the current regulatory guideline by July 2015.[44, 45] Other strategies considering both affinity and kinetic indicators have also been proposed in order to improve the assessment of arrhythmic side effects for drug candidates.[46, 47]

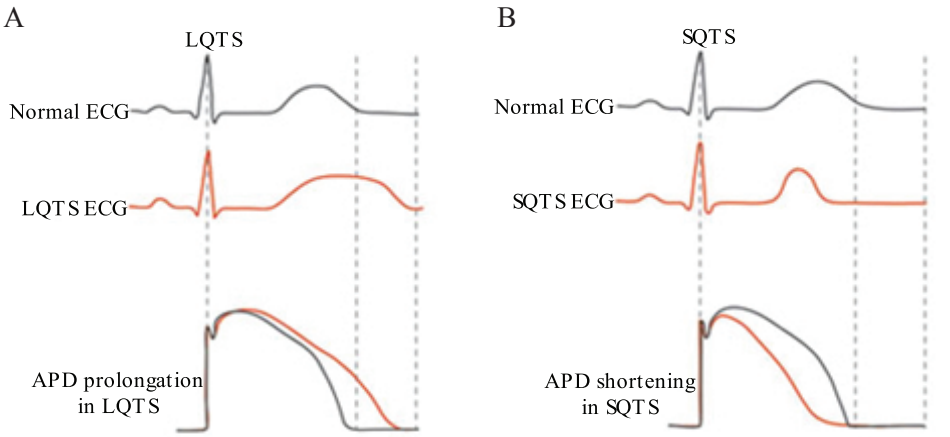

Figure 3. Schematic diagrams depicting the correlation of ventricular action potential duration (APD) to body-surface electrocardiogram (ECG) in normal, LQTS and SQTS patient groups. **(A)** Representation of an LQTS; **(B)** Representation of an SQTS.[48]

Gating kinetics of the K_v11.1 channel

Voltage gated potassium channels regulate the selective conduction of K^+ across biological membranes by opening and closing the pore via structural rearrangement of the activation and/or inactivation gates. These gates are located at both intracellular and extracellular entrances of the SF, and the mediating process is referred to as gating kinetics.[49]

The two gates of the K_v11.1 channel control the potassium flow through the channel. The activation gate is arranged at the intracellular side and formed by four S6 segments with slow movement to open and closed states, while the inactivation gate is located at the extracellular region of the pore and constituted by four SF segments with rapid movement to an inactivated state. The pore of the K_v11.1 channel is more stable in the open conformation compared to other voltage-gated potassium channels.[50] The voltage-sensor of the K_v11.1 channel consists of positively charged residues on the S4 domain and mediates the channel opening.[5] Additionally, the K_v11.1 channel has three extra negative charges in the VSD, which makes it different from other voltage-gated potassium channels that possess only three negative charges on S2 and S3 segments.[51] All these negative charges stabilize the positive charges on the S4 segment by forming salt bridges, influencing the movement of the S4 relative to other transmembrane segments during the gating.

Compared to most voltage-gated potassium channels, the gating kinetics of the K_v11.1 channel are unique: the transition rates of inactivation and recovery from inactivation are more rapid than the kinetics of activation and deactivation

(**Figure 4**).[51] The time constant of inactivation for the K_v11.1 channel aligns from 6.5 to 2.2 ms in the voltage range of -10 to +30 mV, whereas its activation time constant is more than 50 ms at a voltage of +50 mV. By comparison, the durations of inactivation and activation at the same voltage ranges for the Shaker channel were approximately 2000 ms and less than 2 ms, respectively. The quick removal of inactivation at the depolarizing pulse as well as a slow deactivation leads to the formation of a "hook" (tail) current following repolarization, which is typical for the K_v11.1 channel and can be observed in various macroscopic I_{Kr} recordings.[52]

Activation and deactivation

The activation and deactivation processes of the K_v11.1 channel are relatively slow compared to other voltage-gated potassium channels. The slow activation gating is caused by the slow movement of the VSD. The electrostatic interaction between Asp540 and Arg665 mediates the coupling between the VSD and S6 segment. Mutations on these residues were found to affect the activation and deactivation rates of the K_v11.1 channel, implicating that they play an important role in stabilizing the intermediate states in the activation process.[53] The slow deactivation kinetics is maintained by the unique cytosolic domains of the channel, in particular the proximal N-terminal domain.[54] Smith *et al.* confirmed that deleting the NH_2 terminus and adding a recombinant protein corresponding to the PAS domain accelerated the channel deactivation rate. Comparable to deletion of the entire NH_2 terminus, disruption of the α-helix at the NH_2 tail by substitutions of glycine or serine led to an increase of the K_v11.1 deactivation rate. Similarly, K_v11.1 channels, which have the N-terminal and PAS domains removed or Asp774, Glu788 and Asp803 mutated by arginine, exhibited much faster deactivation gating kinetics in contrast to the wild type channels.[55] However, this amendment did not alter the activation gating kinetics of the K_v11.1 channel. Of note, voltage dependency of activation and deactivation was insensitive to the potassium concentrations at the extracellular face of the channel.[52]

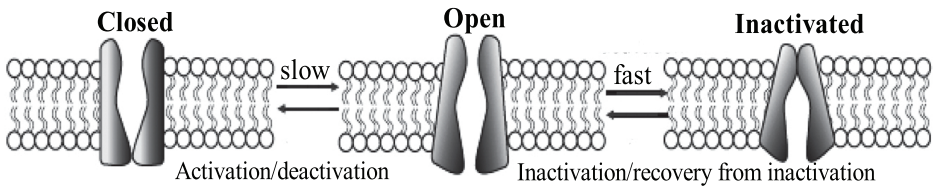

Figure 4. Different K_v11.1 channel conformations in the closed, open and inactivated states.[56]

Inactivation and recovery from inactivation

Inactivation kinetics of the K_v11.1 channel comprise the forward (inactivation) and backward (recovery from inactivation) transitions between the open and inactivated states.[32, 51] These procedures are extremely fast and voltage sensitive. At depolarized potentials the channel inactivation becomes faster than activation resulting in a steady-state rectification at positive voltages, whereas the channel deactivation is much slower compared to its recovery rate from inactivated state forming a hooked tail current that is specific for the K_v11.1 channel.[5, 57] The K_v11.1 channel only demonstrates a C-type inactivation, which is different from the N-type inactivation existing for other voltage-gated ion channels. The N-type inactivation of potassium channels is induced by an occlusion of the ion-conducting pore by the intracellular N-terminal domain, while the C-type inactivation of the K_v11.1 channel permits the interconversion between two conformations of the stable conducting and non-conducting SFs. Kinetic analyses have underlied the early and late pore helix motions during the inactivation process of the K_v11.1 channel.[58] To date, the C-type inactivation of ion channels has been better understood than N-type inactivation.[32]

Effect of gating kinetics on I_{Kr}

Ion conduction is highly dependent on the SF, which is composed of four peptides with a conformational arrangement pointing toward the pore axis of the K_v11.1 channel. The gating kinetics influence the contributions of I_{Kr} to the AP repolarization phases.[59] The K_v11.1 channel is activated during the AP upstroke and plateau phases, while a fast inactivation process of the channel greatly limits the amount of outward current at positive voltages (inward rectification). As the cell membrane repolarizes to negative voltages, the K_v11.1 channel recovers from inactivation and reenters the open state leading to an outward "resurgent" current that facilitates further repolarization. Thereafter, the outward I_{Kr} declines at the end of the AP due to the channel deactivation as well as a decreasing driving force for outward currents. The mechanism of K_v11.1 rectification is closely related to voltage-gated fast inactivation. Since the inactivation of channels takes place at a much faster rate than their activation, no significant outward currents are observable during the depolarization to a high membrane potential. The rapid inactivation of the K_v11.1 channel and the resulting rectification are partially responsible for the prolonged plateau phase which is typical of ventricular APs.[7]

Factors modulating K_v11.1 gating kinetics

Apart from voltage, the gating kinetics of the K_v11.1 channel are susceptible to many other factors such as temperature, extracellular pH, K^+ and Ca^{2+} concentrations.[60] An increase in temperature shifted the voltage dependence of activation to a hyperpolarizing direction, whereas that of inactivation was shifted towards a depolarizing direction.[61] Moreover, the rates of activation and deactivation increase with higher temperatures, but less markedly than inactivation and the reverse recovery rates. In addition, Vandenberg *et al.* emphasized that the results obtained at room temperature could not be simply extrapolated to those of 37 °C by using a single temperature scale factor for the K_v11.1 channel due to the temperature dependence of gating kinetics.[61] When it comes to the extracellular pH, the I_{Kr} amplitude was decreased and kinetics of activation and deactivation were increased when the external pH was lowered from 7.4 to 6.0, whereas activation was accelerated by alternations of the external pH from 7.4 to 8.0.[62] Regarding the extracellular potassium concentrations, increasing the K^+ concentration from 4 to 10 mmol·L^{-1} produced a +10 mV shift in voltage-dependent inactivation of the K_v11.1 channel and slowed the inactivation time course, while reducing the K^+ concentrations 4 to 1 mmol·L^{-1} had little influence on inactivation voltage dependence, but accelerated the inactivation time course.[63] By contrast, an increase in Ca^{2+} concentration from 1.8 to 10 mM did not significantly influence the inactivation kinetics of the K_v11.1 channel, whereby it resulted in a reduction of I_{Kr} amplitude and activation kinetics but increased the deactivation rate.[64] Thus, Ca^{2+} may interact with externally accessible channel residues and further alter the membrane potential sensitivity of the voltage-sensor responsible for activation and deactivation, while this interaction does not change the inactivation gating mechanism.

Influences of ligands on K_v11.1 gating kinetics

K_v11.1 activators are able to increase the ventricular I_{Kr}, which can be used to treat LQTS associated ventricular arrhythmias. These activators act primarily on the C-type inactivation mechanism that is strictly related to the inward rectification. Based on different action modes, K_v11.1 activators are classified into type 1 and 2 groups.[15] The type 1 activators enhance the K_v11.1 function by attenuating the C-type inactivation and slowing down the deactivation rate of the channel. The study of RPR260242 revealed that this activator exerted a concentration-dependent effect on reducing the deactivation rate of the K_v11.1 channel and also produced a positive shift in the voltage dependent inactivation, leading to an increase in I_{Kr} amplitude.[65] RPR260242 was found to interact with two clusters

of residues located on separate domains of the channel. The first cluster had its residues located in regions S5 (L553 and F557) and S6 (N658 and V659). Interaction with this cluster affects the channel's rearrangements that are associated with the deactivation and inactivation gating of the channel. On the other hand, residues for the second cluster are located in the S4-S5 linker (V549 and L550) and S6 (I662, L666 and Y667) segment. Binding of RPR260242 to this cluster is essential to mediate the slow deactivation kinetics induced by its interaction with the first cluster residues. Type 2 K_v11.1 activators augment the channel activity by primarily attenuating its C-type inactivation via slowing down the rapid onset of inactivation and shifting the half maximal inactivation voltage to more depolarized potentials. Perry *et al.* reported that PD-118057 shifted the half-point for inactivation of the K_v11.1 channel by +19 mV and increased the peak outward current of the K_v11.1 channel by 136%.[66] Scanning mutagenesis and molecular modeling suggested that PD-118057 bound to a hydrophobic pocket on two different channel subunits: L646 in the S6 domain for one subunit and L622 and F619 in the pore helix for an adjacent subunit.[15, 66]

Apart from these activators, a number of K_v11.1 blockers alter the gating kinetics of the K_v11.1 channel. Based on the existence of distinct binding regions for ligands at the channel, K_v11.1 blockers can be divided into small molecules and peptide analogs which interact with intracellular and extracellular binding pockets, respectively. The anesthetic drug midazolam was recently unraveled to inhibit I_{Kr} by binding to the same classical binding site within the channel pore as that for prototypical blockers.[67] This compound resulted in a small negative shift of the activation curve of the K_v11.1 channel, while it did not significantly impact the channel's steady-state inactivation. On the other hand, BeKm-1 is a peptide inhibitor interacting with the extracellular amino acid residues close to the external pore region of the K_v11.1 channel.[42] Zhang *et al.* found that BeKm-1 drastically shifted the midpoint of the activation curve towards positive voltages, decreased the activation rate but accelerated the deactivation rate.[68] These effects can be explained by the fact that binding of the BeKm-1 molecule to the channel contacted with the S5-P linker, impacting activation and deactivation kinetics of the K_v11.1 channel.

Binding and corresponding kinetics of ligands at the K_v11.1 channel

Methods for measurements

Patch clamp technique

Voltage-gated ion channels are primarily studied using the voltage clamp/patch clamp technique, which enables the clamping of the cell membrane potential to particular voltages using square or more complex waveforms that allows the measurement of specific activities in the ion channels.[69] Since this method allows a direct and real-time measurement of ion channel activity, it has been regarded as the golden standard in performing electrophysiological investigations on ion channels. There are various patch clamp techniques and systems available, different from one another but relying on the same principle.

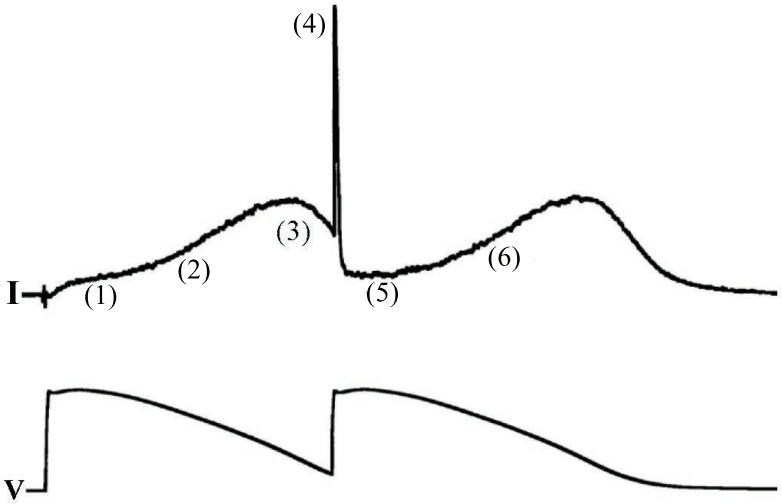

Figure 5. Example of a so-called action potential voltage clamp applied to transfected CHO cells expressing $K_v11.1$ channels ($CHOK_v11.1$ cells). Two pre-recorded action potentials from paced isolated ventricular myocytes (APs, lower panel) were used as the command voltage waveform for whole cell patch clamping the $CHOK_v11.1$ cells. The first AP in this example is followed by a second one elicited by a premature stimulus after 90% repolarization of the first AP. The resulting $K_v11.1$ currents recorded from the $CHOK_v11.1$ cells closely resemble the behavior of I_{Kr} in the native myocytes during the first and second AP (K^+-current, upper panel).[12]

As shown in **Figure 5**,[12] the profile of current flow (upper panel) through the channel can be split into six phases: (1) the initial phase is characterized by a slow opening and fast inactivation of the $K_v11.1$ channel, limiting the current flow; (2) decreasing the voltage enables the channels to recover from inactivation, increasing the current flow; (3) a diminishment in current flow is determined by the slow deactivation and a decrease in driving force for potassium ions. At this phase, many of the channels are still in open states but pass little current; (4) applying a premature stimulus induces an increase in outward current; (5) the outward current has a rapid decay due to the rapid channel inactivation at depo-

larized potentials; (6) similar to the first phase, the large outward current in phase 6 opposes the cellular depolarization and suppresses the propagation of a premature beat. The advantage of this AP voltage clamp technique is that one can study the behavior of a single type of ion channel currents during APs without being hampered by other (often larger) ion currents that are also activated during an AP in native cells. Herein, the AP voltage clamp example implicates a mechanism foundation for other standard patch clamp recordings on the K_v11.1 channel.

The major difference between the K_v11.1 channel and other voltage-gated potassium channels is the presence of a very prominent tail current during the repolarization phase. **Figure 6** shows an example for the application of a standard voltage patch clamp assay in determining the concentration-dependent effects of cisapride on the K_v11.1 channel in transfected CHO cells.[70] From a holding potential of -80 mV, the cell is shortly clamped at -50 mV to test a leak current at this potential when all K_v11.1 channels are in closed states. Since this potential is not enough to activate the K_v11.1 channel, this recording is performed to correct for small background currents that do not originate from the K_v11.1 channel. The recorded current at -50 mV is referred to as a baseline step or leak current. This step is followed by a depolarization to +40 mV, where the K_v11.1 channels are alternating between the open and non-conductive, inactivated states. When repolarizing back to -50 mV, the I_{Kr} reaches its highest peak also known as the "peak tail current" due to the fast recovery of inactivated ion channels. Afterwards, the channels slowly close (de-activate). A cell responding to this voltage clamp protocol (**Figure 6A**) with a typical current signature shown in **Figure 6B** is regarded to express the K_v11.1 channel.

The drug concentration required to inhibit 50% of the tail current (IC_{50}) is usually determined by fitting the Hill equation to the compiled dose-response data. After the channels are exposed to compounds, a subsequent washout experiment is performed to characterize the reversibility of the inhibition. The patch clamp technique is able to identify the activity of moderately potent K_v11.1 blockers which could otherwise have been ignored in other assays leading to false negative results. Although the patch clamp technique is considered to be the "golden standard" in investigating the affinity and kinetic parameters of drugs at the K_v11.1 channel, there are several limitations to this technique. For instance, Hill *et al.* identified a limitation of any kinetic studies on drug blockade at the K_v11.1 channel, which is the lag time associated with drug application and washout.[10] Most systems used for kinetic investigations support a lag time between 10 to 40 milliseconds that still allows recording slow time constants but causes difficulties with capturing fast time constants. Thus, drugs having low affinity and faster unblock time constants at the K_v11.1 channel, demonstrate more inaccurate estimates of

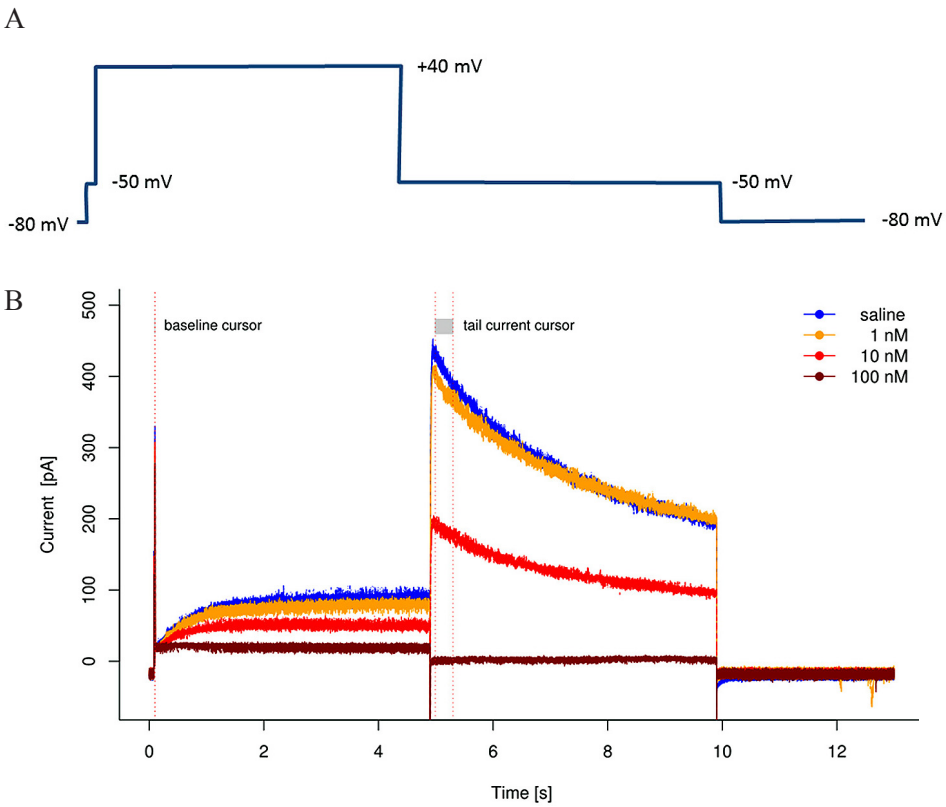

Figure 6. Example of K_v11.1 recordings showing the concentration-dependent effect (0-100 nM) of cisapride: **(A)** the voltage clamp protocol for I_{Kr} measurements; **(B)** I_{Kr} recordings after administration of different dosages of cisapride and saline (0 nM cisapride).[70]

their fast components. Additionally, this technique is low throughput and requires a skilled operator, which hamper profiling of large compound series.[71] Therefore, higher throughput assays are warranted to characterize the binding parameters of drugs candidates at the K_v11.1 channel in the early phase of drug development. Recently, automated patch techniques have revolutionized the research field of experimental electrophysiology, and enabled a substantial increase in throughput without compromising the data quality.[70, 72] Moreover, the throughput of patch clamp screening can be further improved by introducing the patch clamp electrophysiology robots.[70]

Radioligand binding assay

Since radioligand binding assays can provide reasonably accurate and efficient estimates of K_v11.1 affinity for compounds, they have become a different *in vitro* screening tools used by the pharmaceutical industries.[5] These assays rely on

competitive binding of test ligands with a high-affinity, radiolabelled blocker to study the affinity and kinetics of unlabeled ligands at the receptor. Up to now, radioligand binding assays at the K_v11.1 channel have been developed for several specific and potent K_v11.1 blockers including [^{3}H]dofetilide, [^{3}H]astemizole, [^{3}H] MK-499 and [^{3}H]BeKm-1. In the [^{3}H]dofetilide binding assay, the radioligand is used in conjunction with cell lines or membrane preparations expressing the K_v11.1 channel followed by incubation with the test compound which then competes for the binding site that is occupied by [^{3}H]dofetilide.[73] This can yield the experimenter a wealth of information whether the screened compound competitively displaces [^{3}H]dofetilide, its relative potency and, with further experimentation outlined in this thesis, the ligand's kinetics of interaction with that binding site. Diaz *et al.* demonstrated that the binding K_i values for 22 K_v11.1 blockers obtained in a [^{3}H]dofetilide binding assay correlated well with their functional IC_{50} values derived from whole cell patch clamp studies.[74] Chiu *et al.* found that all their selected K_v11.1 blockers inhibited the binding of [^{3}H]astemizole in a competitive manner, and consequently, developed a [^{3}H]astemizole binding assay that they claimed to be robust with adequate sensitivity.[75] This assay is comparable to [^{3}H]dofetilide binding experiments in terms of measured binding affinity (K_i and K_d values), with only 2- to 5-fold variations. Competitive radioligand binding assays apparently provide a rapid and highly predictive tool, which can be utilized together with electrophysiological studies to identify compounds that inhibit the K_v11.1 channel. Nevertheless, the binding assays have the disadvantage of being prone to produce false negative outcomes, for instance when the examined ligand binds to the channel at an allosteric site. Additionally, these assays may also provide false positives as channel modulation may need more than compound-channel binding. Accordingly, the role of radioligand binding assays in screening the K_v11.1 liability of drug candidates is still under discussion, and more researches should be conducted to improve the predictability of these assays.

Ion flux assay

Radioactive ion flux assay

Radiotracers are used to quantitate the flux of ions moving in or out of a cell through ion channels present on the cell membrane.[71] The radiotracers of ions are specific for each class of ion channels, and $^{86}Rb^+$ is commonly used for the voltage-gated potassium channels. However, $^{86}Rb^+$ flux assays are heterogeneous, and also very slow due to the inconvenient radiotracer loading and wash steps. Furthermore, the radioactive tracers can only be applied to measure the steady-

state functions of ion channels. Additionally, an incomplete removal of extracellular tracers after loading or a continuous leak of the tracers out of cells results in a low signal-to-noise ratio for this kind of assays. Hence, although radioactive ion flux assays have been used for more than 30 years in ion channel investigations, their application for screening drug activity at ion channels like the K_v11.1 channel has become limited.

Non-radioactive ion flux assay

Ion flux assays with non-radioactive ion tracers using atomic adsorbance spectrometry (AAS) have been explored for a variety of voltage-gated ion channels.[71] For this analysis, non-radioactive tracer ions are loaded into cells expressing the targeted ion channels (i.e. the K_v11.1 channel). Ion flux is initiated after channel activation performed by depolarizing the cell membrane with a high K^+ concentration buffer. Then, the concentration of the tracer ions in the supernatants and/or cells is measured, and the percentage of ion efflux (or influx) is calculated. This screening method has a moderate throughput, and it has been predominantly focused on potassium channels (e.g., Rb^+ efflux assay for the K_v11.1 channel).[76] The non-radioactive Rb^+ efflux assay is a high-capacity method for functional analysis of the K_v11.1 channel, which is particularly useful for the identification and characterization of K_v11.1 blockers and activators. Since this efflux assay is a direct measure of the channel activity, it is likely not prone to disturbances that usually occur in other indirect procedures, such as false positives in radioligand binding assays. Tang *et al.* compared the IC_{50} values of five known K_v11.1 inhibitors (dofetilide, terfenadine, cisapride, sertindole and astemizole) obtained from both the Rb^+ efflux assay and patch clamp technique.[77] The former displayed 5- to 20-fold higher IC_{50} values than the latter, yet the rank order of measured IC_{50} values for these compounds was identical in these two different experiments. This illustrates the capacity of the Rb^+ efflux assay to rank unknown compounds that block the K_v11.1 channel by their potency, meeting the requirements for compound profiling. In addition, Chaudhary *et al.* used the Rb^+ efflux assay to evaluate the K_v11.1 liability of astemizole, E-4031, cisapride, terfenadine, risperidone, quinidine and sotalol, and compared the results to those acquired in the patch clamp technique and [^{3}H]dofetilide binding assay.[78] IC_{50} values of test ligands gathered with the efflux assay were increased compared with those from the electrophysiological technique, while the Rb^+ efflux assay was less sensitive in determining the potency of compounds but had a higher throughput to identify potent K_v11.1 blockers than the radioligand binding assay. Hence, the Rb^+ efflux assay allows for the rapid screening of compounds in a non-invasive manner. However, this nonradioactive ion flux assay has a low temporal resolution indicative of less

information compared to the voltage clamp assay. Meanwhile, a high expression level of K_v11.1 channels is necessary to achieve an acceptable signal-to-noise ratio for this technique.[79] Additionally, Rb^+ appeared to inhibit the inactivation of the K_v11.1 channel resulting in an underestimation of potency for most drugs, which impairs its application in drug screening for K_v11.1 liability.[76]

Other techniques

The electrophysiological properties of cardiomyocytes expressing various ion channels can also be investigated by micro-electrode arrays (MEAs). This novel technology is based on the integration of cardiac cell cultures or vibratome slices from cardiac tissues on microchips in order to monitor the changes in extracellular electrograms.[80] The detected field potential durations (FPDs) correlate well with the APDs of cardiomyocytes from patch clamp assays as well as with QT intervals in clinical ECGs, while the measured beat frequencies are in agreement with the heart rates.[81, 82] Moreover, MEAs utilizing networks of cells demonstrate a higher complexity, which circumvents the danger of retrieving false positives encountered by single-cell based assays.[82] In addition, the non-invasive nature of MEAs allows the study of drug effects on beating rate and rhythm, and enables the detection of arrhythmia.[81] However, the MEA devices are not suitable for stimulating and recording the electrophysiological parameters of single cells compared to patch clamp techniques. Furthermore, implantation of a micro-electrode array might have negative effects on the measurements due to various biological responses of cells upon the external insertion.[83] Altogether, screening compounds in cardiomyocytes, in particular human induced pluripotent stem cell-derived cardiac myocytes (hiPSC-CMs), with the MEAs technology is still awaiting optimization and will make a substantial contribution to safety pharmacology, which provides a promising means for preliminary drug profiling in the drug discovery pipeline.[80, 84]

xCELLigence Real-Time Cell Analyzer (RTCA) Cardio System is another innovative label-free real-time platform, which can be utilized to measure the beating functions of cardiomyocytes like hiPSC-CMs based on impedance measurement.[85] This assay is performed using specially designed microtiter plates with interdigitated golden electrodes to dynamically monitor the impedance changes of cardiomyocyte layers during rhythmic contractions.[85, 86] This new technology displays a higher throughput than patch clamping, allowing for screening the cardiotoxic liability of compounds earlier during the drug research process. Furthermore, the xCELLigence system enables the monitoring of ligand influences on cardiac functions in real time over prolonged intervals, which supports its application in revealing compounds that cause arrhythmia via inhibiting the

protein trafficking of ion channels.[85, 87] In addition to such advantages, there are several challenges for the application of xCELLigence RTCA Cardio System in preclinical cardiac safety assessment.[87] For example, the predictive values of proarrhythmic drugs derived from the extracted parameters in this technology are not explicit, and are required to be further elucidated in the future.

Factors affecting the measurements

All the methods used for investigating the affinity and kinetic parameters of ligands at the K_v11.1 channel are influenced by a range of external factors. Herein, we mainly focus on the impacts of assay conditions, species diversity and temperature on the data interpretation for the K_v11.1 channel.

The patch clamp technique is a highly flexible method that can be performed in a variety of configurations and modes, providing a direct, precise and detailed measurement of the activity for an ion channel.[79] Specific bath and pipette solutions, voltage and current protocols based on research aims are essential to obtain reliable results. For instance, Kirsch *et al.* found that there was a 2-fold underestimation of the potency for pimozide at the K_v11.1 channel when the step-pulse protocol was changed from 2 s to 0.5 s at 35 °C.[88] In radioligand binding assays, BSA concentration in the assay buffers might considerably reduce the speed of filtration and further result in the loss of a low affinity binding component for the K_v11.1 blockers. Consequently, variability amongst the results acquired in different laboratories may stem, in part, from the varied assay conditions.

Species diversity is another common factor that influences the ligands' activity at the K_v11.1 channel in all techniques. Different binding and kinetic parameters of drugs at the K_v11.1 channel could be observed with cells from different species or tissues. There have been reported differences in IC_{50} values between the patch clamp measurements in *Xenopus* oocytes versus transfected mammalian cell cultures (i.e. HEK293 and native K_v11.1 channel in cardiomyocytes).[89] Next to protocol variations, these are also attributed to the restricted drug diffusion at the oocytes surface membrane and yolk sac absorption, which does not occur in mammalian cells. Moreover, even when the K_v11.1 channel is assessed in the same species but in different cell cultures, variations might be noticeable in the activity and functionality of the channel. This could be related to the varied contents or isoforms of the K_v11.1 proteins in different cell cultures. In this circumstance, tissue linearity experiments can be conducted to standardize the distinct results from different cell cultures.[75]

Temperature is the most important factor that impacts the interpretation of data from different assays. Physiological temperature is supposed to be more

suitable to determine drug safety at the K_v11.1 channel, since channel gating, receptor binding and drug accumulation might be temperature-dependent. In the patch clamp study the measured IC_{50} values of sotalol and erythromycin at 35 °C were decreased by 2.9- and 7.1-fold respectively compared to these determined at room temperature.[88] Furthermore, the binding kinetics of ligands at the ion channel can be remarkably affected by the temperature. For instance, erythromycin had an extremely slow onset of block and the time required to reach half-maximal effect was 156 s at 22 °C, whereas the same compound displayed a much faster onset of block and the time needed to approach half-maximal effect was 18 s at 35 °C.[88] The incubation temperature affects the time for ion exchange in ion flux assays,[90] and also plays a paramount role in measuring the affinity and kinetic parameters of ligands in radioligand binding assays.[91]

Binding of ligands and their kinetics at the K_v11.1 channel

Small molecules that act at the intracellular side

Astemizole, desmethylastemizole and norastemizole

Astemizole is a second generation histamine H_1-receptor antagonist.[89] In addition to antihistamine effects, it causes a QT interval prolongation and cardiac arrhythmia due to the unintended blockade of the K_v11.1 channel. Oxidative *O*- and *N*-demethylations of astemizole generate two metabolites desmethylastemizole and norastemizole, respectively. Desmethylastemizole and astemizole initiated equipotent blockade of the K_v11.1 channel, while norastemizole had weaker potency at the channel.[89] Taglialatela *et al.* investigated the concentration-dependent effect of astemizole on *Xenopus* oocytes transfected with the K_v11.1 channel by an extracellular perfusion, and calculated its IC_{50} as 480 nM.[92] This value was 10 times higher than that previously reported by Suessbrich *et al.*,[93] which was explained by a longer incubation time in the latter study leading to higher intracellular drug concentrations and thus stronger potency. Binding of the parent drug and its metabolites occurs primarily in the open and inactivated states of the channel with a minimal block in the closed state.[89] Blockade of astemizole and desmethylastemizole occurred faster at higher action frequencies. Recovery from astemizole blockade was minimal, while a partial recovery for desmethylastemizole was observed after a washout period of 30 minutes. However, the inhibition of norastemizole was incomplete and only occurred at high concentrations after channel activation. Astemizole and norastemizole were found to bind in the open state of the channel in the region of the hydrophobic inner vestibule after the assembly of the channel tetramers.[94] A mutation to cysteine at the F656 pore

residue lining in the inner vestibule dramatically reduced the blockade of these two compounds.

Clozapine

Hill *et al.* performed the first study on directly measuring the binding and unbinding kinetics of the typical antipsychotic clozapine at the K_v11.1 channel expressed in CHO cells using the patch clamp technique with a Dynaflow automated compound delivery system.[10] They revealed two kinetically distinct blocking effects consistent with different binding kinetics to the open and inactivated states of the channel, while the binding affinity of clozapine for the two different states were almost identical. The block and unblock time courses of clozapine were not monophasic, which encompassed a fast and a slow phase. The fast component of block was related to the fast component of unblock, and vice versa. The current recovery rate from clozapine block during the washout experiment was independent of the drug dosages applied in the system, whereas it was exposure time dependent with a slower recovery rate after a longer application of the drug. The molecular mechanism of clozapine blockade at the K_v11.1 channel has not been elucidated. However, it is believed that clozapine binds at the pore site towards the lumen on residues Tyr652 and Phe656 of the S6 domain, which is comparable to other prototypical K_v11.1 blockers.[95]

Clofilium and ibutilide

Clofilium and ibutilide, which are two structurally related class III antiarrhythmic drugs, were found to have different time-dependent kinetics of block at the K_v11.1 channel in whole cell voltage clamped *Xenopus* oocytes.[18] The binding potency of ibutilide at the channel showed an IC_{50} value of 28 nM, while a precise measurement of the IC_{50} value for clofilium was difficult due to its extraordinarily slow onset of block. The steady-state inhibition of clofilium to the K_v11.1 channel was not approached even after a prolonged exposure to more than 1 hour. Nevertheless, the onset of block was reported to be faster when clofilium was applied from the intracellular side of excised membranes in excised patch clamp experiments. Regarding the unblock rate, the inhibition of ibutilide was almost completely recovered after a 10-minute washout, whereby only 19% I_{Kr} was restored from clofilium block within the same washout period. This utra-slow recovery rate of clofilium from K_v11.1 blockade is similar to the findings for other potent channel blockers which demonstrate slow unbinding profiles because of drug trapping within the central cavity of the channel after closure of the activated gate upon membrane repolarization.[96, 97] To investigate the trapping hypothesis,

both clofilium and ibutilide were tested at the K_v11.1 D540K mutant that permits untrapping at a hyperpolarized state by allowing the gate to remain open.[18] Again, recovery from K_v11.1 blockade in the case of ibutilide was almost complete, yet little current recovery from clofilium inhibition to the channel was observed. This ruled out the possibility of drug tapping within the channel cavity, also known as a "foot in the door" mechanism, for clofilium. The binding sites of clofilium and ibutilide at the K_v11.1 channel were the same by alanine-scanning mutagenesis of Tyr652, Phe656, Thr623, Ser624 and Val625 residues. The S624A mutant located at the base of the pore helix of the K_v11.1 channel was the only one that was found to enable the rapid I_{Kr} recovery from clofilium blockade.

Terfenadine

Terfenadine is a potent blocker of the K_v11.1 channel in the open state. The IC_{50} of terfenadine in *Xenopus* oocytes expressing the K_v11.1 channel was 350 nM at 22 ºC.[93] Extracellular potassium concentrations significantly altered the K_v11.1 potency of terfenadine with an IC_{50} of 3.5 mM at 96 mM K^+ reduced to 350 nM in 2 mM K^+.[98] The steady-state block of the K_v11.1 channel by terfenadine was approached within 7 minutes superfusion of Ringer's solution containing terfenadine, and the blockade was frequency-, voltage- and use-independent. The recovery of terfenadine from block at the closed state took place after a washout period of more than 30 minutes, which can be explained by drug trapping inside of the central cavity of the K_v11.1 channel.[99] Unlike clofilium, the closure-deficient K_v11.1 D540K mutant was experimentally confirmed to facilitate the I_{Kr} recovery from terfenadine block at the K_v11.1 channel. Focusing on the molecular binding perspective, terfenadine interacts with S624, Y652, F656 and T632 residues, while amino acids Y652 and F656 are the most important determinants for the K_v11.1 sensitivity to terfenadine.[99]

Cisapride

Cisapride, a prokinetic agent, is a potent K_v11.1 blocker.[99] The IC_{50} value of cisapride at the K_v11.1 channel on the *Xenopus* oocytes was 630 nM. The onset of block of cisapride was rapid and the recovery from block was complete within approximate 2 minutes.[100] Mutation studies for the binding site of cisapride at the K_v11.1 channel implicated that π-π interactions with Y652 and F656 residues and additional hydrophobic interactions with Y652 were essential for the channel sensitivity to cisapride.[101]

Propafenone

Witchel *et al.* used a concentration of 50 mM propafenone to induce a profound K_v11.1 channel blockade.[102] The tail current was blocked by 96% at -70 mV while the blockade dropped to 5% in the F656A mutant in *Xenopus* oocytes. This indicates that the F656 residue is a critical determinant for the blockade of propafenone at the K_v11.1 channel, similar to other methanesulfonanilides like dofetilide and MK-499. Recovery of the K_v11.1 channel from propafenone blockade was very rapid in the open state, whereas it was virtually undetectable in the time scale tested in the closed state. A further study performed by Windisch *et al.* revealed that propafenone derivatives with short side chains were trapped in the closed channel and displayed a very slow unblock rate at the K_v11.1 channel, which was in contrast to the long side chain analogues displaying insignificant trapping.[96] Altogether, the study provides direct evidence for a "foot in the door" type blockade of ligands at the K_v11.1 channel.

Peptides that act at the extracellular side

APETx1 and Saxitoxin

APETx1 is a peptide purified from sea anemone, which acts as a gate modifier of the K_v11.1 channel.[103] APETx1 inhibited the I_{Kr} in a heterologous system with an IC_{50} value of 34 nM at room temperature, while the inhibition was maximal at 300 nM leaving 20% current unblocked even at concentrations up to 1 µM in COS-7 cells transfected with K_v11.1 channels. Its kinetics of block profile were characterized by a rapid onset of block, and the steady-state inhibition was reached within 1 minute. The block of APETx1 to the K_v11.1 channel was totally reversible upon a washout period of 100 s. Similar to the binding sites for gating modifier toxins of other voltage-gated potassium channels, mutations in the S3 region of the K_v11.1 channel like G514C and E518C had dramatic impact on the responsiveness of the channel to APETx1.[104]

Saxitoxin inhibited the I_{Kr} by more than 90% at 3 µM in HEK293 cells expressing the K_v11.1 channel at 36 ± 1 ºC.[105] Current reduction reached steady state quickly, and the block was reversible upon washout with dissociation characteristics opposing the simple first-order process. The concentration-dependency of saxitoxin alongside its kinetics of block onset and recovery suggested a complex binding of multiple molecules of saxitoxin to the extracellular side of the K_v11.1 channel. Co-administration of methanesulfonanilide MK-499 showed that the K_v11.1 inhibition effects of the two drugs were additive, further indicating that saxitoxin did not bind to the intracellular side of the channel as MK-499.

BeKm-1

BeKm-1 is a scorpion toxin, which selectively blocked the I_{Kr} current with an IC_{50} value of 3.3 nM in a patch clamp study in transfected HEK93 cells at room temperature.[106] The current block and unblock rates of wile type BeKm-1 at the K_v11.1 channel were 2.5 $\times 10^6$ $M^{-1} \cdot s^{-1}$ and 0.017 s^{-1}, respectively.[107] In radioligand binding assays the association and dissociation kinetics of [^{125}I]-BeKm-1 were extremely fast at room temperature, while its on- and off-rate constants at 0 ºC were 3.8×10^7 $M^{-1} \cdot s^{-1}$ and 0.00051 s^{-1}, separately.[107] Additionally, wild-type BeKm-1 displaced the binding of [^{125}I]-BeKm-1 with a half-maximal inhibitory concentration of 44 pM at 0 ºC.[107] Collectively, discrepancies exist between quantitative results acquired in patch clamp and radioligand binding assays, albeit to the comparable rank orders of measured parameters from these two different techniques. BeKm-1 preferentially inhibited the I_{Kr} current through a closed state channel blockade mechanism,[108] and interacted with the K_v11.1 channel at the extracellular site.[42]

Conclusions

The K_v11.1 channel is a voltage-gated potassium channel that shares structural characteristics with the voltage-gated potassium channel family but displays unique gating kinetics. It is involved in a number of physiological conditions, and has the potential to become a new biomarker and therapeutic target for cancer, schizophrenia and other important diseases due to its high expression in diseased cells instead of normal cells. More importantly, the K_v11.1 channel plays an important role in maintaining cardiac stability under normal conditions being the target of class III antiarrhythmic drugs, but at the same time, when dysregulated, causes notorious side effects of severe arrhythmias. A surprisingly diverse group of drugs including cardiac and non-cardiac medications have been withdrawn from or restricted for use in the market due to their blockade of the K_v11.1 channel. Therefore, it is routine practice for pharmaceutical companies to screen the K_v11.1 liability of drug candidates before they are approved to enter the market by regulatory agencies.

In this review, we summarized the generation, degradation, structure and function of the K_v11.1 channel, and its corresponding intentional and unintentional roles in drug development. This provides valuable information and theoretical foundations for investigations on the K_v11.1 channel as well as a comprehensive overview of the channel.

The unique gating kinetics of the K_v11.1 channel contribute to its important

functions in physiological conditions. Almost all K_v11.1 ligands affect the gating kinetics of the channel during their interaction with the channel. We reviewed the closed, open and inactivated states of the channel and also the transitions among these different states. Moreover, the impact of several assay conditions and K_v11.1 ligands on the channel kinetics was discussed. This emphasizes the importance of considering the gating kinetics of the K_v11.1 channel in seeking for K_v11.1 activators and compounds with less K_v11.1-related cardiotoxicity. In addition, the differences between laboratories in evaluating the K_v11.1 ligands should be standardized by taking distinct assay conditions into consideration.

Currently, the estimation of the risk of a drug to cause LQTS is only based on its IC_{50} value at the K_v11.1 channel. When a drug or drug candidate exhibits an IC_{50} value 30-fold higher than its effective therapeutic plasma concentration, this drug is considered to have a safe cardiac profile. Although this rule ensures the exclusion of potentially harmful drugs, it could also lead to the preliminary withdrawal of certain drug candidates that may be unproblematic. Furthermore, drug inhibition of the K_v11.1 channel is a dynamic process in which binding and unbinding events occur over time. Therefore, we also examined in this review the binding and corresponding kinetics of different series of ligands to the K_v11.1 channel. Different techniques used to determine the binding and kinetic parameters of these ligands at the channel were summarized as well. All in all, we strongly plea for incorporation of kinetic assessment into ligand affinity at the channel in order to better predict the proarrhythmic propensities of K_v11.1 blockers. To date, the roles of binding (association) and unbinding (dissociation) of ligands with respect to their K_v11.1 cardiac side effects have not been well elucidated, and thus, more attention should be paid to the studies on these kinetics in the future.

Overall, a thorough analysis of the K_v11.1 channel, in particular the gating kinetics and ligand binding kinetics, enables the improvement of drug targeted design and reduction of unwanted arrhythmic side effects on this channel. The outcome of these investigations would also substantially contribute to the development of homology and mathematical models for better predicting the interactions of K_v11.1 ligands with the channel, which could effectively facilitate the investigations on the K_v11.1 channel in the near future.

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