Atrial Fibrillation in Aging: Mechanisms and Potential Therapeutics

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Atrial Fibrillation in Aging: Mechanisms and Potential Therapeutics

by

Mengmeng Chang

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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List of Abbreviations

Action potential (AP)
Sodium (Na$^+$)
Potassium (K$^+$)
Calcium (Ca$^{2+}$)
Chloride (Cl$^-$)
Resting membrane potential (RMP)
Excitation contraction coupling (E-C coupling)
Sinus atrial node (SA node) Atrioventricular node (AV node)
L-type calcium channel (LTCC)
Delayed rectifier potassium current ($I_k$)
Hyperpolarization-activated cyclic nucleotide-gated channel (HCN)
Inward rectifying potassium current ($I_{K1}$)
Connexin (Cx)
Transient outward K$^+$ current ($I_{to}$)
Slow delayed rectifier K$^+$ current ($I_{Ks}$)
Rapid delayed rectifier K$^+$ current ($I_{Kr}$)
Acetylcholine activated inward rectifying K$^+$ current ($I_{KACh}$)
Adenosine triphosphate (ATP)
ATP sensitive K$^+$ current ($I_{KATP}$)
Transverse tubules (t-tubules)
Calcium induced calcium release (CICR)
Type 2 ryanodine receptors (RYR2)
Sarcoplasmic reticulum (SR)
Adenosine diphosphate (ADP)
Sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA pump)
Beat per minute (bpm)
Catecholaminergic polymorphic ventricular tachycardia (CPVT)
Brugada syndrome (BrS)
Sudden cardiac death (SCD)
Electrocardiogram (ECG)
Spontaneous calcium release (SCR)
Long QT syndrome (LQTs)
Short QT syndrome (SQTs)
Sick sinus syndrome (SSS)
Atrioventricular conduction block (AV block)
Intracardiac electrophysiology study (EPS)
Supraventricular tachyarrhythmia (SVT)
Ventricular tachycardia (VT)
Non-sustained VT (NSVT)
Ventricular fibrillation (VF)
Torsade de pointes (TdP)
Implantable cardioverter-defibrillator (ICD)
Atrioventricular nodal re-entrant tachycardia (AVNRT)
Atrioventricular re-entrant tachycardia (AVRT)
Atrial tachycardia (AT)
Wolff-Parkinson-White (WPW)
Multifocal atrial tachycardia (MAT)
Atrial fibrillation (AF)
Early afterdepolarization (EAD)
Delayed afterdepolarization (DAD)
Ultrarapid delayed rectifier current ($I_{Kur}$)
Chronic atrial fibrillation (cAF)
Acetylcholine (ACh)
Small-conductance Ca^{2+}-activated K^{+} channel (SK)
Two-pore K^{+} channels (K_{2P})
Reactive oxygen species (ROS)
Xanthine oxidase (XO)
Nitric oxide synthase (NOS)
Nicotinamide adenosine dinucleotide (NADPH)
NADPH oxidase (NOX)
Superoxide (O^{2-})
Hydrogen peroxide (H_{2}O_{2})
Hypochlorite (HOCl)
Nitric oxide (NO^{·})
Peroxynitrite (ONOO^{·})
Hydroxyl radicals (·OH)
Effective refractory period (ERP)
Inward rectifying potassium channel (Kir, IRK)
Type 2 muscarinic receptor (M_{2})
Phosphatidylinositol 4,5-bisphosphate (PIP_{2})
Protein kinase C (PKC)
4-β phorbol ester 12-myristate-13-acetate (PMA)
Diacylglycerol (DAG)
Phosphatidylserine (PS)
Caveoline-3 (Cav3)
Protein kinase C epsilon (PKC_{ε})
Wild type (WT)
Knockout (KO)
TertiapinQ (TPQ)
Intracardiac electrogram (ICE)
Sinus rhythm (SR)
Phosphate-buffered saline (PBS)
Dithiothreitol (DTT)
Phenylmethylsulfonyl (PMSF)
Protease phosphatase inhibitor (PPI)
Bicinchoninic acid assay (BCA)
N-acetyl-cysteine (NAC)
Immunoglobulin G (IgG)
Abstract

Atrial fibrillation (AF) is one of the most common cardiac arrhythmias seen in the clinics, and currently available antiarrhythmic pharmacotherapies in AF are not very effective. Although AF has been recognized as an aging-mediated disease, our understanding of the electrophysiological pathways that link aging and AF remain incomplete, which limits breakthroughs in the development of novel antiarrhythmic treatments for this disease. Studies have shown that aging increases the generation of reactive oxygen species (ROS) in the heart, and high levels of ROS have been associated with development of AF in animals and patients. Additionally, in some forms of AF, the acetylcholine activated inwardly rectifying potassium current ($I_{K_{ACh}}$) is constitutively active, possibly in a protein kinase C epsilon (PKCε) dependent manner. However, at present, there are no direct and fundamental mechanistic links between aging, constitutively active $I_{K_{ACh}}$, and AF. In this thesis, I will utilize a PKCε knock out mouse, molecular, electrophysiological and protein engineering approaches to test the hypothesis that in the heart, aging leads to constitutively active $I_{K_{ACh}}$ via PKCε, and thus to the perpetuation of AF. My results demonstrate that the increased susceptibility for AF in the aging heart is in part dependent on a PKCε mediated constitutively active $I_{K_{ACh}}$ and that blocking $I_{K_{ACh}}$ with a novel and highly potent blocker that we designed reduces the susceptibility to AF in the aging heart. It is my sincere hope that the work I present in this thesis could provide insights into the molecular underpinnings of a novel
pathway that contributes to the atrial arrhythmogenicity of aging. I also hope that my work would open new avenues for the design of novel ion channels blocking modalities that can be used as a new generation of effective antiarrhythmics.
Chapter One: General Introduction

I will begin my thesis by describing the fundamental concepts that underlie the generation and propagation of the action potential in the heart, and their relation to normal and abnormal cardiac electrical impulse propagation.

1.1 Ionic Basis of the Action Potential

The major physiological function of the heart is to act as a pump that provides continuous circulation of blood throughout the body. The heart achieves this by the coordinated contraction and relaxation of billions of cardiomyocytes. The excitation of the heart is accomplished by the generation and propagation of electrical impulses known as action potentials (AP). Due to the analysis of the squid axon action potential performed by Hodgkin and Huxley[1], and the development of the patch clamp technique[2, 3], it has become clear that it is the movement of electrical charge carriers like sodium (Na\(^+\)), potassium (K\(^+\)), calcium (Ca\(^{2+}\)), and chloride (Cl\(^-\)) ions across the phospholipid bilayer of the cell membrane that results in the AP generation. Since the lipid bilayer of cell membrane is not permeable to ions, ionic movements across the lipid bilayer are made possible by specialized transmembrane proteins such as ion channels, exchangers, and pumps[4]. Cell membrane exchangers and pumps actively push ions
across the membrane and establish concentration gradients while ion channels allow ions to move across the membrane down those concentration gradients. The resulting charge distribution across the membrane therefore creates a voltage difference between the inside and outside of the cell, which is called membrane potential. The relatively static and negative membrane potential of quiescent cells is called the resting membrane potential (RMP)[5]. Differences in ion channels expression and regulation result in cells with quite distinct membrane potential properties and give rise to the differing action potential morphologies that are present in the excitable cells such as neurons, skeletal myocytes, and the cardiomyocytes in the different chambers of the heart. The opening and closing of ion channels can induce a departure from RMP. In excitable cells, the response to depolarizing stimuli depends on the intensity of the stimulus. Low-intensity depolarizing stimuli produce graded responses. However, when the degree of depolarization exceeds a critical value, called threshold[6, 7], an AP is fired, during which the membrane potential changes rapidly and significantly for a short time, often reversing its polarity. It is important to know that above threshold, the amplitude, and waveform features of the AP are independent of stimulus intensity. It is an all-or none response[8, 9]. The Nobel Prize break-through work by Hodgkin and Huxley (1952b) characterized the generally applicable AP across species and accurately describe the AP waveform, which has 5 phases as shown in figure 1.1. Before the stimulus occurs, the membrane potential stays static at a state, the RMP. A stimulus results in opening of voltage-gated sodium channels when the membrane
potential reaches the threshold for sodium channel activation and causes a large influx of sodium ions, initiating the upstroke of the AP. This phase is called the depolarization, where the inside of the cell becomes increasingly electropositive, until the potential gets closer the electrochemical equilibrium for Na\(^+\) of +61 mV. The phase of positivity above 0 mV is the overshoot phase. After the overshoot, the sodium permeability suddenly decreases due to the inactivation of its channels, the peak of the AP representing inactivation of Na\(^+\) channels. While the voltage-gated potassium channels are opening, there is a K\(^+\) efflux, decreasing the cell electropositivity. This phase is called repolarization phase, which leads to the restoration of the resting membrane potential. In some cells, the membrane potential can hyperpolarize beyond the resting values, resulting in a more negative membrane potential than the RMP. This phase is called afterhyperpolarization, following which the membrane returns to the RMP.
1.2 Generation and Propagation of the Action Potential in the Heart

The coordination of billions of cardiomyocytes in the heart is achieved through a hierarchical generation and propagation of APs resulting in excitation-contraction coupling (E-C coupling) across the myocardium in a specific temporal sequence to maximize the efficiency of the heart as a pump. Under normal circumstances, the contraction of cardiac muscle is initiated by the AP generated in the sinoatrial node (SA node), which is positioned in the right atrium, near the entrance of the superior vena cava. The cluster of myocytes in SA node are modified cardiomyocytes, possessing rudimentary contractile filaments, but contract relatively weakly compared to the cardiac contractile cells[10]. They possess the intrinsic ability to generate APs spontaneously, also known as
pacemaker activity, and thus set the rhythm and rate of the heart[11, 12]. The normal sinus rhythm is constantly modified by the autonomic nervous system. The atrioventricular node or AV node, located in the lower atrial septum close to the coronary sinus ostium is composed of cells that also have the ability to spontaneously fire APs. The discharging rate of AV nodal cells is slower than that of the SA node, therefore, the AV node is regarded as the secondary pacemaker. The ventricular specialized conduction system is comprised of the His bundle, the left and right bundle branches, and the Purkinje fibers could also produce spontaneous APs, but at a rate slower than that of the AV node. If the SA node does not function properly and is unable to drive the heart rate, the AV node will take over the pacemaker activity. If both the SA node and AV node fail to function, the cells from the left and right branches of Bundle of His, and Purkinje fibers can become pacemakers. It is important to realize that the SA node cells have the quickest rate of spontaneously depolarization and are considered the primary pacemakers. The AP generated by the SA node reaches the other potential pacemaker cells (AV node) to initiate APs before these cells have had a chance to generate their own spontaneous action potential, thus they are overridden.
The action potential of pacemaker cells has only three phases as shown in figure 1.2, there is no obvious phase 1 or 2, and they do not have a resting membrane potential; instead, there is a prominent phase 4 depolarization.

Pacemaker cells are never at rest and are characterized by their spontaneous depolarization, which is also known as pacemaker potential. In phase 4, the pacemaker cells spontaneously depolarize, causing the membrane potential to slowly become more positive. When the membrane depolarizes to about -40 mV, reaching threshold potential, an action potential is generated. The depolarization phase, called phase 0 or upstroke is slow because it is mainly caused by the activation of L-type calcium channels (LTCC) that open when the threshold is reached[13]. There is no considerable fast sodium current in these cells due
to inactivation of the current and low level of expression of sodium channels. Phase 3 repolarization is generally triggered by inactivation of LTCC and the activation of the delayed rectifier potassium current (IK)[14]. The sodium/potassium ATPase pump restores the distribution of sodium and potassium ions by pumping sodium out of the cell and pumping potassium into the cell, restoring their concentrations. This enables the cell to reset and repeat the process of spontaneous depolarization leading to activation of the next action potential. The pacemaker potential is thought to be caused mainly by a group of channels, known as hyperpolarization-activated cyclic nucleotide-gated (HCN) channels[15-21], which are activated by hyperpolarization at voltages more negative than -40/-45 mV, and allow an inward non-specific K+ and Na+ current into the cells at its activation range. This current is referred to as the funny current[22-24]. The calcium clock is regarded as another mechanism for phase 4 depolarization. Increased intracellular Ca2+ activates the sodium-calcium exchanger resulting in the increase in membrane potential, as 3 Na+ is being brough into the cell, but only 1 Ca2+ is pumped out of the cell, resulting in a net positive charge entering the cell[25]. According to Hodgkin-Huxley model, the resting membrane potential in excitable cells is caused by a continuous outflow or “leak” of potassium ions through ion channel proteinssuch as the inwardly rectifying potassium current (IK1). However, in the pacemaker cells, such potassium channels are not highly expressed. Once the pacemaker cell AP is initiated in the SA node, the electrical impulse will then depolarize the neighboring atrial cells via gap junctions.
Figure 1.3. The cardiac conduction system. The cartoon shows the anatomy of the cardiac conduction system and the path of the action potential propagation (arrows) including the time delay observed at the AV junction (green zigzag). (Adapted from Monteiro et al., Regen Med, 2017)

Figure 1.3 shows the paths for propagation of the action potential from the SA node to the right atrium, then through Bachmann’s bundle the AP reaches the left atrium, resulting in synchronized atrial contraction. Then the AP propagates through the anterior, middle, and posterior internodal tracts, reaches the AV node, where propagation is slow, resulting in a conduction delay, allowing for the diastolic filling of the ventricles. The AP is then conducted through the left and right Bundles of His to the respective Purkinje fiber networks of the right and left ventricles, exciting the endocardium at the apex of the heart, then transmurally towards the epicardium, causing ventricular contraction[26, 27].

In the cardiac muscle, the AP travels between myocytes through gap
junctions. Gap junctions are specialized intercellular channels that allow the direct exchange of ions, metabolites, and other messenger molecules between adjacent cells [28]. The connexons hemichannels in a cell dock with those in the adjacent cell forming a gap junction[29]. A connexon is comprised of six connexins that form a central pore[30, 31]. In the heart, connexins 40(Cx40), 43(Cx43) and 45(Cx45) are the major connexins that form gap junctions between cardiomyocytes with differential distributions in chamber specific and region specific manners[32]. Connexins have four transmembraneregions. Gap junctions allow for a synchronous depolarization of the myocardium and the subsequently rapid and coordinated contraction of the cardiac muscle as a syncytium[33].

Unlike the SA node AP, atrial and ventricular action potentials have 5 phases as shown in figure 1.4, although their waveforms are different[34]. Figure 1.4 and Table 1.1 show the major ionic currents and their pore forming α subunit genes and proteins. The differential distribution and properties of the specific type of ion channels expressedresult in the differing atrial versus ventricular AP morphologies. Phase 0, the depolarization, consists of a rapid, positive change from the resting membrane potential, lasting a few milliseconds[35]. It is produced primarily by the activation of cardiac Na⁺ channels. For instance, an AP which propagates from the neighboring cell through gap junctions increases the voltage within the cell to the threshold potential (~70 mV) and causes Na⁺ channels to open. This produces a large influx of sodium into the cell driving the quick depolarization phase.
Figure 1.4. Phases of the atrial and ventricular action potential with underlying currents. (Adapted from Jost at al., Advances in Cardiomyocyte Research, 2009)
Table 1.1. The alpha subunits of the major currents that underlie the cardiac action potential. (Adapted from Grant et al, *Circ Arrhythm Electrophysiology*, 2009)

<table>
<thead>
<tr>
<th>Current</th>
<th>Description</th>
<th>AP Phase</th>
<th>Protein</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I</em>&lt;sub&gt;Na&lt;/sub&gt;</td>
<td>Sodium current</td>
<td>Phase 0</td>
<td>Na,1.5</td>
<td>SCN5A</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;Ca,L&lt;/sub&gt;</td>
<td>Calcium current, L-type</td>
<td>Phase 2</td>
<td>Ca,1.2</td>
<td>CACNA1C</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;Ca,T&lt;/sub&gt;</td>
<td>Calcium current, T-type</td>
<td>Phase 2</td>
<td>Ca,3.1</td>
<td>CACNA1G</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;to,f&lt;/sub&gt;</td>
<td>Transient outward current, fast</td>
<td>Phase 1</td>
<td>KV 4.2/4.3</td>
<td>KCND2/3</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;to,s&lt;/sub&gt;</td>
<td>Transient outward current, slow</td>
<td>Phase 1</td>
<td>KV 1.4/1.7/3.4</td>
<td>KCNA4/KCNA7/KCNC4</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;Kr&lt;/sub&gt;</td>
<td>Delayed rectifier, ultrarapid</td>
<td>Phase 1</td>
<td>KV 1.5/3.1</td>
<td>KCNA5/KCN1C</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;Kr&lt;/sub&gt;</td>
<td>Delayed rectifier, fast</td>
<td>Phase 3</td>
<td>HERG</td>
<td>KCNH2</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;ks&lt;/sub&gt;</td>
<td>Delayed rectifier, slow</td>
<td>Phase 3</td>
<td>KVLQT1</td>
<td>KCNJ1</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;K1&lt;/sub&gt;</td>
<td>Inward rectifier</td>
<td>Phase 3,4</td>
<td>Kir2.1/2.2</td>
<td>KCNJ2/12</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;KATP&lt;/sub&gt;</td>
<td>ADP activated K&lt;sup&gt;+&lt;/sup&gt; current</td>
<td>Phase 1,2</td>
<td>Kir6.2 (SURA)</td>
<td>KCNJ11</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;KACa&lt;/sub&gt;</td>
<td>Muscarinic-gated K&lt;sup&gt;+&lt;/sup&gt; current</td>
<td>Phase 4</td>
<td>Kir3.1/3.4</td>
<td>KCNJ3/5</td>
</tr>
<tr>
<td><em>I</em>&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Pacemaker current</td>
<td>Phase 4</td>
<td>HCN2/4</td>
<td>HCN2/4</td>
</tr>
</tbody>
</table>

During the transient repolarization period, Phase 1, as the membrane potential starts to repolarize, there is a rapid inactivation of the Na<sup>+</sup> channels, and the concomitant activation of the transient outward K<sup>+</sup> (*I*<sub>to</sub>) current. This period is also referred as the “notch”. The plateau phase is phase 2, during which the membrane potential remains almost constant. This is produced by the balance of small, but non- inactivated Na<sup>+</sup> and the L-type Ca<sup>2+</sup> current and the outward repolarizing K<sup>+</sup> currents. Afterwards, the reduction of L-type Ca<sup>2+</sup> and Na<sup>+</sup> currents and the increasing K<sup>+</sup> currents contribute to phase 3 repolarization. Here, the potassium channels that are active are the slow delayed rectifier K<sup>+</sup> current (*I*<sub>ks</sub>), the rapid delayed rectifier K<sup>+</sup> current(*I*<sub>Kr</sub>) and the inwardly rectifying K<sup>+</sup> currents (*I*<sub>K1</sub>), and under specific circumstances, the acetylcholine activated inward
rectifying K⁺ current (I_{K_{ACf}}), and the adenosine triphosphate (ATP) sensitive K⁺ current (I_{K_{ATP}}). This causes a net outward current, leading the cell to repolarize. Phase 4, the resting membrane potential, occurs when the cell is at rest, where the membrane voltage is constant, at around -80 mV. During this phase the membrane is most permeable to K⁺, mainly through the background I_{K1} current[36].

1.3 Excitation-Contraction Coupling

The action potential leads to contraction of the cardiac myocytes by a process called excitation-contraction coupling (E-C coupling), which is achieved by converting a chemical signal into mechanical energy[37]. It is suggested that cardiac transverse tubules (t-tubules) play an important role during E-C coupling. T-tubules are highly branched invaginations of the cardiomyocyte sarcolemmal membrane and are rich in LTCC. They allow the action potential to travel into the cell center, regulating the cardiac E-C coupling through calcium induced calcium release (CICR)[38, 39]. The LTCCs located on the t-tubular membrane are activated by the action potential, allowing Ca^{2+} to enter the cell. Ca^{2+} then binds to and activates the type 2 ryanodine receptors (RYR2) located on the membrane of the sarcoplasmic reticulum (SR)[40]. Since SR is a Ca^{2+} store [41], the activation of RYR2 causes Ca^{2+} release into the cell, causing a 3 orders of magnitude increase in sarcoplasmic Ca^{2+} concentration. As the ryanodine receptors open, Ca^{2+} is released from the SR into the local junctional space and diffuses into the bulk cytoplasm [42]. It is important to realize that the LTCCs located in the t-tubules are positioned in close proximity to RYRs, at the junctional membrane of SR. The closely positioned t-tubule and the terminal cisterna of the sarcoplasmic reticulum
form calcium-releasing units, Dyads, which play an important role in coordinating E-C coupling [43]. The released Ca\(^{2+}\) will then initiate the contraction of cardiac muscle through the basic contractile unit, sarcomere, which is composed of actin and myosin. These two main protein filaments are the active structures, and the sliding interaction between them leads to sarcomere shortening, and thus to the overall contraction of the muscle. This is well known as sliding filament theory, initially proposed in 1954 by two groundbreaking studies[44, 45], stating that active force is generated as actin filaments slide past the myosin filaments, resulting in force generation and shortening of an individual sarcomere. This theory has remained impressively intact. The studies described the basic organization of the sarcomere with repeated arrangement as shown in figure 1.5, where the “A band” in the center, contains the thick filament of myosin. The “I band” sits beside “A band”, rich in thinner filaments made of actin. Located at the lateral ends of each “A band”, is the region of overlap of actin and myosin. At the center of the “A band”, there is the “H zone”, which contains only thick filaments and is also shortened during contraction. The sarcomere unit is defined as the distance between two consecutive “Z lines”, where actin is tethered. During contraction, the “A band” remains relatively constant in length while the “I band” shortens its length along with the sarcomere. Muscle contraction is also regulated by two other proteins, troponin and tropomyosin, the activity of which requires Ca\(^{2+}\). In the resting sarcomere, tropomyosin would block the binding of myosin to actin. When Ca\(^{2+}\) gets releases, it binds to and changes the conformation of troponin, which then subsequently shifts the position of tropomyosin and moves it away from the myosin-binding sites on actin, effectively unblocking the binding site.
Figure 1.5. Sarcomeric structure. Schematic of sarcomere (above) and sarcomere ultrastructure observed by electron microscopy (below). The sarcomere is an elastic scaffold that consists of structural proteins lined out from Z-disk to M-band, including actin (black), myosin (green), and titin (grey) that extends through the half-sarcomere. Scale is 100 nm. (Adapted from Crocini et al., Biophysical Reviews, 2021)

Once the myosin-binding sites are exposed, a cross-bridge cycling (Figure 1.6) is initiated. During this process, ATP produced by mitochondria is used as a source of energy. Myosin has the binding site for ATP. Its enzymatic activity hydrolyzes ATP to adenosine diphosphate (ADP), releasing an inorganic phosphate molecule and energy. Myosin binds to actin via the S1 region, the globular end of myosin protein that is closest to actin, which has multiple hinged segments, and have been demonstrated to play an important role in myosin binding and moving or “walking” along actin[46, 47]. The binding of the myosin head to actin is known as cross-bridge. The contraction of myosin S1 segment is called power stroke. During the power stroke, the myosin head reaches forward, binds to actin, bends, and ADP and phosphate are released; afterwards, a new molecule of ATP attaches to the myosin head, causing the cross-bridge to detach.
ATP is then hydrolyzed to ADP and phosphate, which returns the myosin to the cocked position. Myosin then binds to another part of the actin and produces another power stroke, shortening the muscle further. This process continues, with the myosin head moving in a motion similar to that of an oar rowing boat, until the cytoplasmic Ca\(^{2+}\) level decreased[48]. Contraction ends when Ca\(^{2+}\) is removed from the cytoplasm to either the extracellular space, or back into the SR. When this happens, the troponin changes back to its original shape, blocking the binding sites on actin and preventing cross-bridge formation.
Ca$^{2+}$ removal from the cytoplasm is brought about by sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA pump), which pumps Ca$^{2+}$ back into the SR, the sarcolemmal sodium-calcium exchanger, which pumps one Ca$^{2+}$ out of the cell, in exchange for 3 sodium ions being pumped into the cell, the sarcolemma Ca$^{2+}$-ATPase, which uses ATP to pump Ca$^{2+}$ directly out of the cell and the mitochondrial Ca$^{2+}$ uniport system, which pumps Ca$^{2+}$ into the mitochondria. In addition, the increased intracellular Ca$^{2+}$ produced by CICR binds to the intracellular side of the LTCC, inactivating the channel and preventing further influx of Ca$^{2+}$ through LTCCs into the cell[49].
1.4 Arrhythmias

From the above, it is clear that the cardiac cycle is based on the normal periodicity, regularity, and coupling of electromechanical activity of the heart[50]. Therefore, interruptions at any levels of integration could cause cardiac dysfunction, which may present as abnormal heart rhythms or cardiac arrhythmias. I would also like to add that both intrinsic and extrinsic factors, such as age, genetics, lifestyle, co-morbidities, and environment could increase the susceptibility for developing cardiac arrhythmias[51].

Cardiac arrhythmias can be classified into two types: bradycardia, where the heart beats at less than 60 beats per minutes (bpm), and tachycardia, where the heart rate at rest is over 100 bpm. Cardiac arrhythmias can also be classified by the initiation location. If the arrhythmia begins above the ventricles, it is called supraventricular arrhythmia. If it originates in the ventricles, it is known as ventricular arrhythmia. Below, I will briefly introduce inherited arrhythmia syndrome before I discuss arrhythmias in terms of their subtypes, underlying ionic mechanisms, and currently available therapeutic approaches.

1.4.1 Inherited cardiac arrhythmias

Inherited cardiac arrhythmias result from mutations in several genes that encode ion channels or proteins involved in electrical impulse generation and propagation. Such mutations can cause long QT syndrome, short QT syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT) or Brugada syndrome (BrS). They are often the underlying cause of sudden cardiac death (SCD) in young, and otherwise healthy individuals[52].
1.4.1.1. Brugada syndrome

As one of the most common genetic arrhythmias[53], Brugada syndrome (BrS) has the high possibility of increasing the risk of VT and SCD. It affects individuals in their 40s[54]. BrS is usually characterized by the presence of elevated, coved ST-segments in leads V1-V3 on the of electrocardiogram (ECG), a history of SCD in the absence of structural heart diseases[55], and pseudo-right bundle branch block[56, 57].

The arrhythmogenesis of BrS has been proposed to be explained by two major hypotheses[58]. The repolarization hypotheses which suggest that the coved ST segment elevation represent an accentuated “J-wave” due to genetically mediated ionic current imbalances that create pathological ventricular transmural voltage gradient[59, 60]. The depolarization hypothesis suggested that increased fibrosis and reduced expression of the gap junction protein connexin-43 and decreased density of Na\(^+\) channels cause BrS, which results in a slowed and dispersed conduction[61, 62]. But it is very important to realized that neither hypothesis has been established as a single underlying mechanism, and these two hypotheses are not regarded as mutually exclusive.

1.4.1.2. Catecholaminergic polymorphic ventricular tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT)[63], is characterized by ventricular tachycardia induced episodic syncope during exercise or emotional stress or in response to β-adrenergic receptor stimulation in individuals without structural cardiac abnormalities[64]. CPVT is known to increase the risk of SCD in young individuals. CPVT is caused by mutations in
genes that encode proteins regulating calcium handling during E-C coupling[63], which mainly include gain-of-function mutations in RYR2 (encoding ryanodine receptor calcium release channel)[65], mutations in CASQ2 (encoding cardiac calsequestrin)[66], TRDN (encoding triadin)[67], and CALM1, CALM2, CALM3 (encoding calmodulin proteins)[68-70]. Mutations in other genes that are integral to SR calcium handling could also potentially cause CPVT[71-74]. It is well-accepted that CPVT mutations, under adrenergic stimulation, result in SR Ca$^{2+}$ release via RYR2, randomly and spontaneously during late diastole in the absence of the normal trigger provided through L-type Ca$^{2+}$ channel activation during a normal sinus rhythm action potential[75]. This causes pathological SR calcium release termed spontaneous calcium release (SCR). SCR is also favored by other cardiac pathological conditions producing Ca$^{2+}$ overload such as ischemia, increased sympathetic nerve activity, hypertrophy, or heart failure[76-78]. The SCR during diastole rises cytosolic calcium, which activates the sodium-calcium exchanger, generating the arrhythmogenic transient inward current. This subsequently induces cell membrane depolarization termed delayed afterdepolarization[79], which is the underlying cellular mechanism of CPVT.

1.4.1.3. Long QT syndrome

Long QT syndrome (LQTs), a life-threatening congenital cardiac channelopathy, is characterized by the prolongation of the QT interval (>480 ms) and T-wave abnormalities on the ECG. Its occurrence is commonly associated with syncope, or cardiac arrest, which is mainly induced by emotional or physical stress[80, 81]. LQTs can result from either ion channel mutations or from
acquired causes. Multiple factors have been reported to increase the risk of developing LQTs, such as age, electrolytes abnormality, and drugs which can block $I_{Kr}$ [82-84]. The QT interval represents the global depolarization and repolarization of the ventricles. The long QT interval could be caused by increased depolarizing inward sodium or calcium currents or decreased repolarizing outward potassium current, which represent the pathophysiological substrate for LQTs[80]. Currently, human genetic studies have shown that 15 genes are associated with LQTs. LQTs is classified into 17 subtypes [85]. Mutations in KCNQ1, KCNH2, and SCN5A respectively cause LQT1, LQT2, and LQT3. They are the most common LQTs genes, accounting for approximately 90% of all genotype-positive cases[86, 87].

KCNQ1 gene encodes the α-subunit of the voltage-dependent potassium channel (Kv7.1) within the cell membrane of cardiomyocytes, which mediates the slowly activating delayed rectifier potassium current ($I_{Ks}$). Loss-of-function of KCNQ1 results in LQT1, the most common subtype of LQTs[88, 89]. KCNH2 gene codes the α-subunit of the voltage-gated rapidly activating delayed rectifier potassium channel (hERG)[90], which mediates the generation of the repolarizing $I_{Kr}$ current. LQT2, the second most common subtype of LQTs, arises from Loss-of-function mutations of the hERG[91]. The mutation could reduce the $I_{Kr}$ amplitude, resulting in the prolongation of the cardiac repolarization[89]. SCN5A gene encodes the α-subunit of the cardiac sodium channel NaV1.5, which mediates the rapid depolarizing inward sodium current during the upstroke of the cardiac action potential. Gain-of-function mutations in SCN5A cause a continuous influx of
sodium ions (late sodium current) during the plateau phase of the action potential, which delays the repolarization of cardiomyocytes, resulting in the QT interval prolongation, and underlying LQT3, the third most common subtype of LQTs[92, 93].

1.4.1.4. Short QT syndrome

Short QT syndrome (SQTs), an inheritable primary electrical cardiac abnormality, is typically characterized by a short QT interval (<300 ms) on the ECG and a propensity to atrial fibrillation and SCD even in young patients and newborns. It was initially described in 2000[94], and since then, mutations in 3 different genes that encode for cardiac ion channels have been identified to be associated with this disease.

Gain-of-function mutations in KCNH2 gene, which result in increased \( I_{Kr} \) current was shown to cause heterogenous abbreviation of action potential duration (APD) and refractoriness and underlies SQT1[95]. Gain of function mutations in KCNQ1 gene result in increased \( I_{Ks} \) which also abbreviates the QT interval and lead to SQT2[96]. Gain of function mutations in KCNJ1 which codes for Kir2.1, the main protein mediating the strong inward rectifying potassium current (\( I_{K1} \)), increase the current and abbreviate the action potential duration, resulting in SQT3[97].

Inherited cardiac arrhythmias can cause SCD even in young and healthy individuals. Presently, management of these inherited cardiac arrhythmias mainly consists of pharmacological therapy with \( \beta \)-adrenergic receptor blockers and antiarrhythmics. If pharmacotherapy is ineffective, surgical interventions,
including left cardiac sympathetic denervation and implantation of a cardioverter-defibrillator are warranted[98].

1.4.2. Bradycardia

Bradycardia is defined as a resting heart rate of less than 50-60 bpm in adults[99]. But it is important to known that the heart rate of healthy and elderly people, healthy well-trained athletes, and during sleeping could be lower than 50-60 bpm which does not require treatment. Because both normal aging and disease progression could result in slower heart rates due to changes in SA nodal action potential generation and propagation, bradycardia is more commonly identified in the elderly. For instance, symptomatic bradycardia in the elderly may cause dizziness, weakness, fatigue, or syncope, which could be attributed to hypoperfusion in the various tissues, including the brain. Abnormalities of the sinus node, atrial tissue, atrioventricular nodal tissue, and the specialized conduction system can all contribute to bradycardia, and to a discordant timing of atrial and ventricular depolarization. In addition, multiple pathophysiological processes like myocardial ischemia or infarction, infiltrative diseases, fibrosis, surgical trauma, endocrine abnormalities, individually or in combination, can compromise impulse initiation and propagation, increasing the likelihood of bradycardia[100-103]. Bradycardia could be divided into three main types: sinus bradycardia, atrioventricular conduction block, and intraventricular conduction abnormalities[104].
1.4.2.1. Sinus bradycardia

Sinus bradycardia occurs due to the decreasing frequency of AP generation in the SA node. This could be due to several reasons including the degeneration of SA node caused by aging, conditions where the vagal tone is predominant, adverse effects from antiarrhythmic drugs like class II beta blockers, or some class IV antiarrhythmic calcium channel blockers like verapamil. Table 1.2 lists the different classes of antiarrhythmics based on the Vaughan Williams classification and gives few examples of drugs in each class. Sinus pauses are due to failure of AP generation in the SA node or block of AP in the atrium. It can usually last for several seconds and may thus cause syncope. Sick sinus syndrome (SSS) or sinus node dysfunction is caused by various factors involving complex electrophysiological and structural remodeling. It is most often related to age-dependent, progressive, degenerative fibrosis of the SA nodal tissue and surrounding atrial myocardium[100, 105, 106], damaging the pacemaker cells in the SA node resulting in aperiodic firing[107]. Additionally, mutations of ion channels and ion channels interacting proteins that regulate heart rate are also suggested to play roles in SSS development[108-114]. The clinical manifestations of SSS can be as benign as mild fatigue, or as serious as syncope, which depends on the severity of the bradycardia or the pause duration. Bradycardia-tachycardia syndrome is also variant of SSS where both, slow and fast heart rates alternate and is commonly referred to as “tachy-brady syndrome".
Table 1.2. Vaughan-Williams classification of antiarrhythmic drugs.

<table>
<thead>
<tr>
<th>Class</th>
<th>Moderate blockade of sodium channels</th>
<th>Moderate reduction in phase 0 slope; increase APD; increase ERP.</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Moderate blockade of sodium channels</td>
<td>Moderate reduction in phase 0 slope; reduce APD; decrease ERP in depolarized/ischemic tissue.</td>
<td>Quinidine Procainamide</td>
</tr>
<tr>
<td>IB</td>
<td>Weak blockade of sodium channels</td>
<td>Pronounced reduction in phase 0 slope; no effect on APD or ERP.</td>
<td>Lidocaine Mexiletine</td>
</tr>
<tr>
<td>IC</td>
<td>Strong blockade of sodium channels</td>
<td>Reduced heart rate and conduction/excitability in nodal tissue.</td>
<td>Flecainide Propafenone</td>
</tr>
<tr>
<td>II</td>
<td>Beta adrenergic blockade</td>
<td>Delay repolarization (phase 3); increase action potential duration and effective refractory period.</td>
<td>Carvedilol Propranolol Metoprolol</td>
</tr>
<tr>
<td>III</td>
<td>Potassium channel blockade</td>
<td>Blocks L-type calcium-channels; reduces heart rate and conduction/excitability.</td>
<td>Amiodarone Sotalol Dofetilide</td>
</tr>
<tr>
<td>IV</td>
<td>Calcium channel blockade</td>
<td>Verapamil Diltiazem</td>
<td></td>
</tr>
</tbody>
</table>

Because patients with bradycardia, especially SSS, are typically stable and minimally symptomatic, no acute therapy is usually required, and evaluation of SSS and assessment for potentially reversible causes can be performed in an outpatient setting. Bradycardia caused by acute myocardial infarct, atrial arrhythmias, electrolyte abnormalities, hypothyroidism, medications or drug toxicity, infections, and metabolic abnormalities could be reversed by treating the underlying conditions[115]. If the bradycardia causes hemodynamically significant instability, such as prolonged and symptomatic pauses and when all the potential treatable or reversible etiologies have been excluded, permanent cardiac pacing may be necessary[104].
1.4.2.2. Atrioventricular conduction block

Atrioventricular conduction block (AV block) is an interruption or delay of impulse conduction from the atria to the ventricles. The electrical conduction delay or block could occur in the AV node, within the His-bundle, or below the His-bundle, and can be determined by invasive intracardiac electrophysiology study (EPS). Both congenital and acquired disease could affect the atrioventricular conduction system resulting in AV block. The most common causes seen in the clinical practice are degenerative diseases, including idiopathic fibrosis and sclerosis of the conduction system, and ischemic heart disease. Moreover, inflammatory diseases, infiltrative disorders, metabolic disorders, increased vagal tone, valvopathy, cardioactive drugs (beta-blockers, calcium channel blocker, digoxin), congenital heart disorders could also lead to AV block[116]. But the AV block could also happen in the young well-trained athletes during sleep due to increased vagal tone. Symptoms related to atrioventricular block vary and depend largely on the degree of AV block, the ventricular rate, and the frequency of its occurrence[117].

AV blocks are divided into three types: first, second, and third-degree blocks. 

First-degree AV block means that the electrical conduction from the atria to the ventricles through the AV node is delayed, but no true block is present. Patients with isolated first-degree AV block are usually asymptomatic except sometimes during exercise. Thus, the prognosis of first-degree AV block is benign. Profound first-degree atrioventricular block can lead to symptoms of fatigue or exertional intolerance. Second-degree AV block is characterized by delay, or interruption of
atrial impulse conduction to the ventricles through the AV node[118]. Second-degree AV block is further sub-classified into Mobitz I second-degree AV block and Mobitz II second-degree AV block. Mobitz I second-degree AV block is characterized by a progressive prolongation of the atria-ventricle conduction interval, which finally results in AV transmission failure and the subsequent ventricular contraction failure. Enhanced activation of the parasympathetic nervous system is regarded as the common cause. Patients are often asymptomatic, but if occurring frequently or during exercise, it can cause symptoms of exertional intolerance or dizziness. Mobitz I usually has a good prognosis. Mobitz II second-degree AV block is caused by an unexpected non-conducted atrial impulse, which carries a high risk of progressively developing into complete heart block. *In the third-degree AV block* (complete AV block), the impulses generated in SA node do not propagate to ventricles at all. The most common cause of complete AV block is coronary ischemia. Syncope or presyncope are the more typical symptoms seen in patients.

The principles for AV block treatments are very similar with the treatments or managements of bradycardia. No treatment is required for the asymptomatic patients. As for the symptomatic patients, with timely identification and removal of potential causative factors, permanent pacing might be considered if necessary.

**1.4.3. Tachyarrhythmia**

Tachyarrhythmia (or Tachycardia) is used to describe a rapid heart rate over 100 bpm[119]. Patients with paroxysmal tachycardia are often asymptomatic
on presentation. When symptoms are present, they may include palpitations, fatigue, lightheadedness, chest discomfort, dyspnea, presyncope, and syncope. In general, tachycardia is classified as supraventricular tachyarrhythmia (SVT), or ventricular tachyarrhythmia (VT), based on the site of origin. It is reported that syncope is observed in about 15% of patients with SVT, while patients with ventricular arrhythmias more often present with presyncope, syncope, or even cardiac arrest. A history of syncope should warrant immediate referral to an electrophysiologist[120].

1.4.3.1. Ventricular tachycardia

Ventricular tachycardia (VT) is due to improper electrical activity in the ventricular conduction system, or the working myocardium[121]. Many arrhythmogenic factors have been characterized, which include changes in autonomic nervous system activity, metabolic disturbances, myocardial ischemia due to coronary artery diseases, channelopathies, structural heart disease, proarrhythmic drug toxicity. Such factors can lead to the abnormal electrical activity that underly VT[122-126]. As discussed before, arrhythmias usually result from abnormalities in the initiation of electrical impulses or in the conduction of these impulses or a combination of both. Abnormal impulse initiation could be caused by either increased automaticity or triggered activity. Triggered activity is usually caused by afterdepolarizations that occur either during repolarization (early afterdepolarization) or after repolarization is completed or nearly complete (delayed afterdepolarization). Abnormal impulse conduction could lead to reentrant activity occurrence, which requires both slowed conduction and unidirectional
conduction block[127, 128]. Reentry is regarded as an important mechanism of VT[129]. According to the episode duration, VT could be classified into non-sustained VT (NSVT), sustained VT, ventricular fibrillation (VF) and torsade de pointes. NSVT is defined as a rapid heart rate of at least 120 bpm with at least 3 or more consecutive ventricular beats in fewer than 30 seconds[130]. NSVT is short-lasting, it may appear or disappear suddenly, therefore, the patients are often asymptomatic. The heart rate and duration of the NSVT are two main determinants for the symptom severity. Atria-ventricles synchrony could be compromised by heart rate faster than 150bpm, which dramatically reduces ventricular filling time, causing compromised cardiac output. Sustained VT is a rapid heart rate greater than 120 bpm, up to 250 bpm, lasting at least for 30 seconds or requiring termination due to hemodynamic instability[118]. Its characteristics are very similar to NSVT. Without intervention, VT can degenerate into life-threatening VF [131, 132]. VF is the most common, malignant cardiac rhythm disturbance characterized by rapid (350-450 bpm) and irregular electrical activity of the ventricles[130] which subsequently result in sudden cardiac death[133]. VF has been reported to be associated with various diseases, like ischemic or nonischemic heart diseases (e.g., coronary artery disease with myocardial infarction, hypertrophic or dilated cardiomyopathy), nonstructural heart disease (e.g., inherited channelopathies) and noncardiac diseases (e.g., pulmonary embolism)[134-136]. Torsade de Pointes (TdP), translated as “twisting of peaks”, is an atypical form of VT with a variable and very rapid rate at 250-350 bpm[137]. On the ECG, it is characterized by the absence of discernible QRS complexes and T waves. The occurrence of this
arrhythmia could be increased by a drug induced, or channelopathy induced prolongation of the QT interval [138].

Sustained VT could be subdivided into several subtypes based on the ECG morphology, including monomorphic and polymorphic VT[121]. Thus, it is important to identify the VT subtype before the correct therapy strategy can be identified. In general, administration of class III antiarrhythmic agents such as amiodarone are considered as the first line of defense in the hemodynamically stable patients. As for the hemodynamically unstable patients, with a palpable pulse, cardioversion is required, which is followed by the administration of antiarrhythmics such as class Ia procainamide, or class III drugs such as amiodarone, or sotalol. In pulseless VT, immediate defibrillation followed by the administration of vasopressin or epinephrine is usually promptly performed. Implantable cardioverter-defibrillator (ICD) placement may be strongly considered to prevent sudden cardiac death[139].

1.4.3.2. Supraventricular tachycardias

Supraventricular tachycardias (SVT) involves tissue from the His bundle or above[140, 141]. Traditionally, SVT has been used to describe all tachycardias apart from ventricular tachyarrhythmias and atrial fibrillation. SVT can impact the quality of life, which varies according to the frequency and the duration of SVT episodes, and whether symptoms occur not only with exercise but also at rest[142, 143]. SVT symptom onset often begins in adulthood, which includes palpitation, chest pain, syncope, and even SCD[144]. However, it is reported that light-headedness is common, and true syncope is infrequent with SVT. In addition
to classification by the QRS duration as narrow (<120ms) or wide(>120ms) QRS
tachycardia, SVT could also be traditionally classified into AV junctional
tachycardias, atrial tachycardia (AT), atrioventricular nodal re-entrant tachycardia
(AVNRT), atrioventricular re-entrant tachycardia (AVRT)[145, 146]. Below, I will
discuss several subtypes of SVTs which are relatively common in clinical practice.

1.4.3.2.1. Atrioventricular nodal re-entrant tachycardia

AVNRT is the most common regular SVT [147]. It is usually seen in young
adults without structural heart disease or ischemic heart disease. In AVNRT, the
ventricular rate ranges from 110 bpm to > 250 bpm, although it often ranges from
180 bpm to 200 bpm[148]. Patients with AVNRT describe symptoms of “shirt
flapping” or “neck pounding”[148, 149], that may be related to pulsatile reverse
flow when the right atrium contracts against a closed tricuspid valve. The anatomic
substrate of AVNRT is the dual AV node pathways. It is demonstrated that AVNRT
is caused by a reentrant circuit in the region of the AV junction[150, 151], the part
of the atrioventricular-specialized conducting system comprising the transitional
cell zone, the AV node and its extensions, and the penetrating part of the bundle
of His[152]. Although there are several models of the AVNRT circuit that have
been proposed[153, 154], the most well-accepted model is that of two
anatomically distinct AV nodal pathways, one of which conducts rapidly and the
other slowly. In sinus rhythm, the electrical impulse is conducted through the fast
pathway in the AV node from the atrium to the ventricles. However, a typical
AVNRT occurs when there is a unidirectional block in the fast pathway, and the
impulse can only propagate through the slow pathway in the AVN, in order to get
to the ventricles. The impulse conducted through the slow pathway could then travel retrogradely from the ventricles to the atria through the fast pathway. This creates a reentrant circuit within the AV node[146], allowing for the simultaneous activation of the atrium and ventricles. Propagation through the slow pathway is the down antegrade limb of the circuit, and the fast pathway is the up retrograde pathway.

Adenosine intravenous administration may terminate the rhythm. Adenosine has a half-life of only 9 seconds and terminates the circuit by causing transient block in AV node conduction due to depression of excitability by activating the $I_{K_ACh}$ potassium current. $I_{K_ACh}$ will be discussed in detail in subsequent sections. Long-term therapy with antiarrhythmic class II beta-blockers or class IV calcium channel blockers can prevent recurrence by inhibiting the AV nodal excitability. If pharmacological approaches are not successful and AVNRT is recurrent, radiofrequency ablation is the treatment of choice and has a low risk of complication[140].

1.4.3.2.2. Atrioventricular re-entrant tachycardia

AVRT has been reported as the second most common SVT and is also called extra nodal accessory pathway[155]. Bypassing the AV node, there is an accessory pathway connecting the myocardium of the atrium and the ventricle, which may be capable of conducting in anterograde or retrograde directions or both[120]. Two pathways, one formed by the AV node/His-Purkinje fiber and one formed by the accessory pathway compose the reentrant circuit[140]. According to the conducting direction of the reentrant impulses in the circuit, AVRT is divided
as orthodromic and antidromic AVRT. In the case of orthodromic AVRT, the most common mechanism of AVRT[156], the reentrant impulses travel through the AV node and the His-Purkinje system to result in ventricular depolarization, and then they travel retrogradely through the accessory pathway to the atria forming a reentrant circuit. When there is antidromic AVRT, the reentrant impulses travel antegrade through the accessory pathway and reentry into the atria is via the His-Purkinje system and the AV node. Vagal maneuvers and adenosine are usually recommended for acute treatment of AVRT, both of which result in $I_{K_{ACh}}$ activation. Cardioversion may be performed, and catheter ablation of the accessory pathway is recommended for the ongoing management of AVRT[140, 145].

In the case of Wolff-Parkinson-White (WPW) syndrome, a congenital cardiac preexcitation syndrome, an accessory pathway that connects the atrium and ventricle and bypasses the AV node also exists in the heart, allowing cardiac electrical activity to bypass the conduction delay of the atrioventricular node, and arrive early at the ventricle, leading to premature ventricular depolarization. This preexcitation also bypasses the fast conducting His-Purkinje system and results in early but slowly propagated ventricular depolarization, which gives rise to the ECG pattern of a short PR interval and prolonged QRS with a “slurred” upstroke known as delta wave. This is caused by the fusion of ventricular preexcitation through the accessory pathway and normal electrical conduction[157].

Patients with known WPW pattern and prior current symptomatic episodes are at a higher risk of recurrent arrhythmias, and they should be treated. The treatment of choice is catheter ablation of the accessory pathway[158].
1.4.3.2.3. Atrial tachycardia

Atrial tachycardia (AT) is another abnormally fast heartbeat and is recognized as the least common supraventricular tachycardia. It occurs when the electrical signal that controls the heartbeat starts from an unusual location in the atria and activates the tissue at fast rates. There are three major types of atrial tachycardia: focal atrial tachycardia, multifocal atrial tachycardia and re-entrant atrial tachycardia[159], each of which has unique arrhythmic substrates and characteristics.

1.4.3.2.3.1 Focal atrial tachycardia

In the case of focal AT, the atrium is activated rhythmically at a discrete origin (focus), from which the impulse spreads out centrifugally. The ventricular rate could vary depending on AV nodal conduction[145]. Patients could symptoms such as palpitations, shortness of breath, chest pain, and rarely syncope or presyncope. Focal AT could be caused by increased automaticity, triggered activity, or microreentrant activity. Acute therapy may be initiated with beta-blockers or calcium channel blockers, which may terminate focal ATs or slow the ventricular rate[160-163]. Additionally, adenosine (i.v.), class Ia, Ic, and III antiarrhythmic drugs may also be effective under some specific cases[164-167]. Cardioversion is usually effective in acutely terminating focal AT. Catheter ablation is the treatment of choice for recurrent focal AT, especially for incessant AT, which is reported to have a 75%-100% success rate[162, 168]. Currently, there are limited studies about chronic therapy, but there are suggestions that class II beta-blockers and class IV calcium channel blockers may be effective and with low risk.
of side effects, class Ic drugs may also be effective if first-line therapy has
failed. Ivabradine, a blocker of the funny current If, may also be effective in focal
AT, and ideally should be given with a beta-blocker[169]

1.4.3.2.3.2 Multifocal atrial tachycardia

Multifocal atrial tachycardia (MAT), where the atrial rate is greater than 100
bpm, is defined as a rapid, irregular cardiac arrhythmia caused by multiple sites of
competing atrial activity. It is characterized by the atrial activity with at least three
distinct morphologies of P waves in the same lead on the surface ECG[170]. Each
unique P wave corresponds to a different site of atrial origin. There are also
irregular PP intervals and an isoelectric baseline between P waves. MAT is
typically seen in elderly patients with a variety of underlying conditions, especially
with significant lung disease, the most common of which is chronic obstructive
pulmonary disease. It is also associated with conditions such as coronary artery
disease, congestive heart failure, pulmonary artery hypertension, and
diabetes[170-173]. Re-entry, abnormal automaticity, and triggered activity are
proposed to be the possible underlying mechanisms of MAT, but no theory has
been demonstrated conclusively yet[174-176].

Managing the underlying condition is the first-line treatment. Intravenous
administration of magnesium may be helpful, even in patients with normal
magnesium levels[177]. Though antiarrhythmic medications are not helpful in
suppressing MAT[178], some drugs do show efficacy in patients. Antiarrhythmic
class IV drugs such as verapamil or class II beta-blockers, by slowing conduction
at the AV node to control heart rate, have some efficacy in patients with multifocal
AT who do not have ventricular dysfunction, sinus node dysfunction, or AV block[179-182].

1.4.3.2.4. Macro-re-entrant atrial tachycardia

Macro-re-entrant atrial tachycardia (atrial flutter) is characterized by an organized rhythm at a rate between 250 and 350 bpm and occurs via a reentry mechanism[183]. Macro-reentrant atrial arrhythmias development is conditioned by the presence of fixed (anatomical or acquired) or functional barriers of conduction with areas of slow conduction at critical atrial sites. It may occur with or without underlying heart disease[184]. Patients typically present with a 2:1 AV conduction block producing a ventricular rate of about 150 bpm. Patients may also present with a variable AV block producing an irregular rhythm.

Acute management focuses on ventricular rate control using AV node blocking agents such as class II beta-blockers or class IV calcium channel blockers. Cardioversion is also very effective for patients who are hemodynamically compromised, and ablation may also be beneficial[185]. In patients with atrial flutter for more than 48 h, anticoagulant therapy is normally started, and an echocardiogram should be obtained prior to cardioversion, to rule out the presence of an intracardiac blood clot[185].

1.4.3.2.5. Atrial fibrillation

Atrial fibrillation (AF) was first described by William Harvey in 1628 as "auricular fibrillation". It was recognized later as an irregular pulse reflective of abnormal conduction, with the first case report published in the 1900s[186]. It is the development of ECG by Willem Einthoven in 1902 that allowed the recording
of the electrical events that represent AF[187]. In 1909, Thomas Lewis coined the classic “absence of P waves” and “irregularity of the F waves” that define AF[188].

AF is the most frequent arrhythmia in clinical practice. It is estimated that there are more than 33 million individuals with AF in the world, emphasizing that AF has become a global burden[189]. AF incidence and prevalence rate have been progressively increasing. It is thought that by 2030, there will be more than 50 million AF patients in the world based on a dynamic age-period cohort simulation progression model. It is thus proposed that AF is endemic and is a major public health care problem[190-193].

On the ECG, AF is characterized by rapid and irregular activation in the atria without consistent and discrete P waves, but with rapid oscillation or fibrillatory waves that vary in amplitude, shape, and timing[194, 195]. This causes the uncoordinated atrial activation with consequent deterioration of atrial mechanical function. As demonstrated in many studies, AF is frequently associated with cardiac or noncardiac comorbidities. Hypertension is the most prevalent comorbidity, followed by heart failure, diabetes, and obesity[196-198]. AF is also suggested to increase the risk of other diseases, including stroke, heart failure, dementia, and even sudden cardiac death[199-203]. Consequently, AF increases the mortality and morbidity of patients and worsens the long-term prognosis[204]. In addition to causing a wide variety of symptoms, including fatigue, reduced exercise tolerance, and significantly impaired quality of life[205], AF also augments the health care cost[206]. Despite more than a century of research and speculation, the comprehensive mechanisms underlying AF are not
well established, and presently available therapeutic approaches remain suboptimal. Anticoagulation is important in order to prevent the formation of clots. Significant adverse effects, like proarrhythmia, and AF recurrence has been linked with currently available therapies that include antiarrhythmic drugs for rhythm control (class Ia, Ic, and III), rate control (class II and IV antiarrhythmics), or cardioversion, and ablation [207-211]. Therefore, it is of great significance to study the molecular and cellular mechanisms of AF, which can ultimately help in improving strategies for AF prevention, diagnosis, and treatment.

1.4.3.2.5.1. Classification

Although various classification systems have been proposed for AF, here, I will describe AF in terms of the duration of episodes [212, 213], as shown in table 1.3.
Proper AF classification or accurate AF subtype recognition is important within the context of decision making for treatment strategies. Paroxysmal AF is defined as recurrent AF episodes that terminate spontaneously or with intervention within 7 days of onset. Persistent AF is defined as continuous AF that is sustained beyond 7 days. Early persistent AF is a new term defined as continuous AF of more than 7 days but less than 3 months in duration. Paroxysmal AF and early persistent AF patients are reasonable candidates for AF ablation procedure, as better results are obtained with AF of shorter durations. Long-standing persistent AF is defined as continuous AF of greater than 12-month duration; the term of permanent AF is used when the patient and clinician make a joint decision to stop further attempts to restore and/or maintain sinus rhythm. Silent AF is defined as asymptomatic AF diagnosed by an opportune ECG. Paroxysmal, persistent, and long-standing persistent AF can be silent. Lone AF has been variously defined but generally applied to young individuals (under 60 y of age).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Paroxysmal AF</td>
<td>Spontaneous termination within 7 days and more often within 48 hours. Paroxysmal AF may degenerate into a sustained form of AF.</td>
</tr>
<tr>
<td>Persistent AF</td>
<td>Not self-terminating; lasting longer than 7 days, or prior cardioversion. Persistent AF may degenerate into permanent AF.</td>
</tr>
<tr>
<td>Early persistent AF</td>
<td>Continuous AF of more than 7 days but less than 3 months in duration.</td>
</tr>
<tr>
<td>Permanent AF</td>
<td>Long-standing AF (longer than a year) that is not terminated successfully by cardioversion, when cardioversion is not pursued, or has relapsed following termination.</td>
</tr>
<tr>
<td>Silent AF</td>
<td>Asymptomatic AF diagnosed by an opportune ECG or rhythm strip; Paroxysmal, persistent, and long-standing persistent AF can be silent.</td>
</tr>
<tr>
<td>Lone AF</td>
<td>Without clinical or echocardiographic evidence of cardiopulmonary disease; generally applied to young individuals (under 60 y of age).</td>
</tr>
</tbody>
</table>
60 y of age) without clinical orehocardiographic evidence of cardiopulmonary disease[214].

1.4.3.2.5.2. Pathophysiology
Multiple modifiable and nonmodifiable risk factors have been suggested to escalate the likelihood of AF development [195, 212, 215], which includes aging, sex, hypertension, symptomatic heart failure, valvular heart diseases, cardiomyopathies, atrial septal heart defects, coronary artery disease, overt thyroid dysfunction, obesity, diabetes mellitus, chronic obstructive pulmonary disease, obstructive sleep apnea, alcohol consumption, and chronic renal disease. Integration of these risk factors makes it possible to predict short-to-midterm AF risk development[216, 217]. Among these risk factors, age is stipulated to be the most prominent and independent risk factor of AF. According to the Framingham heart studies[218, 219], it is rare to develop AF prior to age 50, but approximately 10% of individuals are diagnosed with AF by age 80. The lifetime risk for AF development is 1 in 4 after age 40. Hence, AF is recognized as an aging-mediate disease. However, the precise pathophysiological basis of the aging-mediated AF is not well-understood.

1.4.3.2.5.3. Natural history
A wealth of studies has evaluated the clinical progression of AF, and suggest that AF is a progressive condition, which usually begins in a paroxysmal form, progressing through persistent to permanent AF[220-222]. It is widely believed that AF progresses in frequency and duration to become permanent over the long term[223, 224]. In the young patients presenting predominantly with
paroxysmal AF, without underlying structural cardiovascular diseases, the progression rate from paroxysmal to more permanent stages is lowest, amounting to 1%-3% per year[222, 225]. Generally, older patients and those with underlying heart disease have faster progression rates where 35% to 40% of patients with persistent AF can progress to permanent AF in a year[226]. Underlying heart disease and AF risk factors accumulation could increase the risk of AF and accelerate the progression of the arrhythmia[222, 227-229].

1.4.3.2.5.4. Genetic causes

Beyond the risk factors and comorbidities discussed above, studies are strongly suggesting that AF might have heritable components. A huge number of genetic studies have demonstrated that AF has a substantial genetic basis[230-233]. With the standard genetic techniques, mutations in genes that encode ion channels, like myocardial potassium[234-238] and sodium channels[239-241], potassium-adenosine triphosphate channels (ABCC9), and that encode cardiac proteins, including nucleoporin-155 (NUP155), and gap junction protein connexin 40 (GJA5) are identified to cause dominantly inherited AF in the absence of traditional risk factors[242, 243]. Moreover, genome-wide associated studies have led to the pinpointing of genetic variants associated with common forms of AF[244]. Such studies provide new inroads into the mechanisms underlying AF, which can lead to new mechanism-targeting therapies and clinical studies.

1.4.3.2.5.5. Mechanisms

Three major components- initiation, maintenance, and progression toward longer-lasting AF forms- are suggested to be involved in the pathophysiology of
AF\[225,245\], although we do not yet fully understand the complete and precise mechanistic basis of these components. There are three principal competing hypotheses attempting to explain the fundamental mechanism of AF [246].

Multiple wavelets hypothesis was proposed by the seminal work of Moe et al\[247, 248\]. It postulated that it is the multiple coexisting electrical wavelets which propagate randomly and uninterruptedly throughout the atria that sustain the fibrillatory activity in AF. Although this idea is ubiquitous in the literature, experimental and clinical studies have argued against the applicability of this hypothesis as a unifying mechanism to explain sustained AF [249].

The second hypothesis is that of the single-source hypothesis, where a rapidly discharging ectopic focus, in the form of a single localized electrical source, is suggest triggering AF[245]. The mechanisms believed to produce ectopic activity via enhanced atrial automaticity (spontaneous diastolic depolarization) or triggered activity[250]. Focal ectopic/triggered activity is likely caused by either early afterdepolarization (EADs) or delayed afterdepolarization (DADs) which is shown in figure 1.7. EADs is associated with action potential duration prolongation, where Ca$_{2+}$ channels will be able to recover from inactivation[251] and thus result in EADs. DADs could be caused by intracellular Ca$_{2+}$ handling abnormalities, which result in the abnormal Ca$_{2+}$ leak from the sarcoplasmic reticulum and activation of the sodium calcium exchanger[252]. The influential work by Haïssaguerre et al. demonstrated that pulmonary veins are the major source of ectopic electrical triggers of AF [253].
Rotors or reentrant circuits are the third theory proposed to underlie the perpetuation and maintenance of AF. This hypothesis proposes that AF is the consequence of a small number of localized functional reentrant sources (rotors). These rotors generate spiral waves, which could emerge at high frequency, and propagate away from the rotor to interact with tissue heterogeneities and giving rise to complex patterns of nonuniform propagation termed “fibrillatory conduction”[254, 255]. Re-entry could be functional, where premature impulses conduct unidirectionally around an initially refractory border. However, re-entry can also occur around an anatomical obstacle when each point in the pathway has sufficient time to regain excitability before the arrival of the next impulse. The tissue’s wavelength, which is the product of conduction velocity and effective refractory period, is an important determinant for likelihood of re-entry[211].

Leading circle concept was formulated by Allessie and colleagues to account for reentrant activity in the absence of an anatomical obstacle[256]. The key feature of the leading circle concept is that the functional reentry is governed by activity in a

Figure 1.7. Early and delayed afterdepolarization. (Adapted from Haissaguerre et al., *N Engl J Med*, 1998)
limited region for which the wavelength occupies virtually the entire pathway of reentry as shown in figure 1.8A. Spiral wave in figure 1.8B is another conceptual interpretation for functional re-entry, which has been proposed by the work of Jalife and colleagues[257].

Figure 1.8. The leading circle and spiral wave concepts. A) The leading circle concept: Activity established itself in the smallest pathway that can support reentry, shown as a large black arrow. Inside the leading circle, centripetal excitation wavelets (small arrows) emanating from it constantly maintain the central core in a refractory state. B) Spiral wave concept: Schematic diagram of a spiral wave with the activation front shown in black and the repolarization tail in red. The point at which the red and black curves meet is usually referred to as the phase singularity point. (Adapted from Comtois et al., *EP European*, 2005)
In general, the spiral wave excitation proceeds around an excitable but unexcited central core, which is also called the phase singularity. The shape of the spiral wave can be described by the activation front starting from the inner area of the spiral and propagating away from the center zone. Curvature of the activation front is an important characteristic, which modulate the velocity of the propagation. The repolarization tail is at the end of the refractory period following the activation front[258].

Although numerous in vivo and in vitro studies have not conclusively defined a mechanism, both triggered/ectopic activity and re-entry have been suggested currently as the major arrhythmogenic mechanisms of AF[245, 249, 258]. Focal ectopic firing is required for the initiation of AF in a vulnerable substrate[259]. In addition, it can maintain AF when occurring repetitively at a high frequency. Multiple circuit re-entry or one or more rotors with fibrillatory conduction are accepted as the most likely mechanisms for the maintenance of AF[260]. Figure 1.9 gives a comprehensive illustration of AF mechanisms.
Figure 1.9. Rendering of left and right atria showing various mechanisms of AF. A, Focal trigger arising from muscle sleeve of pulmonary vein (PV) propagating into the left atrium and initiating AF in the vulnerable substrate. B, Fixed or moving spiral wave, a result of functional reentry, acts as a driver for AF. C, Circus movement around anatomic structures or scar generating micro- and macro-reentrant circuits. D, Perpetual propagation of multiple simultaneous wavelets mediated by both functional and structural reentries. E, Point source with fibrillatory conduction acting as driver for persistence of AF. F, Electrical dissociation between myocardial layers enabling reentry in 3-dimensions. CS indicates coronary sinus; IVC, inferior vena cava; LAA, left atrial appendage; LIPV, left inferior pulmonary vein; LSPV, left superior; PG, parasympathetic ganglia (yellow); RIPV, right inferior pulmonary vein; RSPV, right superior pulmonary vein; and SVC, superior vena cava. (Cited from Staerk et al., CircRes, 2017)
1.4.3.2.5.6. AF begets AF and atrial remodeling

As I already mentioned, AF is a progressive disease. Paroxysmal AF often progresses to chronic stages (persistent and permanent AF), independent of the underlying etiology. The transition from paroxysmal AF to chronic AF occurs in 31% of patients with paroxysms shorter than 2 days versus 46% if the episodes of AF were of longer duration[261]. Clinical studies showed that the success rate of pharmacological or electrical cardioversion was lower in patients with longer AF duration[262-265]. An experimental study in goats found that short, infrequent AF episodes progressively became longer and more frequent, until it being chronic, and thus coined the term “AF begets AF”. This is because as AF progresses, it causes atrial remodeling, which promotes more AF which in turn causes more remodeling[266, 267].

Remodeling of ion channels can account for the different electrophysiological properties of the different atrial regions in terms of conduction velocity, APD, and refractory period[268-271]. Numerous studies investigated the ion channel remodeling in the atria[272], which subsequently lead to APD shortening and faster repolarization, promoting AF, as shown in figure 1.10.
Figure 1.10. Abnormality of refractoriness is a major determinant of reentry in AF. Increased outward currents and decreased inward currents reduce refractory period and APD, promoting AF by accelerating repolarization (dashed line). Major atrial ionic currents and their atrial selectivity are shown underneath the action potential (Modified from Iwasaki et al., Circulation, 2011)

The transient K⁺ outward current ($I_{to}$) and the ultrarapid delayed rectifier current ($I_{kur}$) are decreased, but the slow component of the delayed rectifier K⁺ current ($I_{ks}$) is increased in chronic AF, which results in significant APD shortening, allowing faster spinof the rotor[273-276]. It has also been suggested that that AF remodeling results in increased $I_{K1}$ and $I_{KACr}$. Upregulation of such repolarizing
currents can help in establishing a substrate for stable and very fast rotors[277-279]. In addition to increased potassium currents, depolarizing sodium and calcium currents are suggested to be decreased by AF remodeling via possible reduction in the expression of alpha subunit of the sodium channel (Na\textsubscript{v}1.5) and calcium channel (Ca\textsubscript{v}1.2) respectively[274].

In addition to the ionic determinants that are important for AF initiation and maintenance, atrial structural remodeling is also critical for AF initiation and maintenance. Increased atrial fibrosis and atrial dilation are central features of atrial structural remodeling in AF [260, 280-287], and sympathovagal disbalance, and possible changes to atrial innervation have also been shown to be important for the initiation and for maintenance of the arrhythmia[288-292].

1.4.3.2.5.7. Potential targets for treatment

AF treatment success remains suboptimal. Invasive ablation approaches aiming at curing AF are on the rise, but pharmacotherapy aimed at maintaining sinus rhythm (rhythm control therapy) remains a first line of defense[293]. However, currently available pharmacologic interventions for AF have major limitations, including limited efficacy and risk of life-threatening ventricular proarrhythmic side effects[294-299]. One potential approach to improving pharmacotherapy is the mechanistically driven identification of novel targets. Therefore, modulation of atrial selective currents, which have been shown in figure 1.10, that are remodeled in AF has emerged as a novel and promising therapeutic concept to control AF without significant consequences to the electrophysiology of the ventricles[296, 300]. In patients with chronic AF (cAF)
(persistent and permanent), the increased $K^+$ currents contribute APD abbreviation, a major hallmark of electrical remodeling in AF, which promotes shorter refractoriness and wavelength, and thereby facilitates the maintenance of reentrant circuits[245, 260]. Therefore, it is assumed that inhibition of such repolarizing $K^+$ current may destabilize AF. Conversely, inhibition of ventricular $K^+$ channels may lead to ventricular AP prolongation, which may promote the occurrence of life-threatening arrhythmias such as torsade de pointes [301]. Therefore, inhibition of $K^+$ channels that are atrial specific is a major goal in the development of novel anti-AF $K^+$ channel blockers. Potential candidates of interest have been reported to include acetylcholine (ACh)-activated inwardly rectifying $K^+$ current ($I_{K\text{ACH}}$), ultrarapid delayed rectifier $K^+$ current ($I_{Kur}$), small-conductance $\text{Ca}^{2+}$-activated $K^+$ (SK) channels, and the two-pore $K^+$ channels ($K_{2p}$) and $K_{\nu}1.1$ channels[300, 302-310]. In addition to these atrial specific $K^+$ channels, other atrial specific ion channels such as the atrial specific late sodium current, stretch-activated channels, connexin 40 are also suggested to be potential targets for AF treatments[311,312]. Thus far, targeting many of these channels has not been shown to be optimal in persistent AF.

1.4.3.2.5.8. Aging and AF

Aging is an independent risk factor of cardiovascular diseases, even in the absence of the well-known traditional factors, including obesity, hypertension, and diabetes [313-315]. Since the 1990s, the incidence and prevalence of AF have been reported to increase by advanced age based available epidemiological data, which further suggested that aging is a strong predictor of
atrial fibrillation[316-321]. Clinical and laboratory evidence indicated that aging increases the susceptibility to AF through atrial electrical and structural remodeling. Many factors, including cardiovascular comorbidities, sympathovagal dysfunction, calcium dysregulation, atrial myopathy with apoptosis, fibrosis and oxidative stress, have been shown to be commonly associated with aging and contribute to AF genesis[322].

Oxidative stress, mainly due to an imbalance between the oxidant production and endogenous antioxidant defenses has been demonstrated as one of the primary determinants of aging in the heart[323, 324]. Reactive oxygen species (ROS) are products of oxidative stress. They are generated by a variety of enzymes, including xanthine oxidase (XO), nitric oxide synthase (NOS), and nicotinamide adenine dinucleotide (NADPH) oxidase (NOX)[325]. Examples of ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hypochlorite (HOCl), nitric oxide (NO), peroxynitrite (ONOO$^-$), and hydroxyl radicals (·OH). These are unstable, and highly reactive molecular species that can damage cellular proteins and lipids by oxidation and activate intracellular signaling cascades[326]. In the cardiovascular system, NADPH oxidase is the major source of ROS[327, 328]. In myocardial tissue, increased levels of ROS, such as superoxide and H$_2$O$_2$, have been implicated in the pathogenesis of AF by affecting ion channels, calcium handling, and propagation of the action potential [329-331]. Studies have shown that myocardial oxidative damage is associated with atrial electrophysiological remodeling, resulting in effective refractory period (ERP) shortening, and higher inducibility of AF in animal models of rapid atrial pacing, both of which can be
prevented by treatment with high doses of antioxidant or anti-inflammatory agents[332-334]. In addition, increased superoxide and hydrogen peroxide production have been detected and suggested to play an important role in atrial electrophysiological remodeling observed in patients with AF[335-337]. Hence, oxidative stress is a potential link between AF and atrial remodeling in the aging heart.

1.4.3.2.5.9. $I_{\text{KAC}}$ and AF

Electrical remodeling promotes AF through abbreviating atrial refractoriness by shortening the action potential duration, in part, via increased inwardly rectifying potassium currents such as the background current $I_{K1}$ and a constitutively active form of the acetylcholine-dependent potassium current ($I_{\text{KACH}}$)[303, 338-341]. Inward rectifying potassium channels (Kir, IRK) is a family of mammalian K$^+$ channels with subunit proteins that have structural and functional features distinct from the voltage- gated K$^+$ channel superfamily[342, 343]. Kir channels are made up of two transmembrane helices with cytoplasmic NH$_2$ and COOH termini and an extracellular loop which folds back into the transmembrane space to form the pore-lining ion selectivity filter [342, 343]. It is thought that these channels play an important role in the regulation of membrane excitability [344]. Because they conduct better in the inward direction (at membrane potentials negative to K$^+$ reversal potential) versus outward direction, they are called inward rectifiers. Inward rectification is due to the high-affinity block by endogenous intracellular polyamines, namely spermine, as well as divalent cations such as magnesium ions. For example, at potentials more positive than the K$^+$ reversal
potential, polyamines plug deeply into the channel, at sites in the transmembrane region, occluding the flow of K+, resulting in a decrease in the outward current[345]. Kir channel activity can be modulated by ions, phospholipids, and kinases. Functional Kir channels are composed of four subunits in either a homo- or heterotetrameric arrangement[346]. Cardiac $I_{K\text{ACh}}$ is mainly a hetero tetramer consisting of two Kir3.1 and two Kir3.4 protein subunits[347-349]. In normal physiology, $I_{K\text{ACh}}$ is activated by vagally released acetylcholine (ACh) which in turn activates the type 2 muscarinic ($\text{M}_2$) receptors. This results in the dissociation of the $\alpha$ and the $\beta\gamma$ subunits of the inhibitory $\text{G}_i$ protein and the subsequent activation of $I_{\text{KCh}}$ due to the direct interaction of the $\beta\gamma$-subunits with the $I_{\text{KCh}}$ channel proteins[346, 349, 350]. $I_{\text{KCh}}$ channels can also be gated by internal Na$^+$ ions. Both of the activation mechanisms are reported to be prevented by blocking phosphatidylinositol 4,5-bisphosphate (PIP$_2$), which means the regulation of the $I_{\text{KCh}}$ channel activity may be crucially dependent on PIP$_2$ and phosphatidylinositol[351]. $I_{\text{KCh}}$ on/off is tightly regulated since this ionic current is important for mediating the chronotropic parasympathetic effects resulting in decreasing the heart rate[352, 353]. However, in atrial myocytes from patients with chronic AF and animal models of chronic AF, $I_{\text{KCh}}$ is constitutively active independent of parasympathetic signaling (Figure 1.11). This constitutively active current subsequently works as a background current, which contributes to APD abbreviation, and shortening of the refractory period, which promotes AF initiation and perpetuation[303, 338, 354-357]. Animal and human studies have postulated that in chronic atrial fibrillation, PKC$\varepsilon$ may be responsible for $I_{\text{KCh}}$ constitutive
activity[354, 355].

Figure 1.11. Regulation of $I_{\text{KACH}}$ in normal physiology and chronic AF. In normal physiology, $I_{\text{KACH}}$ is tightly regulated by parasympathetic signaling, where Acetylcholine (ACh) binds to the $M_2$ receptor. This binding results in the disassociation of $G_{\beta \gamma}$ from $G_\alpha$ protein complex. $G_{\beta \gamma}$ then docks into the intracellular domain of the $I_{\text{KACH}}$ channel, which subsequently causes channel activation. However, in chronic AF, $I_{\text{KACH}}$ is constitutively active in an agonist independent way and works as a background current. (Adapted from Dobrev et al, Cardiac electrophysiology: From cell to bedside, 2014)

**1.4.3.2.5.10. PKCε and AF**

Changes of cardiac excitability caused by transcriptional and post-translational modifications of ion channels and gap junction proteins are implicated in cardiovascular diseases (i.e., arrhythmias, heart failure)[358, 359]. Additionally, evidence is showing that post translational modification of ion channel proteins can influence the electrical properties of cardiomyocytes[360, 361], and various kinases have been implicated in mediating pathological cardiac electrophysiological remodeling. Specifically, activation of protein kinase C (PKC) isozymes has been a subject of great attention in pathological settings such as heart failure[362, 363], ischemia[364, 365] and ischemic preconditioning[366-368]. The relevance of PKC isozymes to cardiac excitability was initially shown by
studies demonstrating that prolonged exposure of isolated neonatal cardiomyocytes to 4-β phorbol ester 12-myristate-13-acetate (PMA), a non-selective PKC activator, enhanced the rate of contraction in a dose-dependent fashion[369]. Indeed, several studies have reported the contribution of specific PKC isozymes to cardiac excitability by regulating ion channel expression[370-372] and activity[373-375], and inotropic and chronotropic effects[376].

PKC, identified in 1977 by Nishizuka and coworkers[377], is a group of closely related phospholipid-dependent serine-threonine protein kinases, which are activated as a result of receptor-dependent activation of phospholipase C and the hydrolysis of membrane phosphoinositide[378]. These isozymes are classified based on their structure and activation requirements into three subgroups: conventional PKCs (α, βI, βII and γ), which are Ca\(^{2+}\)-dependent and activated by diacylglycerol (DAG) and phosphatidylserine (PS); novel PKCs (ε, δ, η and θ), which are Ca\(^{2+}\)-independent but activated by DAG and PS; and atypical PKCs (ζ and ι/λ), which are Ca\(^{2+}\) and DAG-independent but activated by PS. For all PKC isoforms, translocation to membrane structures provides a mechanism to regulate access to substrate and has been taken as the hallmark of activation[379]. PKC activation involves translocation of the protein from the cytosolic autoinhibited latent form to the membrane associated active form, where it localizes to the caveolin-3 (Cav3) rich microdomains[380, 381].

PKC isoforms are demonstrated to differentially modulate I\(_{KACH}\), with conventional Ca\(^{2+}\)-dependent isoforms inhibiting and novel isoforms enhancing activity. It is thought that PKC epsilon (PKCε), is active and plays a role in the
electrical remodeling of the fibrillating atria[382]. More specifically, animal and human studies have postulated that the activated PKCε in chronic atrial fibrillation may be responsible for I_{KACCh} constitutive activity[354, 355]. Additionally, oxidative stress has been demonstrated to result in PKCε activation[383].

As discussed above, numerous epidemiological studies have strongly suggested that AF is an aging-mediated disease. However, the complex molecular pathways that are involved in promoting AF in the aging heart are not understood. Presently, the pharmacological and non-pharmacological therapeutic approaches of AF have been shown to be suboptimal. Invasive ablations are successful in terminating persistent AF, however, in the chronic stages of AF ablation becomes less effective[384-386]. Catheter ablation for atrial fibrillation has emerged as an important rhythm control strategy and is by far the most common cardiac ablation procedure performed worldwide. Current guidelines recommend the procedure in symptomatic patients with paroxysmal or persistent AF who are refractory or intolerant to antiarrhythmic drugs. The procedure might also be considered as a first-line approach in selected asymptomatic patients. In the 1990s, it became increasingly clear that some forms of AF, particularly paroxysmal AF, is triggered by a focal source, most commonly from muscular sleeves originating in the posterior left atrial wall, that extend into the pulmonary veins (PV-LA junction). In addition to acting as a source for AF triggers, the PV-LA junction has also been found to be an important substrate for the maintenance of AF[386]. Therefore, pulmonary vein isolation using point-by point radiofrequency or cryoballoon ablation remains the cornerstone technique in AF ablation[387].

Pharmacotherapies with rhythm controlling drugs such as antiarrhythmics have
been found to have limited long-term efficacy, off target side effects, and drug induced proarrhythmic side effects[388]. Accordingly, greater comprehension of the molecular mechanisms underlying AF is strongly required in order to develop potentially novel, mechanically driven, and more effective therapeutic strategies geared towards AF in the aging heart.

It should be noted that AF associated cardiac remodeling is very complex and includes various and complex players[389, 390]. However, in my thesis, I will focus on aging-mediated AF through a very specific pathway so that I may investigate, in a focused manner, the direct mechanic links between aging, ROS, PKCε, I_{\text{KACH}} and AF. Therefore, in chapter 2, I will test the novel hypothesis (Figure 1.12) that aging leads to PKCε activation, resulting in constitutively active I_{\text{KACH}}, shortening of the action potential, and perpetuation of AF. Then, in chapter 3, I will explore whether blocking I_{\text{KACH}} with novel, bioengineered protein modalities can terminate AF in the aging mouse heart.
Figure 1.12. Central hypothesis.
Chapter Two: \( \text{IK}_{\text{ACh}} \) is Constitutively Active via PKC\( \varepsilon \) in Aging-Mediated AF

2.1 Introduction

Epidemiological studies have shown that the natural history of AF is progressive[391, 392], and that aging is the single greatest risk factor for AF[393]. Unfortunately, the underlying mechanisms of aging-mediated AF are not well understood, and therefore, current pharmacological and more invasive therapeutic interventions have numerous limitations and remain inadequate in treating this disease[394].

Cardiac aging is a complex process, characterized by a progressive increase in cardiac remodeling[395], where increased generation or accumulation of reactive oxygen species (ROS) is one of the major contributors[396]. Growing evidence suggeststhat progressive atrial remodeling underlies the process of AF development. Such remodeling is defined by persistent changes in atrial structure and function, including changes in atrial refractoriness, atrial conduction, aberrant impulse formation, and fibrosis[397]. These factors constitute the appropriate electrophysiological and anatomical substrates conducive for the initiation and perpetuation of AF. In addition, oxidative stress is proposed to likely play a role in the pathogenesis of AF[398]. In myocardial tissue, increased levels of ROS such as superoxide \( (\text{O}_2^-) \) and hydrogen peroxide \( (\text{H}_2\text{O}_2) \) have been found to be
associated with AF[335-337, 399].

Electrical remodeling promotes AF through abbreviating atrial refractoriness by shortening the action potential duration (APD), in part, via increased inwardly rectifying potassium currents such as the background current $I_{K1}$ and a constitutively active form of $(I_{KACH})[303, 338-341]$. It has been shown in atrial myocytes from patients with chronic AF and animal models of chronic AF, that $I_{KACH}$ is constitutively active independent of parasympathetic signaling. This constitutively active current subsequently works as a background current that can participate in APD abbreviation, and shortening of the refractory period, which promotes AF initiation and perpetuation[303, 338, 354-357].

A set of collaborative studies in both animal and human suggested that the constitutively active $I_{KACH}$ in chronic atrial fibrillation could be attributed to the PKCε modulation [354, 355, 400]. Moreover, oxidative stress can activate PKCε[383], and has been demonstrated to play a critical role in the electrical remodeling of fibrillating atria[382].

Presently, it is not known whether PKCε and $I_{KACH}$ play a role in aging-mediated AF. Here, we directly examined if the PKCε/$I_{KACH}$ interplay is important as a novel mechanism for aging mediated AF. We thus tested the hypothesis that in the aging heart, $I_{KACH}$ is constitutively active via a PKCε mechanism, leading to the perpetuation of AF.
2.2 Materials and Methods

2.2.1 Animals

All animal care procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Use and Care of Animals of the University of South Florida. Both male and female mice were used. PKCε knockout (KO) mice were purchased from the Jackson Laboratory. Experiments were performed in male and female young and old WT and KO animals. The average age of young animals was 3.9 ± 0.08 months and that of old mice was 20.9 ± 0.43 months. For euthanasia, CO2 was used to induce loss of consciousness, and after loss of pinch reflex, swift cervical dislocation was performed, followed by rapid harvesting of the heart via thoracotomy. This method is consistent with the guidelines of the American Veterinary Medical Association.

2.2.2 Single Cell Isolation

Atrial myocytes were enzymatically dissociated from young and old WT and KO mice. Immediately after cardiac excision, the heart was cleaned, and the aorta was cannulated. The heart was then retrogradely perfused at 2 ml/min at 36±0.5 °C, for 3 min with Ca2+ free Tyrode solution (in mM): NaCl 137, KCl 5.4, HEPES 10, MgCl2 1, and glucose 10 (pH 7.3) until the effluent was clear of blood. Then the heart was perfused with the same solution containing 1 mg/ml collagenase Type A (Roche, Germany), and 0.08 mg/ml protease Type XIV (Sigma-Aldrich, USA) for 8 to 11 min, followed by Tyrode solution containing 0.2 mM CaCl2 for 5 min. Single cells were then obtained by dissociation via gentle agitation of digested atrial tissues. Atrial myocytes suspensions were filtered through a nylon mesh, and cells
were stored at room temperature in Tyrode solution. All solutions used for
dissection and perfusion were continuously bubbled with 100% \( \text{O}_2 \).

### 2.2.3 Cellular Electrophysiology

**Atrial myocytes:** The \( I_{\text{KCh}} \) current in atrial myocytes was measured with standard
whole-cell voltage-clamp technique. The Multiclamp 700B (Molecular Devices)
amplifier, an A/D converter (Digidata 1550B plus Hum Silencer, Molecular Devices),
and the pClamp 10.6 PC software (Molecular Devices) were used for current
acquisition. Clampfit 10.6 (pClamp, Molecular Devices) and OriginPro 2018b
software packages (OriginLab Corp) were used for data analysis. Whole-cell
measurements were performed at room temperature using borosilicate glass
microelectrodes with tip resistances of 2.5-3 M\( \Omega \) filled with pipette solution (in mM):
KCl 140, MgCl\(_2\) 1, HEPES 10, EGTA 5, Mg2ATP 5, and GTP 0.1, pH adjusted to 7.2
with KOH. Basal current was recorded by holding the atrial myocyte at -40 mV,
followed by 1000 ms steps from -140 mV to +40 mV in 10 mV increments. The
extracellular solution contained (in mM): NaCl 100, KCl 50, MgCl\(_2\) 1, HEPES 5, and
D-glucose 5.5, adjusted to pH 7.4. To measure the constitutively active \( I_{\text{KCh}} \) current
in atrial cardiomyocytes, 100 nM Tertiapin-Q (TPQ, Alomone Labs) was applied to
the extracellular solution to block the constitutively active \( I_{\text{KCh}} \) current.

**HEK cells:** HEK293 cells stably transfected with Kir3.1/Kir3.4 were a gift from Dr.
Bayliss, University of Virginia. This cell line displays a basal \( I_{\text{KCh}} \), without the need
for muscarinic stimulation. \( I_{\text{KCh}} \) was recorded in these cells and the internal pipette
solution contained (in mM): K-aspartate 100, NaCl 10, KCl 40, Mg-2ATP 5, EGTA
2, GTP-Tris .1, HEPES 10, pH 7.4. The bath solution contained (in mM): NaCl 90,
KCl 50, CaCl$_2$ 1, MgCl$_2$ 2, HEPES 10, glucose 10, pH was adjusted to 7.4 with NaOH. The current was evoked in response to a ramp from -130mV to 40mV, from a holding potential of 0 mV, and subtracted from the residual current remaining after adding 1 mM BaCl$_2$ to the bathsolution. In HEK293 cells stably expressing Kir3.1 and Kir3.4, SiRNA was used to silence PKCε (PKCε siRNA, Santa Cruz Biotechnology) according to the manufacturer’s protocol.

2.2.4 In-Vivo Electrophysiology

Mice were anesthetized with 1.8% isoflurane. The ECG was recorded in Lead II configuration, and a 1.1 F octapolar Millar electrophysiology catheter, with an injection port for intracardiac delivery (Mikro-Tip® catheter, ERP-801, Millar, USA), was placed in the right atrium through jugular access. The ECG and the intracardiac electrograms were simultaneously recorded using the Advanced Instruments platform as shown in figure 2.1.
Figure 2.1. Experimental set up for in vivo atrial electrophysiological testing in mice. A. The external jugular vein is isolated and the electrophysiology catheter is introduced through the vein and advanced to the right atrium (B and C). The catheter tip has 8 electrodes (D), that are used in a bipolar configuration for intracardiac electrogram recording (blue and pink traces) and for electrical stimulation. During experiments, ECG is recorded as shown in the red trace simultaneously with the intracardiac electrograms.

The cardiac signals were recorded via Animal Bio Amp (AD Instruments, USA) and digitized via the PowerLab data acquisition system (AD Instruments, USA). LabChart Pro 7.2 software (AD Instruments, USA) was used for acquisition and analysis of the cardiac electrical signals. To study AF inducibility and duration, intracardiac programmed electrical stimulation of the right atrium was performed via the catheter. The pacing protocol consisted of a 2-second burst, 2 x diastolic threshold, 2.5 ms pulse duration, increasing from 28 to 60 Hz in 2 Hz increments. AF was defined as a period of rapid irregular atrial rhythm lasting at least 2 seconds [401]. Animals showing AF episodes longer than 2 seconds were considered as inducible for AF. To test the effects of I_{KACH} block on AF, saline (50
μL) or TPQ (5 μM, in 50 μL saline) was injected through the catheter port. Figure 2.2 shows an example of ECG and right atria intracardiac electrogram (ICE) recorded in old WT mice. Upper panel shows that two seconds burst of stimuli at 40Hz induced an 88.43 second AF episode after which the heart reverted to sinus rhythm (SR). Saline (200μL) was injected control. However, with TPQ (5μM,200μL) given, AF was not induced in the WT old mouse as shown in bottom panel.

Figure 2.2. In vivo electrophysiology study in old mice injected with saline (Upper) and TPQ (Bottom). AF was induced in old mouse injected with saline but not with TPQ. Red traces: ECG; blue traces: intracardiac electrogram near ventricle; pink traces: intracardiac electrogram in atria. SR: sinus rhythm. Stim: stimulation. ICE: intracardiac electrogram.

2.2.5 Optical Mapping Studies

Optical mapping in the isolated Langendorff-perfused mouse heart was
performed as described before[279, 402, 403]. Briefly, the excised hearts were rapidly cannulated and retrogradely perfused with normal Tyrode solution at 37 °C. The heart was then placed in the well of a custom-made chamber maintained at 37 °C. Optical mapping of the right atrial epicardial surface was carried out using a high-resolution CCD camera (1000 frames/s) and Di-4-ANEEPPS (Sigma, St. Luis, MO, USA). The preparation was monitored using volume-conducted ECG in lead II configuration, via Animal Bio Amp (AD Instruments, USA) and digitized via the PowerLab data acquisition system (AD Instruments, USA). LabChart Pro 7.2 software (AD Instruments, USA) was used for acquisition and analysis of the cardiac electrical signals. Motion uncoupling was achieved with 7 µM blebbistatin (Abcam, USA). Action potential duration maps were generated as previously reported[279, 402, 403].

2.2.6 Total Internal Reflection Fluorescence Microscopy

HEK293T cells were seeded on coverslips. 24 hours later, cells were transfected with mCherry-CRY2-PKCεCAT-HA using polyethyleneimine (0.75 µg DNA:3 µL PEI for 2 hours). Total internal reflection fluorescence (TIRF) microscopy experiments were conducted the following day. mCherry was used as a proxy to detect the translocation of mCherry-CRY2-PKCεCAT-HA to the cell membrane. Cells were incubated with 100 µM H$_2$O$_2$ in a solution comprised of (in mM): NaCl 130, KCl 4, MgCl$_2$ 1.2, CaCl$_2$ 2, HEPES 10, pH was adjusted to 7.4 with NaOH. mCherry was excited with a 561 nm laser (Thor Labs) at 5 mins, 15 mins and 30 mins after treatment with H$_2$O$_2$. Images were captured at 5 s intervals to prevent bleaching and data were saved as separated stacks; the background was
subtracted. Data were analyzed with ImageJ.

2.2.7 HL-1 Cell Culture

HL-1 cells are a cardiac cell line derived from a mouse atrial cardiomyocyte tumor lineage. This widely used cell line maintains the ability to contract, retains some phenotypic characteristics of the atrial cardiomyocytes, and can be serially passaged[404, 405]. HL-1 cells were cultured in Claycomb medium (Thermo Scientific, USA) with 10% fetal bovine serum (FBS, Gibco™, Thermo Scientific, USA), 100mg/mL penicillin/streptomycin (Thermo Scientific, USA), 0.1mM (norepinephrine Sigma-Aldrich, USA) and 2µM L-glutamine (Gibco™, Thermo Scientific, USA). 70-80% cell confluency was required for experiments.

2.2.8 Flow Cytometry

To measure the effect of H₂O₂ on HL-1 cells, the cells were dislodged by 0.05% trypsin, washed in phosphate-buffered saline (PBS) and centrifuged at 1000rpm, for 5 minutes at 4°C. Cell pellets were resuspended with warm cell medium (mentioned in Cell Culture) and divided into two groups: non-treated (control), 1mM H₂O₂ (equate™) treated for 30 minutes. Then the ROS fluorescent indicator CellROX® (Invitrogen by Thermo Fisher Scientific) was added to the cells (500nM), and incubated for 30 minutes at 37°C, 5% CO₂, protected from light. 3µM DAPI (www.AppliChem.com) was then added, and the samples were read immediately on a BD LSR II Cytometer (using 488nM and 405nM lasers for the excitation of CellROX® and DAPI respectively, and the emission collected using a 530/30BP and 450/50BP filter respectively).
2.2.9 Western Blot

To measure the effect of oxidative stress on PKCε translocation, cells were treated with 0, 1, 10, 100 µM H₂O₂ for 30 minutes before cell lysate collection. To verify the PKCε translocation was caused by H₂O₂, pretreatment with 10 mM N-Acetylcysteine (NAC, Sigma-Aldrich) was done for 30 minutes before the H₂O₂ treatment. As a positive control, cells were treated with 300nM Phorbol 12-myristate 13-acetate (PMA, Sigma) for 15 minutes. Cells were rinsed twice with cold phosphate-buffered saline (PBS, pH7.4). The cells were then scraped and centrifuged at 1000 rpm, 4°C for 5 min, and the supernatant was removed. The pellet was resuspended and lysed in ice-cold hypotonic buffer (in mmol/L: HEPES 10 (pH 7.9), KCl 10, MgCl₂ 1.5, EDTA 0.1 (pH 8.0), EGTA 0.1, DTT 1, PMSF 1, 1:100 protease inhibitor cocktail (Sigma). After homogenization using an ultrasonicator and centrifugation (13000g for 10 min, at 4°C), the supernatants were transferred into Beckman coulter centrifuge tubes. The supernatants were then centrifuged at 40000g for 40 min, at 4°C to separate the cytosolic fraction (supernatant) and membrane fraction (pellets). The pellets were resuspended with SDS-Tris buffer (in mmol/L: Tris-HCl 50 (pH 7.6), SDS 1%, NaCl 0.15, MgCl₂ 1.5, EDTA 0.1 (pH 8.0), EGTA 1, DTT 1, 1:100 protease inhibitor cocktail (Sigma), and PMSF 1. Both fractions were analyzed for protein concentration. Equal amounts of the purified proteins were then separated by 4-15% pre-stained SDS-PAGE Mini-PROTEAN stain free gels (BIO RAD, USA), transferred onto nitrocellulose membranes (BIO RAD, USA). The nitrocellulose membranes were blotted with TBS intercept blocking buffer (LICOR), immunoblotted with rabbit anti PKC epsilon
monoclonal antibody (H.51.9) (1:500 dilution, Thermofisher Scientific) and mouse anti GAPDH (sc-166574) monoclonal antibody (1:250 dilution, Santa Cruz Biotechnology) overnight at 4°C. After washing, the blots were reacted with secondary antibody IRDye 800CW Donkey anti-Rabbit and IRDye 680RD Donkey anti-Mouse (1:20000, LI-COR) for 2 hours at room temperature. Bands were visualized with Odyssey CLx imaging system. PKCɛ western blots measurements were quantified by using ratios of membrane over cytosolic PKCɛ band intensities, normalized to GAPDH. We also performed immunoblot analysis with WT and KO mice atria to verify the knockout of PKCɛ as shown in figure 2.3. Adult (3-5 months) mice were sacrificed by CO₂ asphyxiation. Hearts were perfused with cold PBS and extracted. Both left and right atria were removed and homogenized in 50µL of RIPA buffer (Thermo Scientific, USA), 10mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF) and PPI were also added. Samples in lysis buffer were rocked for 20 minutes on ice, then centrifuged at 14000 ×g at 4 °C for 15 minutes to remove the debris. Protein concentrations were determined by bicinchoninic acid assay (BCA, Thermo Scientific, USA), and western blot was performed to detect PKCɛ expression.

![Figure 2.3](image)

Figure 2.3. Western blot of PKCɛ (green) in 2 WT and 2 KO mice atria. Red: GAPDH.
**2.2.10 Co-immunoprecipitation**

HL-1 cells were homogenized in ice-cold RIPA lysis buffer (Thermo Scientific, USA) then centrifuged at 14000g for 15 min at 4°C. Antibodies against PKCε or Kir3.1 (1:200, Alomone labs) or Kir3.4 (1:200, Alomone labs) were covalently linked to dynabeads protein A (Invitrogen, USA) according to manufacturer’s protocol. Following 2 hours incubation of lysates with the dynabead-PKCε or dynabead-Kir3.1, or dynabead-Kir3.4 antibodies complexes, dynabeads slurries were washed 3 times with RIPA buffer before elution of bound proteins by boiling at 90°C for 5 min in SDS sample buffer. Samples were loaded and separated by 4-15% pre-stained SDS-PAGE Mini-PROTEIN stain free gels. Resolved proteins were transferred onto nitrocellulose membrane, blocked, and then incubated with mouse anti-caveolin-3 antibody (1:200, Santa Cruz Biotechnology, USA) and secondary antibody IRDye 800CW Donkey anti-mouse (1:20000, LICOR), then analyzed by Odyssey CLx imaging system.

**Statistics**

Data are expressed as mean ± SEM, except for AF duration presented as box plots (Median, 25th and 75th percentiles, minimum and maximum). For statistical analysis, Chi square, Student’s t-test, Mann Whitney Wilcoxon test, one way ANOVA and linear mixed model were used as appropriate. Statistical significance was taken as p<0.05.

**2.3 Results**

**2.3.1 PKCε contributes to AF initiation and perpetuation in the old heart**

To investigate the role of aging in AF initiation and perpetuation, we
conducted a set of in vivo electrophysiological studies with intracardiac programmed electrical stimulation in WT young and old mouse hearts. Figure 2.4A shows the significantly different age (left) and weight (right) of the old compared to the young WT mice. When both genders are considered, the inducibility of AF in old WT mice is not significantly different from that in young WT mice (Figure 2.4B left panel). We then investigated whether there is a gender specific difference in AF inducibility. The inducibility of AF in female mice was not different between old and young WT animals (Figure 2.4B middle), however, inducibility was statistically higher in old WT compared to young WT male (Figure 2.4B right). The result in figure 2.4C shows that the duration of induced AF adjusted for gender is significantly longer in old WT mice compared to young.
Figure 2.4. Aging increases AF inducibility and duration. A: Age (left) and weight (right) are significantly different between WT old and WT young mice. B: AF inducibility comparison between WT old (N=42) and WT young (N=28) mice. (Left panel) AF inducibility was not different between WT old (31 out of 42 mice) comparable to WT young mice (16 out of 28), of both genders. (Middle) No significant difference of AF inducibility was found between WT old female mice (17 out of 24) and WT young female mice (12 out of 18). (Right) AF inducibility of WT old male mice (14 out of 18) is notably higher than WT young male mice (4 out of 10). #p<0.01, Chi-square test. C: AF duration comparison between WT old (N=42) and WT young (N=28) mice. Induced AF duration in WT old mice is remarkably longer than in WT young mice. *p<0.01, linear mixed model adjusted for gender.
Figure 2.5. Knocking out PKCε abrogates the effects of aging on AF. A: Age and weight comparison between WT old and KO old mice. (Left) Age of WT old and KO old mice are comparable. (Right) The weight of WT old is significantly heavier than KO old mice. B: AF inducibility comparison between WT old (N=42) and KO old (N=29) mice. (Left) AF inducibility in WT old mice (31 out of 42) is significantly higher than KO old mice (11 out of 29) of both genders. (Middle) AF inducibility in WT old female mice (17 out of 24) is significantly higher than in KO old female mice (3 out of 11). (Right) AF inducibility in WT old male mice (14 out of 18) is significantly higher than KO old male mice (8 out of 18). #p<0.01, Chi-square test. C: AF duration comparison between WT old (N=42) and KO old (N=29) mice. AF duration in WT old mice is longer than in KO old mice. *p<0.01, linear mixed model adjusted for gender.

To investigate the possible role of PKCε in AF initiation and perpetuation in the old heart, we assessed AF inducibility and duration in old PKCε KO versus old WT animals. Figure 2.5A indicates that the age of old WT and old KO animals was not different, but there was a difference in body weight. As figure 2.5B shows, AF inducibility is significantly reduced in old KO mice compare to old WT mice of both genders. Additionally, the duration of induced AF in the old KO mice is significantly shorter than that in the old WT mice, as shown in figure 2.5C.
Knocking out PKCε abrogated the effects of aging on AF inducibility and duration. These results suggest that PKCε plays an important role in the inducibility and stability of AF in the aging heart.

2.3.2 Constitutively active \( I_{K_{ACh}} \) is arrhythmogenic in the old heart

To investigate whether constitutively active \( I_{K_{ACh}} \) plays a role in aging-mediated AF, we assessed the in-vivo inducibility and duration of AF in old WT mice after jugular vein administration of the \( I_{K_{ACh}} \) blocker tertiapinQ (TPQ). TPQ is a 21 amino acid synthetic peptide originally isolated from the European Honeybee toxin. In the heart, TPQ has been shown to be a potent and selective blocker of \( I_{K_{ACh}} \). Moreover, TPQ prolongs the effective refractory period and terminates AF that is dependent on constitutively active \( I_{K_{ACh}} \), or AF that is dependent on \( I_{K_{ACh}} \) activated with an M2 agonist[406, 407]. TPQ administration significantly reduced the duration and the inducibility of AF in the old WT mice compared to saline vehicle control (figure 2.6A), suggesting that \( I_{K_{ACh}} \) is constitutively active in the old heart. However, in KO old mice, TPQ did not affect AF duration and inducibility, indicating that \( I_{K_{ACh}} \) is not constitutively active in the atria of old KO mice (figure 2.6B).
Figure 2.6. The effects of blocking $I_{KAC\text{h}}$ on AF in old mice with TPQ. A: AF duration and inducibility in WT old mice injected with TPQ or Saline. Left: AF duration is reduced by TPQ in WT old mice. Right: AF inducibility is decreased by TPQ in WT old mice (2 out of 10 vs 8 out of 12). Box & whiskers, Min-Max. B: The effect of TPQ on AF duration and inducibility in KO old mice. Saline is used as vehicle control. Left: In KO old mice, AF duration is not changed by TPQ. Right: AF inducibility is not changed by TPQ in KO old mice (3 out of 10 vs 5 out of 14). Box & whiskers, Min-Max, NS: not significant. *p<0.01, Mann Whitney Wilcoxon test. #p<0.01, Chi square test.

Subsequently, we used patch clamp in atrial myocytes isolated from WT and KO mice to confirm whether $I_{KAC\text{h}}$ is constitutively active in the old atrial myocytes, and if knocking out PKCε abrogates the effects of aging on the development of constitutively active $I_{KAC\text{h}}$. In figure 2.7, the background inward rectifier current was measured in 50mM $[K]_o$ before and after addition of 100nM TPQ to the bath solution as previously reported[357]. In the young WT atrial myocytes (figure 2.7A), TPQ did not affect the background current, indicating that $I_{KAC\text{h}}$ is not constitutively active. In the old WT atrial myocytes (figure 2.7B),
application of TPQ resulted in a significant decrease in the background current, indicating that $I_{K\text{ACh}}$ is constitutively active in the old heart, and that the constitutively active $I_{K\text{ACh}}$ works as a component of the background current in the old cardiomyocytes. In the old KO atrial myocytes (figure 2.7C), TPQ did not appreciably reduce the background current, indicating that knocking out PKCε diminishes the aging effect on constitutively active $I_{K\text{ACh}}$. Figure 2.7D is the quantification of the TPQ sensitive, agonist independent, constitutively active $I_{K\text{ACh}}$ at -140 mV. The constitutively active $I_{K\text{ACh}}$ was significantly larger in the old WT myocytes compared to young WT, and old KO myocytes.

Figure 2.7. Measurement of constitutively active $I_{K\text{ACh}}$. A: IV curves of the background current in young WT atrial myocytes before (black) and after 100 nM TPQ (grey), N=2, n=6. B: IV curves in old WT atrial myocytes before (black) and after TPQ (grey), N=2, n=6. C: IV curves in old KO atrial myocytes before (black) and after TPQ (grey), N=2, n=7. D: Quantification of the TPQ sensitive, constitutively active $I_{K\text{ACh}}$ at -140 mV. Average±SE. *p<0.05, one way ANOVA with Bonferroni correction. (Experiments were done with the help of Dr. Bojji Babu Chidipi).
2.3.3 Knocking out PKCɛ prevents APD shortening in the old heart

To provide further evidence that $I_{Kach}$ is constitutively active in the old atria, in a PKCɛ dependent manner, we proceeded to test the effects of TPQ on the atrial APD in the isolated Langendorff perfused mouse heart, using optical mapping. Our data in figure 2.8 show that 200nM TPQ prolonged the APD in the old WT but not old PKCɛ KO hearts. Figure 2.8A is an APD map at 60% repolarization (APD$_{60}$) of the right atrium in an old WT (top) and old KO (bottom) heart in control, and after TPQ application. TPQ caused an appreciable prolongation of the APD in the WT, but not in the KO heart. Single pixel optical tracings of voltage from the map in 2.8A show that TPQ prolonged APD in WT old but not in KO old hearts (figure 2.8B and 2.8D). Figure 2.8C and 2.8E are compilations of APD$_{60}$ and APD$_{30}$ in 4 old WT and 4 old KO hearts. TPQ caused significant APD prolongation in WT, but not in KO. This experiment shows at the tissue level, $I_{Kach}$ is constitutively active in the old WT heart, in a PKCɛ-dependent manner.
2.3.4 H$_2$O$_2$ increases ROS in HL-1 cells

To establish if oxidative stress in the form of exposure to H$_2$O$_2$ causes PKCε translocation to the membrane in atrial myocytes, we performed flow cytometry with HL-1 cells, which have been treated with 1mM H$_2$O$_2$ for 30 minutes. Our results (Figure 2.9) indicated that H$_2$O$_2$ treatment resulted in a significantly larger ROS positive cell compared to control (p<0.01). However, there...
was no significant difference in the number of dead cells. Accordingly, H$_2$O$_2$ treatment induces ROS production in HL-1 cells without inducing cells death and thus H$_2$O$_2$ concentration of up to 1mM could be used.

Figure 2.9. Flow cytometry in HL-1 cells. HL-1 cells were treated with 1mM H$_2$O$_2$ for 30 minutes or control untreated. H$_2$O$_2$ significantly increased the percentage of ROS positive cells, (left, CellROX) without increasing cell death (right, DAPI). T-test, *P<0.01.

2.3.5 Oxidative stress induces cell membrane translocation of PKCε, leading to increased $I_{KACH}$

Increased levels of myocardial ROS such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) have been found to be associated with AF[335-337, 399]. Additionally, the ability of oxidative stress to recruit PKC isoforms to the cell surface has been shown[380, 408]. To assess the ability of H$_2$O$_2$ to recruit PKCε to the cell membrane, we used the catalytic domain of PKCε tagged with mCherry (mCherry-CRY2-mPKCεCAT- HA) in total internal reflection fluorescence microscopy (TIRFM) experiments. This technique is able to image fluorescence proteins localized to the cell membrane. HEK293 cells transfected with mCherry-CRY2-mPKCεCAT-HA were incubated with H$_2$O$_2$ and imaged at baseline and 5, 15, 30 minutes after H$_2$O$_2$ treatment. mCherry was excited at 561nm. Figure 2.10A
shows images of time dependent increase in fluorescence with 100µM H₂O₂.

Figure 2.10B is a quantification of mcCherry fluorescence and shows a significant increase fluorescence at the cell surface after a 30-minute incubation with 100µM H₂O₂ compared to surface fluorescence at baseline. These data demonstrate that H₂O₂-mediated oxidative stress induced PKCε translocation to the cell surface.

In order to provide a mechanistic link between aging, PKCε and constitutively active I_{K_{ACh}}, we measured the basal I_{K_{ACh}} current in HEK cells stably transfected with Kir3.1 and 3.4, with or without PKCε knockdown, in the presence or absence of 100µM H₂O₂, as a surrogate exposure to simulate oxidative stress associated with aging. The basal I_{K_{ACh}} current, without muscarinic stimulation, was recorded in 50mM extracellular potassium. Figure 2.10C shows in representative I-V curves that 100µM H₂O₂ for 1-hour resulted in a larger I_{K_{ACh}} (red trace) compared to control (black). Silencing PKCε (green trace) prevented the increase of the current brought about by 100µM H₂O₂. In figure 2.10D, data were compiled where H₂O₂ significantly increased the maximum inward current. However, silencing PKCε prevented the increase in I_{K_{ACh}} after treatment with H₂O₂.
Figure 2.10. Effect of H₂O₂ on PKCε translocation and IₖACh. (A) Representative TIRFM images showing recruitment of mCherry-CRY2-mPKCεCAT-HA to the cell surface by 100µM H₂O₂. (B) Summary data showing cell surface localization of mCherry-CRY2-mPKCεCAT-HA at the cell surface following 30 mins of treatment with H₂O₂ (Average ± S.D. of n=10-24 cells per group). (C) IV curves of basal IₖACh (tertiapin sensitive current) measured in control (black), H₂O₂ treated (red), and PKCε silenced and H₂O₂ treated (green). HEK293 cells stably expressing Kir3.1 and Kir3.4, and co-transfected with PKCε SiRNA and GFP. (D) Quantification of maximum inward current. Average±SE. *p<0.05, One way ANOVA with Bonferroni correction. (TIRF was done with the help of Drs. Kirin Gada and Diomedes Logothetis-Northeastern University. Patch clamp was done with the help of Dr. Sami Noujaim).

We then confirmed that oxidative stress activates PKCε in atrial myocytes by inducing its translocation from the cytoplasmic to the particulate fraction. HL-1 cells were treated with 0,1,10,100 µM H₂O₂ for 30 minutes. Western blot showed a concentration dependent decrease of PKCε in cytosolic fraction and a concentration dependent increase of PKCε in membrane fraction were detected (Figure 2.11). We then proceeded to verify whether PKCε membrane translocation induced by H₂O₂ could be prevented by N-acetylcysteine (NAC) pretreatment, a well-known ROS scavenger. NAC is a synthetic precursor of intracellular cysteine
and glutathione, and its anti-ROS activity results from its free radical scavenging property either directly via the redox potential of thiols, or secondarily via increasing glutathione levels in the cells[409]. Therefore, NAC is commonly used to confirm the involvement of ROS[410]. Phorbol myristate acetate (PMA) was used as positive control PKCε translocation. PMA is the most common and potent phorbol ester, which mimics the second messenger diacylglycerol (DAG) to non-selectively activate PKC and promotes PKC membrane translocation[411, 412]. Figure 2.12 shows representative immunoblots of PKCε translocation to the cell membrane. The results show that 1)- H2O2 activate PKCε and promote its translocation to cell membrane and 2)- NAC prevents PKCε membrane translocation induced by H2O2. In figures 2.11 and 2.12, GAPDH is not a perfect internal control for membrane protein loading. Instead of using GAPDH, we suggest that in future studies, better membrane markers such as caveolin-3, or Na+/K+ ATPase could be used instead.
Figure 2.11. Western blot in HL1 cells treated with H₂O₂. Western blot of membrane and cytosolic fractions in HL1 cells treated with 0, 1, 10, 100 µM H₂O₂ for 30 minutes, using rabbit anti-PKCε antibody. A concentration dependent translocation of PKCε to the membrane fraction is evident.

Figure 2.12. Western blot in H₂O₂ treated HL1 cells with or without NAC pretreatment. Western blot of membrane fractions in HL-1 cells treated with 0, 10, 100µM H₂O₂, for 30 minutes with or without pretreatment with 10mM NAC for 30 minutes. PMA is for positive control.
**2.3.6 Kir3.1, Kir3.4, PKCε and caveolin-3 are in a macromolecular complex**

As mentioned earlier, cardiac $I_{K_{Ach}}$ is a heterotetramer of Kir3.1 and Kir3.4 channel protein subunits. Studies have suggested that in cardiomyocytes, activated PKCε translocates to caveolin-3 rich membrane domains [380, 381]. We tested whether PKCε, caveolin-3, Kir3.1, and Kir3.4 are in macromolecular complex. Co-immunoprecipitation using whole cell lysates from HL-1 atrial myocytes suggested that that Kir3.1, Kir3.4, and PKCε are indeed in a Cavolin-3 rich macromolecular complex (Figure 2.13). The specificity of the anti PKCε, Kir3.1 or Kir3.4 antibodies in this experiment has not been properly demonstrated in figure 2.13. A non-specific antibody such as anti β-actin antibody could be used in future follow up studies, as a negative control, to demonstrate the robustness of the co-immunoprecipitation of the PKCε/ Kir3.1/ Kir3.4/ caveolin-3 macromolecular complex.

![Figure 2.13](image_url)

Figure 2.13. Co-immunoprecipitation of PKCε, Kir3.1, Kir3.4 and caveolin-3 in HL-1 myocytes. Upper panels: Immunoprecipitation with rabbit anti- PKCε antibody and probing for caveolin-3. Caveolin-3 in whole cell lysates is shown as input. Bottom panels: Immunoprecipitation with anti-Kir3.1 or anti-Kir3.4 antibodies and probing for caveolin-3. Caveolin-3 in whole cell lysates is shown as input.
2.4 Discussion

Our experiments showed that $I_{KACH}$ is constitutively active in the old atria through a PKCε-dependent mechanism, where 1- knocking out PKCε abrogates the effect of aging on AF and prevents the development of a constitutively active $I_{KACH}$, 2- blocking constitutively active $I_{KACH}$ with TPQ reduces atrial arrhythmogenesis in the old heart, and 3- oxidative stress leads to the generation of a constitutively active $I_{KACH}$ via PKCε activation. This work provides direct evidence that the PKCε/$I_{KACH}$ pathway plays an important role in aging-mediated AF where constitutively active $I_{KACH}$ contributes to AF perpetuation in the aging heart in a PKCε dependent manner.

In the present study, we demonstrated that aging perpetuates AF. Our results correspond well with epidemiological studies suggesting that clinically, AF prevalence increases with advanced age; in fact, aging is the single greatest risk factor for AF.[391- 393]. Our data also showed that aging increased AF inducibility in male but not in female mice. Although this gender-based differences in cardiac electrophysiology are known, they are still poorly understood.[413, 414]. Gender differences are shown on electrocardiographic pattern of cardiac repolarization, which has been suggested to be important in AF. Atrial ERP in response to rapid atrial pacing was significantly less in premenopausal women compared with postmenopausal women and age-matched men, and castrated men showed slower and longer repolarization and virilized women exhibited a shorter and faster repolarization than normal and castrated men. This suggests that estrogen and testosterone play important roles in modulating cardiac repolarization.[415, 416].
The autonomic nervous system is also a key regulator of the cardiovascular system, where the sympathetic and parasympathetic arms of the autonomic system have co-regulatory effects on cardiac homeostasis[417]. There has been increasing evidence supporting the presence of gender differences in autonomic control of the cardiovascular system. They suggest that sympathetic-mediated responses predominate in men, while parasympathetic activation is more significant in women, which has been associated with a possible increased propensity of AF due to extensive vagal innervation of the atria muscle sleeves extending into the pulmonary veins[418]. More recent data also highlight the differential expression and transcriptional remodeling of ion channel subunits in the human heart as a function of gender. The mRNA levels of ion channel subunits, calcium handling proteins, and transcription factors important in cardiac conduction and arrhythmogenesis in the left atria and ventricles of failing and nonfailing human hearts of both genders were quantified and showed differential patterns of gene expression in the female left atrium compared to the male left atrium [419]. In addition, significant gender-based differences in AF-related atrial remodeling is also observed via analyzing tissue samples from men and women with long-standing, persistent AF. Significantly higher degree of fibrotic remodeling was found in women than men[420], which may contribute to higher incidence and recurrence rates of AF. These differences are thought to be driven by differential expression of fibrosis-related genes and proteins, such as transforming growth factor beta, which were upregulated in women with persistent AF. Gender is also suggested to modulate various AF risk factors. Obesity appears to increase the AF
risk more in men than in women, coronary disease and sleep apnea have a higher prevalence in men with AF than women, while hypertension and heart failure with preserved ejection fraction are more prevalent in women with AF[421]. Overall, multiple studies have shown major gender-based differences in the clinical profile and management of AF, whether these are related to differences in biology or represent patient reporting, diagnosis, and management disparities is unknown. This area deserves further study.

The generation and accumulation of ROS have been intimately linked to the cellular processes which underlie aging. By promoting oxidative damage to multiple subcellular and cellular structures, ROS have been suggested to promote the pathogenesis of AF by inducing structural and electrical remodeling including shortening of APD[330, 422-428]. This is in agreement with our cellular studies. Our data showed that ROS activate PKCε, induces its translocation to the cell membrane and increases \( I_{K\text{ACh}} \). Additionally, our patch clamp data showed that \( I_{K\text{ACh}} \) is constitutively active in the old atrial myocytes via a mechanism that depends, at least in part, on PKCε. It has been proposed that in chronic AF, \( I_{K\text{ACh}} \) is constitutively active and could be modulated by PKCε. Makary et al showed that PKC isoforms differentially modulate \( I_{K\text{ACh}} \), with the conventional Ca\(^{2+}\) dependent isoform PKCα inhibiting and novel isoform PKCε enhancing activity[355]. Here, we provide evidence that aging-mediated AF inducibility and duration are reduced in hearts by abrogating constitutively active \( I_{K\text{ACh}} \) via knocking out PKCε or by direct block of the current with TPQ. The pulmonary veins have been recognized as critically important sites to originate arrhythmogenic foci that trigger AF[429-431].
In the present study, we performed programmed electrical stimulation in the right atria to induce AF. Thus, our data may not reflect the spontaneous initiation mechanisms of aging-mediated AF in patients. It is possible that in the aging heart, oxidative stress could activate $I_{KATP}$ current[432] and therefore, it is possible that this current may also play a role in perpetuation of aging-induced AF. In addition, we used a mouse model of PKCε global knockout which might result in confounders. For instance, the body weight of old KO mice is significantly smaller compared to old WT mice. This could be due to the role that PKCε plays in diet-induced glucose tolerance or in metabolism and adipocytes function[433]. An inducible model of cardiac specific PKCε knock down could be used in the future to assess, in a cardiac specific manner, the role of PKCε and $I_{KAC}$ in aging-mediated AF.
Chapter Three: Bioengineered Peptibodies as Novel Blockers of Ion Channels

3.1 Introduction

As delineated in the previous chapters, constitutively active $I_{K_{ACh}}$ is important for the mechanism of AF initiation and perpetuation. Moreover, antiarrhythmic pharmacological treatment of chronic AF is not effective and therefore, novel ion channels blocking modalities are needed for the development of the next generation of antiarrhythmic pharmacotherapies[434].

We engineered and produced an ion channel blocking peptibody, that targets $I_{K_{ACh}}$. Peptibodies are chimeras generated as fusion proteins of the fragment crystallizable (Fc) domain of the human immunoglobulin G (IgG) with a bioactive "warhead" peptide[435, 436]. Peptibodies combine the biologic/therapeutic activity of a given peptide, with the stability of monoclonal antibodies. They are stable and safe molecules that are emerging as viable clinical therapeutics. For instance, the first peptibody in clinical use is Romiplostim, which is approved for the treatment of immune thrombocytopenic purpura. Romiplostim is composed of thrombopoietin mimetic peptides fused to the C terminus of the Fc region of human IgG[437-441].

Our anti $I_{K_{ACh}}$ peptibody was constructed as a fusion protein between the human IgG1 Fc fragment and tertiapinQ, a 21-amino acid synthetic peptidotoxin
originally isolated from the European honeybee venom. In the heart, tertiapinQ has been shown to inhibit $I_{K_{ACh}}[442, 443]$. As detailed earlier, in some forms of AF, including in the aged heart as demonstrated in Chapter 2, it was shown that $I_{K_{ACh}}$ is constitutively active, and behaves as a background potassium inward rectifier, and can thus contribute to the initiation and maintenance of the arrhythmia[303, 444]. Since $I_{K_{ACh}}$ is mainly expressed in the atria and is largely absent from the working ventricular myocardium, it has been proposed that blocking $I_{K_{ACh}}$ can be an atrial-selective rhythm control pharmacotherapy[434, 444, 445].

Here, we describe the bioengineering and characterization of a peptibody that blocks $I_{K_{ACh}}$. We tested the hypothesis that in the heart, the engineered peptibody is a potent blocker of $I_{K_{ACh}}$ in vivo and in vitro and is antiarrhythmic. The goal being to demonstrate that peptibodies can be used as novel cardiac ion channel blockers and can also guide the generation of novel antiarrhythmic modalities.

3.2 Methods

3.2.1 Animals

Our study conformed with the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University Committee on Use and Care of Animals of the University of South Florida. C57BL/6J mice of both sexes were used.

3.2.2 Peptibody construct

The DNA sequence of the peptibody (Fc-TP) construct was based on
that of Romiplostim[435, 439], and commercially synthesized (Genescript, USA).

3.2.2.1. Construct

EcoRI-Kozak Sequence-Artificial leader sequence-hlgG1FC-Linker-
TertiapinQ-Stop codon-HindIII

3.2.2.2. Protein sequence (378 aa)

MGWSCIILFLVATATGVHASTKGPSVFPLAPSSKSTSGTGTAALGCLVKDYPFP E
VTVS
WNSGALTSGVHTFPALQSSGLYSLSSVVTVPSSSLGTQTYICNVNHPSNTKV
DKKV
EPKSCDKHTTCPPCAPELLGGPSVFLLPPPDKPDTLMLISRTPEVTCVVVDVSHE
DPEV
KFNWYVDGEVEVHNAKTTPREEQNYSTYRVSVSTLVHLQDWLNNGKEYKCKVSNK
ALP
APIEKTISAKGQPREPQVYTLPPSRDELTKNQVSLCLVKGFYPSPDIAWEWESNG
QPE
NNYKTTPVLDSDGSFFLYSKLTVDKSRWQQNVFSCSVMSHELHNHYTQKS
SLSP GKG

3.2.2.3. DNA sequence

GAATTC CCGCCGCCACCATGGGCTTGGTCCTGCATCATATTCTGTGTGGTGT
GGC
CACAGGCCACCCGGGTGCACTCTGCCTCTCTCACAAGGGCCCTAGCCTGCTTCCCT
CTG
GCTCCTAGCAGAACTCTAAGACGGGAGAAGACCCCTGCTGCTGTGCTGCCTG
GTC
AAGGATTACTTTCCGCAGCCCTGTGAACCGCTGTCTGCAATAGCGGAGCACTGA
CAT
CTGGGCGTGACACACATTCCAGCCGTGCAGCTGTACGTCTAGCAGGCCTGTAGTC
GAG
CAGCGTGGTCACAGTGCCAAGCTCTAGCCTGGGCACCCAGACCTACATCTG
CAAT
GTGAACCACAAGCCTAGCAACACCAAGGTGGACAAGAAGGTGGAACCCAAG
AGCT
GGCACAAAGACCCACACCTGTCCTGCTCTCGTCTCAGACTCGTCGCGGCG
GACC
TCCCGTGTTCCTGTCTCTCCAAAGCCGTAAGCAGACCCCTGATGATGAGAAA
CCC
CTGAAGTGACCTGCGTGGTGGTGGATGTGTCCCACGAGGATCCCGAAGTG
AGTT
3.2.3 Expression and purification of the peptibody

The Expi293F cell culture (1L total) was maintained in a 37°C incubator with ≥80% relative humidity, 8% CO₂ on an orbital shaker platform. One day before transfection the cells were seeded to a final density of 2.5 × 10⁶ viable cells/mL and allowed to grow overnight. On day of transfection the culture was diluted to 3 × 10⁶ viable cells/mL with fresh, pre-warmed Expi293™ Expression Medium. The transfection was performed using ExpiFectamine™ 293 Transfection Kit according to the manufacturer’s protocol. In short, peptibody plasmid DNA/Opti-MEM™ and ExpiFectamine™ 293/Opti-MEM™ mixtures were prepared separately and incubated at room temperature for 5 minutes. Diluted ExpiFectamine™ 293 Reagent was then added to the diluted DNA and the total complexation mixture
was incubated at room temperature for another 20 minutes and then slowly transferred to the cell culture. 18h post-transfection, ExpiFectamine™ 293 Transfection Enhancer 1 and ExpiFectamine™ 293 Transfection Enhancer 2 were added to the culture. The cell culture supernatant was collected on day 6 and prepared for purification. The peptibody was purified using protein A chromatography (HiTrap rProtein A FF, GE Healthcare) in a 5 ml column pre-equilibrated with PBS. The peptibody was eluted in 0.1 mM acetic acid by FLPC and immediately buffer-exchanged by dialysis in PBS.

3.2.4 Western Blot

The purified peptibody, or recombinant human Fc (Abcam, USA) were then separated by 4- 15% pre-stained SDS-PAGE Mini-PROTEIN stain free gel (BIO RAD, USA), transferred to a PVDF membrane (Merk Millipore), and then probed with biotinylated protein G, 1:10,000 (Thermofisher, USA), and HRP conjugated streptavidin,1:10,000 (Thermofisher, USA) and imaged with Biorad gel documenting system.
3.2.5 Quantification of the peptibody half-life

Mice received 5.5 µg in 50 µl PBS of either peptibody or recombinant human Fc via tail vein injection. Blood samples were taken by a submandibular bleed at 3 min, 1 h, 3 h, 6 h, 1 day, 3 days, and 7 days. Blood samples were held on ice until clotting occurred, then centrifuged at 13,000g for 30 minutes at 4°C, and stored at -20°C. Quantification of the peptibody or Fc in plasma was performed with the human IgG1 Fc ELISA Kit (Aviva Systems). The HRP ELISA assay was conducted as described by the manufacturer's manual and absorbance was read at 450 nm on a Synergy MX plate reader (BioTeK). Half-life was determined via fitting with a one phase decay in Prism 8 (GraphPad, USA).

3.2.6 Patch clamp

We used patch clamp to measure the effects of the peptibody, teriapinQ or Fc on the I_{K_{ACh}} current in Human Embryonic Kidney (HEK293) cells stably transfected with Kir3.1 and Kir3.4[446]. Currents were recorded using the Multiclamp 700B (Molecular Devices) amplifier an A/D converter (Digidata 1550B plus Hum Silencer, Molecular Devices), and the pClamp 10.6 PC software (Molecular Devices). The analysis was performed with Clampfit 10.6 (pClamp, Molecular Devices) and OriginPro 2018b software packages (OriginLab Corp). Patch pipettes were pulled from glass capillaries (Kimble Glass) and had a resistance of 2.5–3MΩ. After gigaohm seal formation, whole-cell recordings were performed at room temperature. In transfected HEK cells, as previously reported[447], the bath solution contained in mM 90 NaCl, 50 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 10 Glucose, and the pH adjusted to 7.4. The pipette internal...
solution contained in mM 100 K-aspartate, 10 NaCl, 40 KCl, 5 Mg-ATP, 2 EGTA, 0.1 GTP-Tris, and 10 HEPES at pH = 7.2. In HEK293 cells, $I_{KAC}$ whole-cell current was elicited by a holding potential at 0 mV for 150 ms followed by steps to -140 mV for 200 ms. Subsequently, the voltage was increased, as a 2-second ramp from –140 mV to +40 mV, followed by a return to 0 mV[447]. $I_{KAC}$ was taken as the 1mM BaCl$_2$ sensitive current. Dose-response curves and IC$_{50}$ for the block at -120 mV were determined using Prism 8 software (GraphPad, USA).

**3.2.7 In-vivo electrophysiological studies in mice**

For measuring the effects of the peptibody on the ECG of mice with carbachol injection, mice were anesthetized (1.8% isofluorane), and the exposed right jugular vein instrumented with a PE tube connected to a 250 µL Hamilton syringe for drug delivery. ECG leads were fixed subcutaneously in Lead II configuration and recorded via Animal Bio Amp (AD Instruments USA) and digitized via the PowerLab data acquisition system (AD Instruments, USA). LabChart Pro 7.2 software (AD Instruments) was used for acquisition and analysis of the ECG. Continuous ECG was recorded, and mice received an i.p. injection of Carbachol (300 µl of 200 µM). Five minutes after carbachol injection, the peptibody or the Fc fragment were delivered via the jugular vein. For atrial fibrillation inducibility, 30 minutes after peptibody or Fc fragment injection, intracardiac programmed electrical stimulation was performed[448] using a Millar 1.2 French octapolar catheter with an injection port (Millar, USA) placed through the exposed right jugular vein into the right atrium, as described in chapter two. Intracardiac electrograms, and ECG Lead II were simultaneously recorded using
the Advanced Instruments platform. Atrial programmed electrical stimulation was carried out with a stimulus isolator unit (AD Instruments, USA). AF inducibility was performed with 2.5 ms square pulses at twice diastolic threshold in animals injected with the Fc fragment or the peptibody. The protocol consisted of 2 seconds trains of burst pacing at 28 to 60 Hz in 2Hz increments until arrhythmia was induced.

3.3 Results

3.3.1 Design, expression and purification of the peptibody

We designed the $I_{K_{ACh}}$ blocking peptibody (Fc-TP) as a fusion protein between the human IgG1 Fc fragment (330 amino acids) and tertiapinQ (TP, 21 amino acids), separated by a linker composed of 8 glycines (Figure 3.1 A). TertiapinQ is a 21-amino acid synthetic peptidotoxin originally isolated from the European honeybee venom, and in the heart, it has been shown to be a specific blocker of $I_{K_{ACh}}$. The N terminus of the peptibody contained an artificial leading sequence composed of 19 amino acids, that drives secretion of the peptibody. This configuration of the peptibody does not interfere with the C terminus of tertiapin-Q, since it has been suggested to be important for $I_{K_{ACh}}$ block[449]. The DNA sequence of the construct synthesized and cloned into pcDNA3.1. The plasmid construct was then used to transfect HEK293 cells. Since the peptibody contained an N terminal leading sequence, the secreted peptibody was subsequently purified from the media via a mono-affinity A resin column. Figure 3.1 B shows a SDS- PAGE gel (left panel) and corresponding western blot (right) of increasing amounts of peptibody and of Fc, under reducing conditions. The
protein gel, and the blot show the peptibody (Fc-TP) and recombinant Fc at about 40 KDa. We then calculated the half-life (t1/2) of the peptibody, and of Fc, injected via tail vein (5.5µg in 50 µl PBS) in mice using an ELISA based human Fc quantification assay (Figure 3.1C). The peptibody half-life and that of Fc were similar (7.6 hours and 7.8 hours respectively), which is in line with the previously measured human recombinant Fc half-life in mice[450].

Figure 3.1. Peptibody design and production. A: Cartoon of the peptibody. An artificial leader sequence (green) is at the N terminus, in order to ensure the secretion of the peptibody. Grey is the Fc fragment of the human IgG1, composed of 330 amino acids followed by an octaglycine spacer, then by the 21 amino acids tertiapin-Q. The peptibody is a dimer. Dimerization is via disulfide bonds formation (SS). B: Protein SDS-PAGE gel (left), and its corresponding western blot (right) of increasing amounts of the peptibody (Fc-TP, 0.2, 0.4, and 1 µg), and 0.8 µg recombinant Fc control. C: Pharmacokinetics analysis of the peptibody.

### 3.3.2 Effect on \( I_{KAC} \) current

We tested in patch-clamp experiments, whether the peptibody inhibits \( I_{KAC} \) (Figure 3.2). Barium-sensitive \( I_{KAC} \) currents were recorded in HEK293 cells stably transfected with Kir3.1/3.4 at 50 mM extracellular \( K^+ \), in response to a 2-second ramp from -140 to +40 mV, from a holding potential of 0mV[446, 447]. These cells overexpressing Kir3.1/3.4 have been shown to display a basal, non-stimulated \( I_{KAC} \) current[446]. At 100 pM, while Fc (panel A) and tertiapinQ (TPQ, Panel B)
had minimal effects on $I_{\text{KAC}}$, the peptibody blocked the current significantly (Fc-TP, Panel C). The concentration-response curves of current block at -120 mV revealed that the peptibody was over 350 times more potent than tertiapinQ. The $IC_{50}$ of the peptibody was 24.9 pM, versus 9.06 nM for tertiapinQ, and the the Fc fragment did not block the current.

Figure 3.2. Block of $I_{\text{KAC}}$ by the peptibody in HEK293 cells stably transfected with Kir3.1 and Kir3.4. The current was elicited by a 2-second ramp from -140 mV to 40 mV. A: IV relationship of $I_{\text{KAC}}$ in a cell at baseline (Ctr), and after addition of 100 pM human IgG1 Fc fragment. B: IV relationship of $I_{\text{KAC}}$ in a cell at baseline (Ctr), and after addition of 100 pM tertiapinQ (TP). C: I-V relationship of $I_{\text{KAC}}$ in a cell at baseline (Ctr), and after addition of 100 pM peptibody (Fc-TP). D: Concentration-response curve of $I_{\text{KAC}}$ block by the Fc fragment, tertiapinQ (TP), and peptibody (Fc-TP). The number of cells at each tested dose is shown. The $IC_{50}$ for tertiapinQ block was 9.06 nM, and that for the peptibody was 24.9 pM. (Experiment was done with the help of Dr. Bojji Babu Chidipi).
3.3.3 In vivo effects

Subsequently, we investigated in mice whether the peptibody displayed cardiac bioactivity by quantifying its effects on heart rate in anesthetized C57BL/6J mice (1.5% isoflurane), with continuous ECG monitoring. Carbachol (300 µL of 200 µM) administered i.p., caused a significant slowing down of heart rate due to I_{K_{ACh}} activation[346] (Figure 3.3 panel A, first column: ECG at baseline control, middle column: after carbachol). The peptibody, Fc-TP, or the Fc fragment were then delivered via jugular vein injection (5.5 µg in 50 µl PBS). At 7 minutes after delivery, Fc-TP reversed the bradycardic effects of carbachol, while at the same dose Fc had a significantly less pronounced effect (Panel A, right column). Panel B is a compilation of 6 mice injected with Fc, and 5 with the peptibody. 5, 7- and 20-minutes following administration, the peptibody significantly blunted the RR prolonging effects of carbachol versus Fc. There were no statistically significant differences in PR, QRS, and QTc intervals between the peptibody and Fc.
3.3.4 Antiarrhythmic activity in old mice

We investigated whether the peptibody displayed anti AF activity in old mice. The animals were assessed for AF inducibility using in-vivo programmed electrical stimulation after jugular vein injection of the peptibody or the Fc fragment (0.1375µg/g in 50 µL PBS). The experiment was done using 1.2 French octapolar catheter with an injection port (Millar, USA) placed through the exposed right jugular vein into the right atrium. Intracardiac electrograms, and ECG Lead II were simultaneously recorded with the Advanced Instruments platform. Right atrial pacing was performed with 2.5 ms square pulses at twice diastolic threshold in animals injected with the Fc fragment or the peptibody. The protocol consisted of 2-seconds trains of burst pacing at 28 to 60 Hz in 2Hz increments. Figure 3.4 A
shows that the weight and age of peptibody injected mice were not different from those of Fc injected animals. In figure 3.4 B, the top panel displays ECG (top) and intracardiac electrogram (bottom) tracings showing that 2-second burst pacing induced AF in an Fc-injected mouse, but not in a peptibody-injected mouse (bottom panel). Panel C shows that AF was inducible in 100% (5 out of 5) of animals that received Fc, while 20% (1 out of 5) of animals treated with the peptibody were inducible for AF (p<0.05, Fisher’s exact test). The peptibody reduced AF inducibility in aged mice, likely via blocking constitutively active I_{KCa}.

Figure 3.4. Antiarrhythmic effects of the peptibody in aged mice. A: Weight and age of mice that received Fc or FcTpQ. B: Top panel: ECG (top trace) and intracardiac electrogram (bottom trace) in a mouse after administration of Fc. Two-second burst pacing induced AF. Bottom panel: ECG (top trace) and intracardiac electrogram (bottom trace) in a mouse after administration of FcTpQ. burst pacing did not induce AF. Inducibility of AF in 5 Fc, and in 5 peptibody mice. *p< 0.05, Fisher’s exact test.
3.4 Discussion

Our work demonstrates that bioengineered anti-ion channel peptibodies can be powerful and produce highly potent ion channels blockers. Peptibodies are new, safe and flexible alternatives to therapeutic antibodies[435, 436]. The peptibody we engineered to block $I_{KAC}$, was designed as a fusion protein between tertiapinQ and the Fc fragment of human IgG1, linked by an octaglycine spacer. The DNA sequence of the peptibody construct was based on that of Romiplostim, a currently used peptibody for the treatment of immune thrombocytopenic purpura[437-440]. The $I_{KAC}$ blocking peptibody was more potent than tertiapinQ and showed in-vivo and in-vitro block of $I_{KAC}$. In patch-clamp experiments, the peptibody was about 350-fold a more potent blocker of $I_{KAC}$ compared to tertiapinQ. In mice where $I_{KAC}$ activation was via i.p. injection of carbachol, the peptibody abrogated the bradycardic consequences of $I_{KAC}$ activation. Moreover, the peptibody decreased the inducibility of AF in old mice.

Peptibodies are stable and safe molecules that are shown to be viable therapeutics. There are two peptibodies in current clinical use: Romiplostim for immunethrombocytopenic purpura, and Dulaglutide, for glucose control in Type 2 diabetes[451,452]. This is in addition to other therapeutic peptibodies in different stages of development[435]. The peptibody Romiplostim is composed of thrombopoietin mimetic peptides fused to the C-terminus of the Fc fragment of human IgG[435-441]. Dulaglutide is a fusion protein consisting of glucagon-like peptide-1 receptor agonist and Fc[451, 452]. Such protein chimeras of a biologically active peptide and the Fc domain of IgG are thought to have an
extended half-life compared to the biologically active peptide, due to the neonatal Fc receptor salvage pathway involved in protecting IgG from degradation, and decreased renal clearance rate because of the larger size of the peptibody compared to that of the biologically active peptide[435, 436]. Peptibodies may offer an increased avidity to the target compared to the biologically active peptide alone. For instance, in the I_{K_ACh} blocking peptibody (Figure 1) the homodimerization of the two Fc moieties results in two tertiapinQ “warheads” per peptibody, and the polyglycine linker which provides flexibility could also result in an increased avidity. Interestingly, and although not yet clear why, certain peptides seem to be more active when fused to the carboxy terminus of the Fc, however, in the case of the anti-I_{K_ACh} peptibody, having the C terminus of tertiapinQ free is desirable since the C terminus is suggested to be important for the channel blocking activity of the peptidotoxin.

Currently, there are no bioactive peptibodies that could potentially be atrial selective antiarrhythmics, in the setting of atrial fibrillation. In some forms of AF, I_{K_ACh} is thought to play a role in the perpetuation of this arrhythmia[303, 434, 444]. We and others have shown that blocking I_{K_ACh} with tertiapinQ or with the small molecule chloroquine, in animals[338, 453] and patients[447, 453-456], could modulate AF. Therefore, targeting this atrial specific current with new tactics that rely on chimeric proteins of Fc and peptide blockers might offer new avenues for pharmacotherapy in atrial fibrillation.

In summary, we demonstrate that engineered peptibodies can be potent, and bioactive ion channel blockers. Our peptibody blocks I_{K_ACh} in vivo and in vitro
and can reduce the inducibility of atrial fibrillation. Given that ionic currents such as
$I_{\text{KAC}_{\text{h}}}$ could play a role in the perpetuation of arrhythmias including atrial fibrillation,
new approaches for antiarrhythmic "biobetter" therapies that rely on anti-ion
channels peptibody engineering warrant consideration.
Chapter Four: Summary and Perspective

Despite the fact that the projected rise in AF incidence is approaching epidemic proportions[202, 457] and despite more than 100 years of basic and clinical research on AF, we still do not fully understand the mechanisms of this arrhythmia, and therefore, pharmacological and ablative treatments are suboptimal and their improvement is urgently needed.

Aging of the atria results in a complex series of pathophysiological events involving a large number of significant players. In chapter 2, my study directly addressed the role of a specific and novel pathway that could be important for aging-mediated AF, where I showed that aging leads to constitutively active $I_{K_A Ch}$ via a PKCε-dependent mechanism. Based on my results and those of others in chronic AF[302, 303, 338, 458], I propose that blocking atrial specific $I_{K_A Ch}$ could work as target for AF treatment in the subset of patients where $I_{K_A Ch}$ might be constitutively active. Since the present therapeutic approaches to AF are suboptimal, in chapter 3, I continued to investigate novel therapeutic approaches to AF. This novel approach relies on engineering, and charactering a peptibody, which is a fusion protein between the human IgG1 Fc fragment and tertiapinQ as a blocker to $I_{K_A Ch}$. My proposed study implied that this peptibody is antiarrhythmic in aged mice by blocking $I_{K_A Ch}$ in vivo and in vitro. Therefore, targeting to the atrial specific $I_{K_A Ch}$ current via chimeric proteins of Fc and a peptide blocker could open
new avenues for AF therapy.

Based on my studies which I presented above, several areas of investigations may be addressed in the future in order to further our understanding of atrial fibrillation in the aged heart. A few of the most prominent areas that warrant further investigations are:

1. **The effect of PKCα isoforms on $I_{K\text{ACh}}$ current in aging-mediated AF**

   Studies have suggested that PKC isoforms differently modulate $I_{K\text{ACh}}$. Atrial tachycardiac remodeling causes a rate-dependent PKC isoform switch, with downregulation of inhibitory PKCα and membrane translocation of stimulatory PKCε, enhancing $I_{K\text{ACh}}$[355]. Currently, no studies are investigating in detail the potential mechanisms of $I_{K\text{ACh}}$ regulation via PKCα. The same methodological approaches as in chapter 2 could be utilized. Additionally, delineating the possible phosphorylation sites of PKCε and PKCα on Kir3.1 and Kir3.4 are needed in order to better understand how different PKC isoforms modulate differently $I_{K\text{ACh}}$. Important information can thus be obtained in order to design better blockers that target constitutively active $I_{K\text{ACh}}$.

2. **The role of other ionic currents in aging-mediated AF**

   Studies show that ROS could also activate ATP-sensitive inward rectifier potassium current ($I_{K\text{ATP}}$)[459, 460]. $I_{K\text{ATP}}$ is another inward rectifier potassium current involved in AP repolarization and can mediate APD shortening. Glibenclamide, a well-known $I_{K\text{ATP}}$ blocker[461], and Kir6.2 knockout mice could be used to probe the contribution of $I_{K\text{ATP}}$ in aging-
mediated AF[462]. Additionally, oxidative stress could also lead to phosphorylation mediated decrease of LTCC[463]. Electrophysiological studies revealed that LTCC plays a crucial role in the electrical remodeling of AF[272, 464], where there is a reduction of LTCC[465], contributing to APD shortening and possibly to AF initiation and perpetuation. Therefore, the role of LTCC in aging-mediated AF could also be studied.

3. The balance between phosphorylation and dephosphorylation of $I_{KACH}$ in AF

It is possible that the constitutively active $I_{KACH}$ is not due to the PKCε activation and its subsequent translocation to the membrane but due to the downregulated expression or enzymatic activity of protein phosphatase 1 (PP1) or protein phosphatase 2A (PP2A), or due to the decreased activity of the phosphatase inhibitor protein in the atria. Studies in experimental AF models also indicate that altered phosphatases regulation contribute to the pathophysiology of AF[466-469]. Additionally, the phosphorylation levels of protein phosphatase 1 regulatory subunit 1A (PPP1R1A), a potent PP1 inhibitor, are suggested to be strongly increased (~10 fold) in chronic AF patients[470], likely resulting in local reductions in PP1 activity. There are also up to date studies suggesting that the global activity of PP1/PP2A is increased in AF patients[470-472]. All these factors indicated that phosphatases may play an important role in AF pathology. Therefore, the roles of PP1 and PP2A in regulating ion channels in aging mediate AF would be exciting to pursue.
4. **Limitations of AF animal models**

The mouse as a model of AF has many limitations[473], including the limited availability of relevant mouse models with spontaneous, persistent AF. Therefore, the studies of chapter 3, regarding the antiarrhythmic potential of the $I_{K_{ACh}}$ blocking peptibody need to be also performed in a more relevant animal model of persistent AF. Sheep, and pig models of tachypacing induced persistent AF have been characterized and consistently used as acceptable models of chronic AF, and thus such models would be very useful in corroborating the mouse findings of chapter 3. As I have discussed in previous chapters, cardiac K+ channels are critical for normal cardiac electrical activity and are critical for modulating the shape and duration of the action potential. The differences in action potential shape between atria and ventricles are mainly due to the atrial-selective expression of K+ channels. Such differences may provide potential targets for atrial-selective antiarrhythmic therapy[474-478]. For instance, increased K+ currents have been shown to contribute to APD shortening in AF and to stabilization of reentrant excitation[245, 298]. The $I_{K_{ACh}}$ and the ultrarapid delayed rectifier potassium current ($I_{K_{ur}}$) [479], are largely exclusive to the atria. Block of $I_{K_{ACh}}$ and $I_{K_{ur}}$ largely affects atrial electrophysiological parameters, which makes these currents a potential target for chamber specific management of atrial arrhythmias[339, 480]. However, studies on the antiarrhythmic effects of blocking $I_{K_{ur}}$ in AF are not consistent. In atrial preparations from sinus rhythm patients, pharmacologic
inhibition of \( I_{Kur} \) elevates the AP plateau but causes abbreviation the APD\[481\], which may facilitate maintenance of reentry excitations\[482, 483\]. Whereas APD shortening may not be a direct consequence of \( I_{Kur} \) block, because inhibition of repolarization outward \( K^+ \) currents is expected to prolong APD. It is thought that the pronounced elevation of the AP plateau results in a stronger activation of other repolarizing \( K^+ \) currents, such as \( I_{Kr} \) and \( I_{Ks} \), thereby shortening the AP\[481, 484, 485\]. In contrast, the usefulness of \( I_{Kur} \) inhibition chronic AF patients has been questioned since it is suggested that \( I_{Kur} \) is in fact downregulated in chronic AF patients, which could limit the effect of \( I_{Kur} \) inhibition in chronic\[486\].

Our lab demonstrated earlier that the small molecule chloroquine reduces the burden of persistent AF and restores sinus rhythm in animal models and in a patient with persistent AF, in part through blocking \( I_{KAC} \)\[447, 453\]. However, like with the majority of antiarrhythmic small molecules, chloroquine is not a specific blocker of \( I_{KAC} \), has a narrow therapeutic window, and can be associated with serious toxicities that include prolongation of the QT interval due to \( I_{Kr} \) blockade\[487\]. NTC-801\[488\], a benzopyrene derivate, and AZD2927\[489\], a benzamide related compound were developed as selective inhibitor of \( I_{KAC} \), which do not block \( I_{Kr} \) and thus do not prolong the QT interval. However, these drugs failed to cardiovert paroxysmal AF and atrial flutter in patients. The results are not surprising given the fact that \( I_{KAC} \) is not constitutively active in patients with paroxysmal AF\[400\], let alone atrial flutter whose mechanism differs from AF\[490\].
It is thus clear from such studies, that ion channel manipulation as a therapy in chronic AF has not been very successful, thus limiting breakthroughs in the development of novel anti-AF pharmacotherapies. Therefore, the treatment of AF remains inadequate, in particular, as the arrhythmia becomes in the chronic stages. Spontaneous, pharmacological or ablative resumption of normal cardiac rhythm in chronic AF stages is infrequent. Nevertheless, antiarrhythmic drug therapy, although suboptimal and marred with deleterious side effects that include QT prolongation and ventricular proarrhythmia, remains the first clinical line of defense.

There is an urgent need to develop a new generation of therapies geared towards effectively preventing chronic AF without affecting ventricular electrophysiology. This has been hampered by an incomplete understanding of the mechanism of chronic AF and how comorbidities such as aging, and how gender modulate this arrhythmia. This is in addition to the lack of therapeutic investigations and clinical trials that are mechanistically driven, and that target pathways that are remodeled in this disease and geared toward chronic AF. With the advances that are being made in the study of this arrhythmia at the genetic, molecular, cellular and organ levels, and with the increasingly interdisciplinary collaborations that are taking place in this field, I hope that the findings of my thesis will serve to advance the mechanistic knowledge of this arrhythmia and help in the development of novel, safe, and effective therapeutics.
References


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