Universidade Católica Portuguesa
Faculdade de Engenharia

Drug side effects on $K_{IR}2.1$ and hERG cardiac channels

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Dissertação para obtenção do Grau de Mestre em

Engenharia Biomédica

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RESUMO

A busca de mais conhecimento sobre a electrofisiologia cardíaca, incluindo o potencial de acção cardíaco e o papel de todos os canais iónicos envolvidos na polarização/despolarização da membrana do cardiomiócito, tem sido uma constante por parte dos investigadores porque nela assenta a explicação cabal dos quadros clínicos de arritmia e o desenvolvimento de novas terapias farmacológicas para patologias relacionadas.

Dois dos canais iónicos com maior relevância neste contexto são o canal de potássio rectificador de entrada $K_{IR}2.1$ e o dependente da voltagem hERG, que parecem contribuir de forma decisiva para a fase terminal da repolarização do potencial de acção (fase 3) e para a estabilização do potencial de repouso da membrana do cardiomiócito através das suas correntes de rectificação de entrada I_{K1} e de rectificação rápida tardia I_{Kr}, respectivamente. Adicionalmente, as patologias inerentes a estes canais podem surgir por problemas de tráfico intracelular, causados, não só por mutações genéticas, mas também por interacções com fármacos particulares.

Concretamente, os efeitos colaterais no sistema cardiovascular dos medicamentos antimicrobianos pentamidina e seu análogo 2EVK e antidepressivo Fluoxetina (Prozac), podem estar relacionados com uma interação sobre o $K_{IR}2.1$ e hERG, especialmente em situações de *overdose* (efeitos agudos). No entanto, pouco se sabe ainda sobre os efeitos destes fármacos nos canais $K_{IR}2.1$ e hERG. Neste estudo, foram analisados, entre outros, os efeitos crónicos da administração de pentamidina, 2EVK e fluoxetina em células HEK-KWGF e HEK-HERG. Ao contrário do esperado, a pentamidina e o 2EVK apresentaram efeitos totalmente diferentes nos ensaios realizados.

A pentamidine e a fluoxetina revelaram-se fortes inibidores da maturação do hERG enquanto o 2EVK não apresentou qualquer efeito. Por outro lado, no que diz respeito ao $K_{IR}2.1$ e após 24h de incubação, o 2EVK e a fluoxetina aumentaram (1,52 ± 0,29 e 1,54 ± 0,16, respectivamente) e a pentamidina diminuiu (0,90 ± 0,20) os níveis da proteína total. Durante este período, o $K_{IR}2.1$ mantém-se na membrana celular e/ou disperso por todo o citoplasma. Curiosamente, ao fim de 48h na presença de fluoxetina é pela primeira vez relatado uma intensa acumulação polarizada deste canal à volta do núcleo, sugerindo, provavelmente, o envolvimento do retículo endoplasmático ou aparelho de Golgi. Apesar dos efeitos destas drogas sobre o $K_{IR}2.1$ e hERG sugerirem uma explicação para os seus efeitos colaterais, não se pode neste momento excluir o envolvimento de outros tipos de receptores e canais iónicos.

Palavras-chave: Canais $K_{IR}2.1$, canais hERG, corrente de rectificação de entrada (I_{K1}), corrente de rectificação rápida tardia I_{Kr}, pentamidine, 2EVK, fluoxetina

ABSTRACT

The correct understanding of cardiac electrophysiology, action potential and behavior of all ionic channels involved in cardiomyocyte membrane polarization/depolarization has become a *Holy Grail* to reach a full explanation of cardiac arrhythmias and development of new pharmacological therapies.

In this context, there are two most relevant ionic channels: the inward rectifier potassium channel $K_{IR}2.1$ and the voltage-dependent hERG, which appear to contribute assertively to the terminal phase of repolarization of the action potential (phase 3) and for the stabilization of the resting membrane potential of cardiomyocytes through its currents – inward rectifier (I_{K1}) and rapid delayed rectifier (I_{Kr}), respectively. Additionally, $K_{IR}2.1$ and hERG inherent pathologies can arise by intracellular traffic problems, caused not only by genetic mutations, but also by interactions with particular drugs.

Actually, antimicrobial or antidepressant drug side effects on the cardiovascular system may be related with interactions on $K_{IR}2.1$ and/or hERG, especially in overdose situations (acute effects). However, little is yet known about these mechanisms of interference. In this study we examined, among others, the effects of chronic administration of pentamidine (antimicrobial), 2EVK (pentamidine analog) and fluoxetine ("Prozac", antidepressant) in HEK-KWGF and HEK-HERG cells. Unexpectedly, pentamidine and 2EVK revealed to have entirely different actions at cellular level.

Pentamidine and fluoxetine proved to be strong inhibitors of hERG maturation, while 2EVK had no effect on this process. On the other hand, $K_{IR}2.1$ protein levels increased with 2EVK and fluoxetine (1.52 ± 0.29 and 1.54 ± 0.16, respectively) and decreased with pentamidine (0.90 ± 0.20) after a 24-hour incubation. During this period and for all drugs, $K_{IR}2.1$ remains at the cell membrane and/or dispersed throughout the cytoplasm.

Interestingly, after 48h in the presence of fluoxetine, a strong polarized accumulation of $K_{IR}2.1$ around the nucleus was reported for the first time, suggesting perhaps the involvement of endoplasmic reticulum or Golgi apparatus. Despite these effects on $K_{IR}2.1$ and hERG may explain, in part, their side effects, the involvement of other receptors and ion channels, cannot be, currently, excluded

Keywords: $K_{IR}2.1$ channels, hERG channels, inward rectifier current (I_{K1}), rapid delayed rectifying current (I_{Kr}), pentamidine, 2EVK, fluoxetine

”When you make a mistake, don’t look back at it long. Take the reason of the thing into your mind and then look forward. Mistakes are lessons of wisdom. The past cannot be changed. The future is yet in your power.”

Hugh White (1773-1840)

ACKNOWLEDGEMENTS

The very meaning of our career choice lies in the fact that, despite the wide range of possibilities, we chose an activity that, ultimately, is geared towards the humanization of man and it's potential regarding a better and fraternal life. This research was only possible to achieve with the help, commitment and dedication of many people.

Firstly I would like to thank the teachers and counselors Marcel van der Heyden and Pedro Simões, the hours spent in meetings, discussing and cooperating in a good routing of this work and for believing that I could always do better.

I also thank to Rosanne Varkevisser, Marien Hotman and Dr. Martin Rook for all the help they gave me in the introduction techniques and for all the support and assistance available continuously ever they had for me.

I want to make a special thanks to Universitair Medisch Centrum Utrecht, for having given me the practical skills so far only theoretical knowledge (most of it) and receiving me warmly for my master's thesis conclusion.

Special thanks for Universidade Católica Portuguesa, its director Manuel Barata Marques, my engineering coordinator Cecília Calado and all the teachers who have crossed my journey and contributed to enhancing my knowledge in various areas.

Also a special thanks to my family for support, pride, trust, care and understanding shown throughout this journey, and for always believing that the result of my commitment, hard work and dedication can achieve mine objectives, and without which no moment of my live would have been pointless.

To my friends, the unforgettable moments of fellowship and sharing - "And if more than receive the joy is in giving, why I do not want to be happy all day?". It is a pleasure, an increase, a rebirth, are a joy ... smiles. Friends who with their presence, have contributed to my personal growth, and that provided, with their advice and friendship, a passage more enjoyable by this stage of my live - "Do not be traded for anything in this life."

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ABBREVIATIONS

AF: Atrial fibrillation

APD: Action potential duration

ATS: Andersen-Tawil syndrome

AV node: Atrioventricular node

CAF: Chronic atrial fibrillation

Ca_{IN}: Calcium intracellular

Ca_v: Voltage-gated Ca^+ channels

COPII: Specific coat protein complex

CPVT: Catecholaminergic polymorphic ventricular tachycardia

DAD: Delayed afterdepolarization

EAD: Early afterdepolarization

EKG: Electrocardiogram

E_K: Equilibrium or reversal potential for potassium

ER: Endoplasmic reticulum

ERP: Effective refractory period

F: Fluoxetine

FAF: Familiar atrial fibrillation

HEK293: Human embryonic kidney 293 cells

hERG: human ether-a-go-go–related gene

HF: Heart failure

HSP90: Heat Shock Protein 90

I/V: Current/voltage

IC_{50}: Half maximal inhibitory concentration

$I_{Ca,L}$: inward L-type Ca^{2+} current

I_{K1}: Inwardly rectifier potassium current

I_{Kr}: Rapid potassium current

I_{Ks}: Slow potassium current

I_{Kur}: Ultra rapid potassium current

I_{Na}: Inward Na^+ current

I_{to}: Transient outward K^+ current

K_{Ach}: Muscarinic-gated atrial potassium channel

K_{ATP}: Adenosine triphosphate sensitive potassium channel

K_{csA}: *Streptomyces lividans* potassium channel

K_{IR}: Inward rectifier potassium channel

K_{OUT}: Extracellular Potassium

K_V: Voltage-gated potassium channels

LD_{50}: Amount of a drug causing the death of 50% of a group of test animals

LQTS: Long QT syndrome

Na_V channels: Voltage-gated sodium channels

PDZ: Combination of the first letters of three proteins – **P**ost synaptic density protein (PSD95), **D**rosophila disc large tumor suppressor (DlgA), and **Z**onula occludens-1 protein (zo-1)

P: Pentamidine

PA: Pentamidine analog (e.g., 2EVK)

PIP_2: Phosphatidylinositol-4,5-biphosphate

SA node: Sinoatrial node

SQTS: Short QT syndrome

SSRI: Selective serotonin reuptake inhibitor

TM: Transmembrane

I. INTRODUCTION

Differences in heart rate and heart dimensions are huge among species. Moreover, even within the same species the shape and duration of action potentials will differ according to its life cycle (for instance, babies have a higher heartbeat than adults).

Cardiac action potential is, as other action potentials, initiated by a depolarization and finalized by a repolarization phase. It is, however, unique in shape due to a prolonged plateau phase lasting several hundred milliseconds. The exact shape and duration is governed by a subtle interplay between different cardiac ion channels. An ion channel is a transmembrane protein complex that spans the entire lipid bilayer through which specific inorganic ions can diffuse, passively, down their electrochemical gradients. These channels allow specific ions to pass through them, limiting the rate of passage through the selectivity filter, the narrowest part of the channel. Ions channels are not continuously open because they are gated, changing between an open and a close state. Ions channels together with exchangers and ion pumps are responsible for the permeability of the cell membrane to specific ions, and for the regulation of both the membrane potential and the cytosolic concentration of ions (Widmaier *et al.*, 2006).

The initial fast depolarization in cardiomyocytes is obtained by opening of voltage-dependent Na^+ channels giving rise to a subsequent opening of voltage-gated Ca^{2+} channels. This depolarization, seen as a result of the inward movement of Na^+ and Ca^{2+} is counterbalanced by two events, the time-dependent inactivation of these channels and the opening of transient outward conducting K^+ channels.

The regulation of cardiac ion channels involves more than just modulation of their synthesis and kinetics, as control on their trafficking and localization is also important. Among those, there are the inward rectifier K^+ (K_{IR}) channels and the human *ether-à-go-go-related gene* (hERG), famous by their important roles in the maintenance and control of cardiac cell excitability and recently used as clinical targets of antifibrillatory-drugs. However, although the body of knowledge about these channels is fairly large, their trafficking pathways are still waiting to be understood.

After knowing the mechanism of intracellular trafficking and modulation of the activity of these channels as well as its complex network of protein-protein interactions, multiple possibilities of designing specific drugs to fight arrythmogenic events will be opened.

Moreover, these potassium channels interactions with cardiac non-specific drugs is also important for understanding their complex heart-related side effects.

II. THEORETICAL COMPONENT

The cardiovascular system consists in two circulatory systems, the systemic and pulmonary circulations and the heart. The heart pumps blood through the "high pressure" – systemic circulation – to deliver blood to individual organs, matching the supply to metabolic demand, and through the "low pressure" – pulmonary circulation – in which gas exchange occurs. Blood pressure and flow are largely controlled by the autonomic nervous system.

The heart comprises four chambers (two atria and two ventricles) divided into two sides, where oxygenated and deoxygenated bloods may circulate without mixing. The atria act as reservoirs for incoming blood, with some pumping action to assist ventricular filling. In contrast, the ventricles are the major pumping chambers, delivering blood to the pulmonary (right ventricle) and systemic (left ventricle) circulations. The left ventricle is conical in shape and has to generate greater pressures than the right ventricle, and so has a much thicker and more muscular wall. Four valves ensure that blood flows only in one way, from atria to ventricles (tricuspid and mitral valves), and then to the arterial circulations (pulmonary and aortic valves). The myocardium consists of working muscle, pacemaker and conducting cells, which have specialized functions.

Since they are different, they also have different characteristics. The first group makes up the muscular walls of atria and ventricles (forms the muscular layers) and possesses specific properties, such as extensibility and contractility, *i.e.*, the ability of the cell to shorten and lengthen its fibers. The second group, the pacemaker cells, generate and conduct spontaneously electrical impulses, cannot contract and their properties are conductivity, excitability and automaticity. The last group, conducting cells are specialized in electrical conductivity.

Taken together, all of these entities work to ensure efficient and rapid movement of highly oxygenated blood to the organs of the body. This has helped in thermal regulation and in rapid, sustained muscle movements (Widmaier *et al*., 2006).

II.1. Cardiac Electrophysiology

The mammalian heart functions as a mechanical dual pump in which the left and right sides of the heart pump blood separately, but simultaneously, into the systemic and pulmonary circuits. This maintains adequate transport of nutrients, removal of waste products, exchange of gases, and circulation of hormones and antibodies, in a constant attempt to fulfill the requisite of

the body. Efficient pumping of blood requires that the atria contract first, followed by almost immediately by the ventricles.

Contraction of cardiac muscle as well as smooth and skeletal muscles, is triggered by a change in voltage (depolarization) of the sarcoplasma membrane, which leads to an action potential. Although contraction may happen spontaneously (ectopic events), it happens normally in response to an electrical impulse. The action potentials spread from one cell to another through gap junctions that connect myocardial cells. Thus, the initial excitation of one cardiac cell eventually results in the excitation of all cardiac cells. This initial depolarization normally arises in a small group of conducting cells, the sino-atrial (SA) node, located in the right atrium near the entrance of the superior vena cava. The action potential then spreads from the SA node throughout the atria, then to the atrioventricular node (AV node), located in the septal wall of the right atrium, and passes down the His bundle branches and Purkinje fibers into the ventricular myocardium, causing almost simultaneous depolarization of both ventricles, approximately 0.2 seconds after the initial impulse has arisen in the SA node – figure 2.1.1. (Grunnet, 2010).

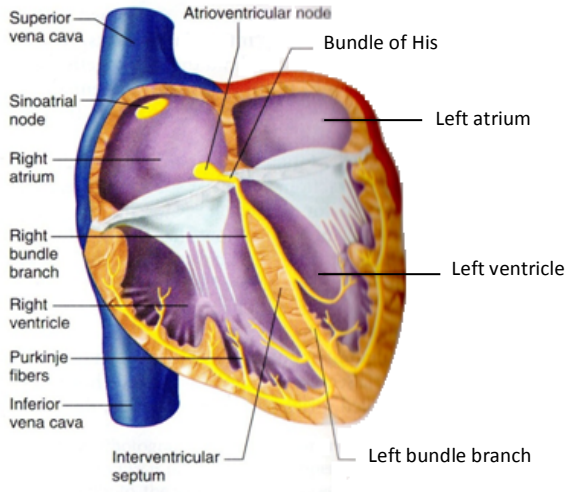

Figure 2.1.1. Conducting system of the heart, in yellow (adapted from Widmaier *et al*., 2006).

Depolarization of the myocardial cell membrane causes a large increase in the concentration of Ca^{2+} within the cell, which in turn causes contraction by inducing a temporary binding between two proteins, actin and myosin. The cardiac action potential is much longer than that of skeletal muscle, and during this time the myocardial cell is unresponsive to further excitation (the refractory period). Proper contraction and thereby pumping of the heart is secured and controlled by electrical signals recognized as cardiac impulses or cardiac action potentials (Grunnet, 2010; Persson, 2007; Widmaier *et al*., 2006).

II.2. The electrocardiogram (EKG)

The action potentials of cardiac muscle cells can be viewed as batteries that cause charge to move throughout the body fluids. These action potentials occurring simultaneously in many individual myocardial cells can be detected by recording electrodes at the surface of the skin. The EKG measures changes in skin electrical voltage/potential caused by electrical currents generated by the myocardium.

The first deflection, the P wave, corresponds to current flow during atrial depolarization. The isoelectric segment between the end of the P wave and the start of the QRS complex reflects the propagation of the electrical impulse through the AV node. The second deflection, the QRS complex, occurring approximately 0.15 sec later, is the result of ventricular depolarization. It is a complex deflection because the paths taken by the wave of depolarization through the thick ventricular walls differ from instant to instant, and the currents generated in the body fluids change direction accordingly. The ST segment and the T wave correspond to the plateau of the ventricular action potential where all ventricular cells are depolarized and to ventricular repolarization, respectively. The duration from the peak to the end of the T wave has been suggested to represent the transmural dispersion of repolarization in the ventricle. Additionally the interval between the start of the QRS complex and the end of the T wave, the QT interval, represents the time from initial depolarization to final repolarization of the ventricle. Repolarization is a process that occurs in cardiomyocytes when the electrical potential across the cell membrane during the action potential returns to that value of the resting state, reaching the resting potential. Although the EKG shows heart rate and rhythm and can indicate myocardial damage, it gives no information on the adequacy of contraction. Curiously, normal electrical complexes can exist in the absence of cardiac pulse, a state known as pulseless electrical activity.

In summary, the EKG is not a direct record of the changes in membrane potential across individual cardiac muscle cells. Instead, it is a measure of the currents generated in the extracellular fluid by the changes occurring simultaneously in many cardiac cells (Rourke *et al.*, 2002; Carmeliet *et al*., 1999).

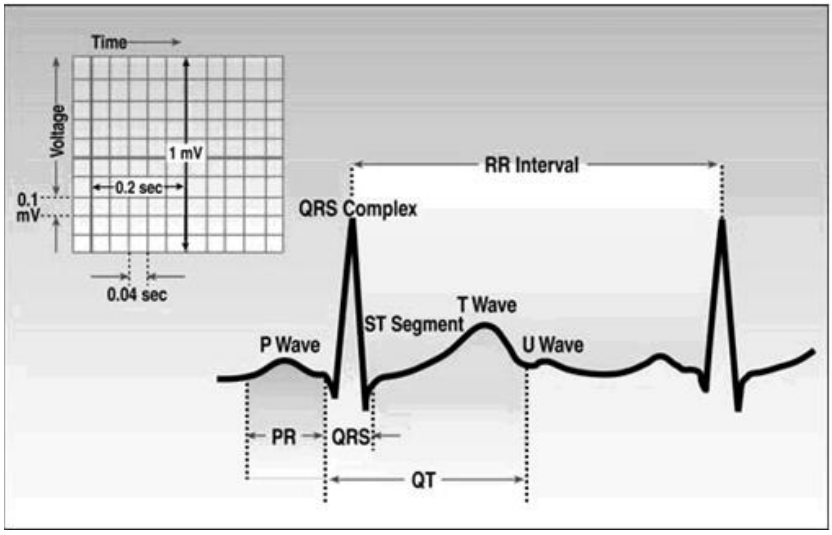

Figure 2.2.1. Segment of a typical electrocardiogram. It can be identified all the waves, intervals, segments and also the scale of voltage-time (adapted from Rourke *et al*., 2002).

II.3. The cardiac action potential

Action potentials are generated by the flow of ions through selective ion channels located in the cell membrane. They consist in large alterations in the membrane potential, from approximately -70 mV (resting potential) to +30 mV that will then repolarize to the former value again. Each channel can open and close, allowing the passage of a particular type of ion (e.g., sodium, calcium, or potassium) across the membrane between the intracellular and extracellular domains. Several nerve and muscle cells as well as endocrine, immune, and reproductive cells have specific ion channels capable of producing excitable membranes, and their ability to generate action potentials is known as excitability. Whereas all plasma membranes can conduct graded potentials, only excitable membranes can conduct actions potentials. As a rule, an outward flow of positive ions acts to reduce the membrane voltage (a process called repolarization) while an inward flow of positive ions elevates the voltage across the membrane (depolarization) (Grunnet, 2010; Nerbonne & Kass, 2005; Roth, 1991).

A precondition for proper coordination of excitation and contraction and thereby cardiac function is that the electrical signals be highly coordinated, both in propagation and in time. From the SA node, in the right atrium, electrical activity is initiated in specialized pacemaker cells and subsequently spread throughout the atria. Atria and ventricles are electrically insulated with the exception of the AV node. Conduction through the AV node is relatively slow thereby securing atria contraction previous to ventricular contraction. Having passed the AV node, the action potential is propagated via the Purkinje fiber conduction system and spread to

endocardial cells in the ventricles. From here the signal is spread via gap junctions in a transmural direction to the outer part of the ventricular wall, the epicardium (Fig. 2.3.1.).

Additionally, the resting membrane potential in atrial, ventricular and Purkinje cells is about -80 mV whereas the resting membrane potential in the pacemaker cells is less negative, -50 to -60 mV in the SA node and -60 to -70 mV in the AV node, due to unique combinations of ion channels that these different cells express. The cardiac action potential is generated by sequential activation and inactivation of ion channels and ion pumps that conduct inward, depolarizing (Na^+ and Ca^{2+}) currents and outward, repolarizing (K^+) currents (Nerbonne et al., 2005). The resting membrane is much more permeable to potassium (K^+) than to sodium (Na^+), due mainly to the presence of a subtype of K^+ channel that is especially leaky at negative membrane voltages. Similarly, the same behavior in the depolarization phase happens. Na^+ entry depolarizes the cell and sustains the opening of more Na^+ channels. At almost the same time, the permeability to K^+ decreases as their leaky channels close, and this also contributes to the membrane depolarization (Persson, 2007; Widmaier *et al*., 2006).

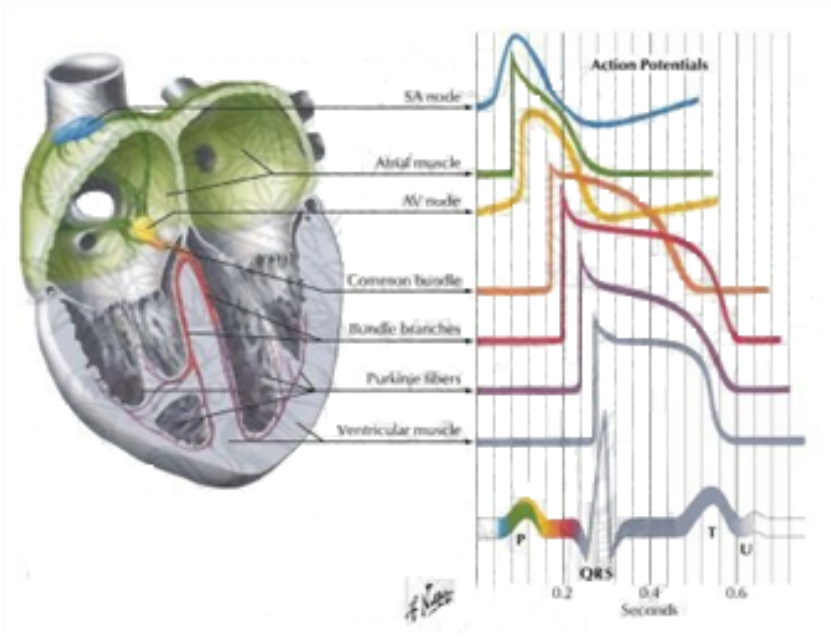

Figure 2.3.1. Electrical activity of the heart with the actions potentials waveforms of different regions (adapted from Hansen & Koeppen, 2009).

Following the passage of an action potential, a certain time period, the refractory period, must pass before a new action potential can be elicited. This is due to the inability of depolarizing ion channels to be activated unless they have first returned to a rested closed state. Consequently, this cycle repeats and alternates between systole and diastole.

The action potentials of atrial muscle cells are similar in shape to those described for ventricular cells, although the duration of their plateau phase is shorter. Three ion channel currents, which are shown in figure 2.3.2., contribute to the pacemaker action potential: i) a progressive increase to Na^+ permeability. Pacemaker cells have a unique set of channels that, unlike most voltage-gated channels, open when the membrane potential is at negative values; ii) a progressive reduction to K^+ permeability and iii) a type of Ca^{2+} channel that opens only briefly but contributes to an inward Ca^{2+} current causing an important final depolarization boost to the pacemaker potential (Zaza, 2010; Widmaier *et al*., 2006; Rourke *et al*., 2002).

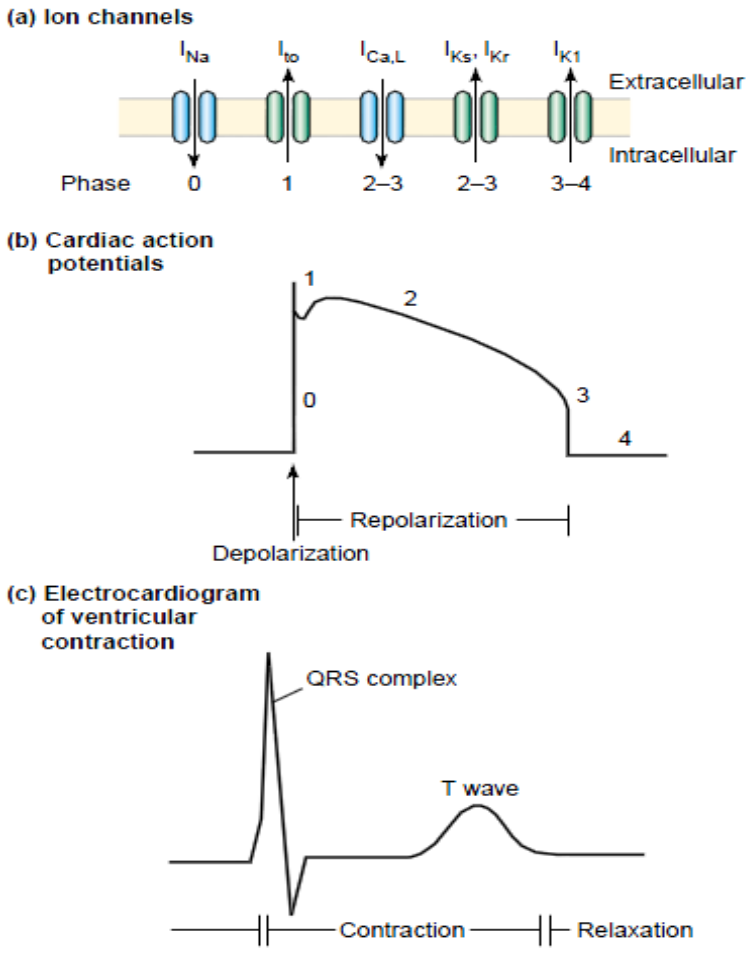

Figure 2.3.2. The relationship between cardiac ion fluxes, the action potential and the surface EKG. (a) The sequence of ion fluxes, with the respective cardiac action potential phase, across the cell membrane. (b) The cardiac action potential of a ventricular myocyte is composed of five phases (phases 0–4), which begin in phase 0 with the upstroke, followed by an early repolarization (the "notch" phase). Phase 2 is the plateau phase, and is generated primarily by inward Ca^{2+} currents ($I_{Ca,L}$), and the outward K^+ currents, I_{Kr} and I_{Ks}. The final phase of repolarization is phase 3 and the last phase, in which the membrane potential returns to the baseline, it is mediated by I_{K1}. (c) An EKG tracing showing depolarization and repolarization of the ventricle. Abbreviations: $I_{Ca,L}$, inward L-type Ca^{2+} current; I_{K1}, inward-rectifier K^+ current; I_{Kr}, the rapid component of the outward delayed-rectifier K^+ current; I_{Ks}, the slow component of the outward delayed-rectifier K^+ current; I_{Na}, inward Na^+ current; I_{to}, transient outward K^+ current (adapted from Cheng *et al.*, 2003).

Every time there is an increase in Na^+ inward current bringing the nodal cell membrane potential above the threshold value, an action potential occurs. The depolarization phase is maintained by calcium (Ca^+) influx through the L-type Ca^+ channel, and after a delay, the opening of K^+ channels repolarize the membrane.

II.3.1. Phase 0

In ventricular and atrial myocytes and in Purkinje fibers (non-pacemaker cells), this phase is fast and corresponds to the upstroke of the action potential, resulting from the activation of voltage-gated Na^+ (Na_v) channels, encoded by the gene SCN5A, resulting in an influx of Na^+ and driving off the membrane potential toward equilibrium potential for this ion. In both nodes (SA and AV) – pacemaker cells – however, phase 0 is markedly slower, suggesting that Na_v channels do not play a prominent role in depolarization.

However, the ability of the cell to open the fast Na^+ channels during phase 0 is related to the membrane potential at the moment of excitation. Here the cell comes from a negative resting potential to positive potentials around +30/+40 mV (Grunnet, 2010; Persson, 2007; Widmaier *et al*., 2006; Klabunde, 2005).

II.3.2. Phase 1

This phenomenon is followed, in non-pacemaker cells, by a transient repolarization, reflecting Na_v channel inactivation and the activation of the fast transient voltage-gated outward K^+ current, which it's a brief repolarization immediately after upstroke due to the closing of the Na^+ gates and the decrease in influx of Na^+ and increase in the efflux of K^+ ions. This repolarization is due to activation of transient outward voltage-gated K_v channels and is termed I_{to}. Two different types of I_{to} current have been described. They are referred to as $I_{to,\ fast}$ or $I_{to,f}$ and $I_{to,\ slow}$ or $I_{to,\ s}$. Both processes are voltage dependent and activation happens at membrane potentials higher than -30 mV (Widmaier *et al*., 2006; Nerbonne & Kass, 2005).

The transient net outward current causing the small downward deflection of the action potential is due to the movement of K^+ carried by I_{to1} current and Cl^- ions carried by the I_{to2} current, respectively. Particularly the first current contributes to the "notch" of some ventricular myocyte action potentials. At the peak of the action potential K^+ efflux is both chemically and electrically favorable (Grunnet, 2010; Persson, 2007; Widmaier *et al*., 2006; Klabunde, 2005).

II.3.3. Phase 2

This is the phase that distinguishes cardiac from neuronal action potentials. This is a long-lasting plateau (L) phase, named by the voltage-gated Ca^{+} channels that open much more slowly than Na^{+} channels and because of the fact that they remain open for a long period, in which the electrical potential remains near 0 mV. This transient repolarization or "notch," can be quite prominent in Purkinje and ventricular cells (Fig. 2.3.2.) and influences the height and duration of the plateau. This is caused by the activation of voltage-gated Ca^{2+} channels (Ca_v) originating by the influx of Ca^{2+} necessary and sufficient to trigger the so-called excitation–contraction coupling in the myocardium. There are two types of Ca_v channels: L-type (L = long-lasting) and T-type (T = transient) – nomenclature based upon biophysical properties. This Ca^{2+} influx through L-type Ca_v channels triggers the release of this ion to the troponin complex. Depolarization also triggers opening of voltage-gated K^{+} (K_v) channels, allowing efflux of intracellular K^{+} ions. The balance between Ca^{2+} influx and K^{+} efflux, keeps the membrane depolarized at the plateau value (Grunnet, 2010; Widmaier *et al*., 2006).

In SA and AV nodes cells, activation of L-type Ca_v channels also contributes to action potential generation, particularly in cells expressing low levels of functional Na_v channels. The driving force for K^{+} efflux is high during the plateau phase of the action potential in ventricular and atrial myocardium and, as the Ca_v channels inactivate, the outward K^{+} currents predominate. In contrast to Na_v and Ca_v currents, however, there are multiple types of voltage-gated K^{+} (K_v) currents, as well as non-voltage-gated, inwardly rectifying K^{+} (K_{IR}) currents, which contribute to myocardial action potential repolarization (Widmaier *et al*., 2006; Klabunde, 2005).

II.3.4. Phase 3

It is the transition from plateau phase to re-establishment of the diastolic resting membrane potential. This repolarization of the action potential happens as a consequence of two opposite events: the Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels and increased conduction of certain K^{+} channels, *i.e.*, increase in K^{+} efflux and decrease in Ca^{2+} influx. The degree on which the different K^{+} channels will affect this repolarization depends on a number of factors such as species, the influence from parasympathetic and sympathetic input, heart rate and the actual compartment of the heart (Klabunde, 2005).

II.3.5. Phase 4

It corresponds to the resting membrane potential, to finalize the process. This is equivalent to the diastolic interval, when the heart is "resting" and the aorta is pumping the blood to the tissues. Under normal circumstances only very limited charged movement will appear during the interval. However, that doesn't stand for that all ion channels are closed. The resting membrane potential is in closest proximity to the equilibrium potential for K^+, meaning that the permeability of K^+ channels is larger than the other two ion channels (Na^+ and Ca^{2+}), which inactivation is time- and voltage-dependents. As a consequence of their biophysical properties, both two-pore and K_{IR} K^+ channels will be active in phase 4 and, therefore, more time is spent in this phase.

Membrane potential is fully repolarized to resting potential (~-80 mV). It approaches but does not quite reach the resting potential for K^+ (~-89mV) reflecting the high conductance to K^+ and also due to small amount of Na^+ influx (Widmaier *et al*., 2006; Klabunde, 2005).

II.4. Potassium channels

The primary role of K^+ channels in cardiac action potentials is cell repolarization. Cardiac K^+ channels determine several characteristics of the heart, *i.e.*, the resting membrane potential, the heart rate, the shape and duration of the action potential.

The diversity of K^+ specific channels exceeds any other group of ion channels. The importance of this super-family of channels is underlined by their responsibility in many regulatory processes. K^+ channels contain alpha subunits, which represents the integral membrane protein responsible for the conduction of these ions across the lipid bilayer, and in some cases auxiliary beta subunits. Based on their molecular architecture different channel classes are distinguished. One of them, composed of voltage-gated K^+ channels (K_v), includes subunits composed of six transmembrane (TM) helices (S1-S6) and one pore-forming region (P-region) and is called 6TM/1P class. Another one, with inward rectifier K^+ channels (K_{IR}) contains subunits with only two transmembrane domains (2TM) and one P-region and is called 2TM. 2TM and 6TM subunits assemble as tetramers – figure 2.4.1. (Anumonwo & Lopatin, 2010; Barford *et al.*, 2007; Prüβ, 2004). The inward rectifier K^+ current (I_{K1}), in cardiac myocytes, plays a significant role in maintaining the resting membrane potential and in shaping the late repolarization phase of the action potential (Lopatin & Nichols, 2001). On the basis of sequence homology, inward rectifier K^+ channels have been classified into seven subfamilies ($K_{IR}1$ to $K_{IR}7$) (Nichols & Lopatin, 1997). Despite seven K_{IR} subfamilies exist, only two of them ($K_{IR}2$ and $K_{IR}3$) play an important role in the cardiac repolarization (Hibino *et al.*, 2010).

Moreover, transcriptional analysis coupled with functional characterization of cloned K_{IR} channels suggests that members of the K_{IR} 2 subfamily are responsible for cardiac I_{K1} (Prüβ, 2004).

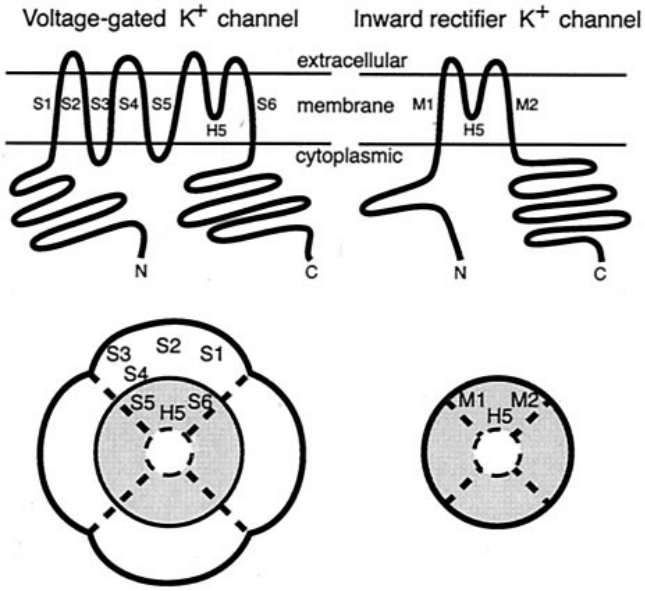

Figure 2.4.1. Voltage-gated K^+ (K_v) channels and inwardly rectifying K^+ (K_{IR}) channels. These channels have in common the pore-forming structure of the K^+ channel families and the tetramer structure. They are comprised of two transmembrane segments and the H5/P loop in between, in each subunit. Between the last two transmembrane segments of these two channels is a highly conserved P region implicated in forming the K^+-selective pore. In an upper view of the K_v and K_{IR} channels it can be observed the similarity in the gross design of their channels and both are amenable to modulation by cytoplasmic factors (adapted from Yi *et al*., 2001).

II.4.1. K_{IR} – inward rectifier K^+ channels

The structural key, causing K^+ selectivity is a highly conserved stretch of eight amino acids (TXXTXGYG), specifically, the GYG motif that allows only K^+ ions to pass through the pore.

Because the equilibrium potential of K^+ is rather negative, all cardiac K^+ channels when activated will carry outward current, repolarized the membrane during the action potential, or stabilize the membrane at a hyperpolarized level. Voltage-activated K^+ currents show activation and inactivation upon depolarization; the rates of these two processes can vary from fast to ultraslow. Ligands can bind to receptors, which then activate the channel via a G protein, or can interact directly with an intracellular site of the channel (Liu *et al.*, 2010; D'Avanzo, 2005; Zhang *et al*., 2004).

There are three different K^+ currents quickly increased, upon depolarization, I_{to} (a transient outward K^+ current that is blocked by millimolar concentrations of 4-aminopyridine

(4-AP)), the ultrarapid (I_{Kur}) and rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed rectifier and I_{K1}. They share a relatively fast inactivation rate. Other channels exist that can be activated by ligand-binding. Ligand-gated channels include those activated by an intracellular concentration of adenosine triphosphate (K_{ATP}) or by acetylcholine (K_{Ach}) – figure 2.4.2. (Tamargo *et al*., 2003; Carmeliet, 1999).

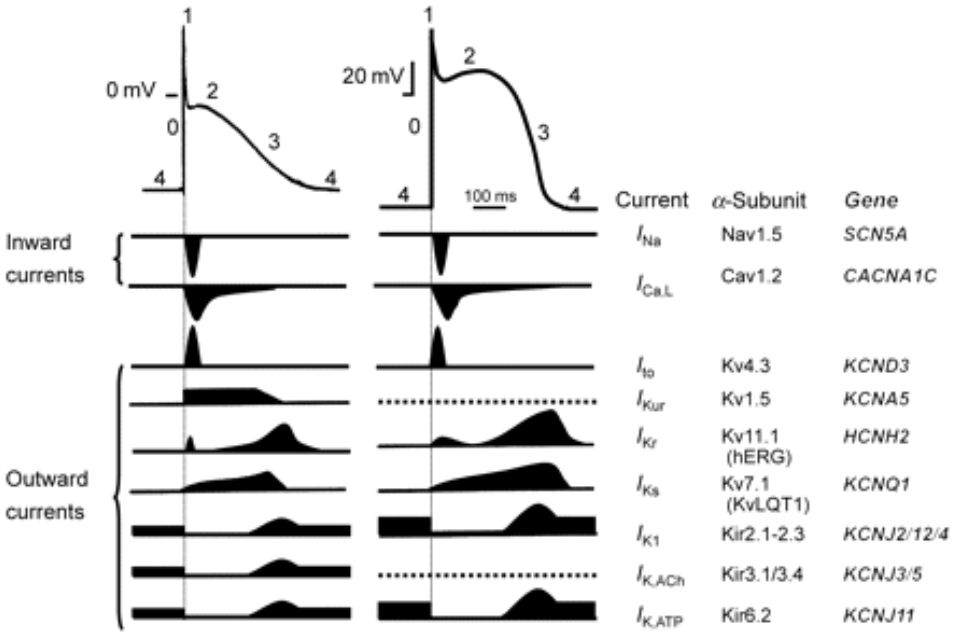

Figure 2.4.2. Inward, depolarizing and outward, repolarizing currents that underlie the cardiac action potential (atrial and ventricular). Inward currents: I_{Na} Na^+ current; $I_{Ca,L}$ L-type Ca^+ current; Outward currents: I_{to} transient outward current; I_{Kur} ultra rapidly activating delayed rectifier current; I_{kr} and I_{ks} rapidly and slowly activating delayed rectifier current; I_{K1} inward rectifier current; $I_{K,Ach}$ acetylcholine-activated K^+ current. Typical cardiac APs consist in five phases and according to their function the cardiac K^+ channels have their classification (adapted from Ravens & Cerbai, 2008).

Inwardly rectifying K^+ (K_{IR}) channels represent the minimal requirement for a K^+ channel function correctly; however, it is involved centrally in physiological functions. The K_{IR} current was first observed electrophysiologically by Katz in 1949 and named an anomalous rectifier due to its unique properties, which were in contrast to the known voltage-dependent delayed-rectifier K^+ channels. In recent years several K^+ channel genes were discovered encoding channels whose properties verify his original measurement. In the usual terminology, members are designated $K_{IR\ x.y}$, whereas 'x' determines the subfamily and 'y' names the subtype within the subfamily (De Boer *et al*., 2010a; Lopatin & Nichols, 2001).

K_{IR} channels share ~60% amino acid identity between individual members within each sub family and ~40% identity between subfamilies (de Boer *et al.*, 2010a).

Although inward rectifiers (K_{IR}) comprise a large family of K^+ channels (figure 2.4.3.a.) and share great structural similarities, there are only two subfamilies, $K_{IR}2$ and $K_{IR}3$, with underlie classical 'strong inwardly rectifying currents'. In the heart, only two similar types of these currents: I_{K1}, a constitutively active K_{IR} current which is more prominent in ventricular tissue and I_{KACh}, a receptor-activated K_{IR} current which is more prominent in atrial tissue, as well as in SA node, play a critical role in the regulation of heart rate by vagal nerve activity, and AV node – figure 2.4.3.b. (Anumonwo & Lopatin, 2010; Lopatin & Nichols, 2001).

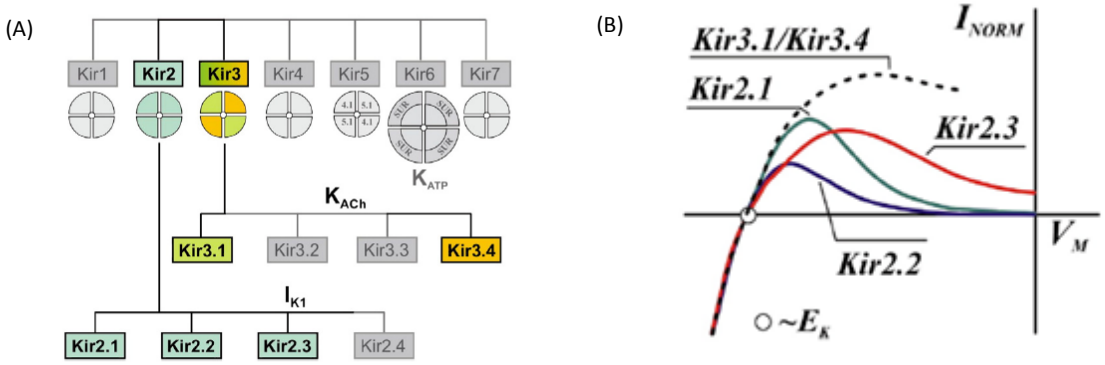

Figure 2.4.3. (A). The family of inward rectifier K^+ channels. $K_{IR}2$ and $K_{IR}3$ subfamilies represent channels carrying classical strongly rectifying currents observed in cardiac muscle (colored boxes). Heteromeric assemblies of $K_{IR}2.1$, $K_{IR}2.2$, and $K_{IR}2.3$ subunits underlie I_{K1} current which is more prominent in ventricular tissue, and heteromeric assembly of $K_{IR}3.1$ and $K_{IR}3.4$ subunits that underlies I_{KACh} current which is more prominent in atrial tissue and in SA node. (B). Rectification profiles of different K_{IR} channels. At similar conditions, when currents amplitudes are normalized at far negative membrane potentials the outward currents are the smallest for $K_{IR}2.2$, very similar for $K_{IR}2.1$ and $K_{IR}2.3$ and the largest for $K_{IR}3.1/K_{IR}3.4$ channels (adapted from Anumonwo & Lopatin, 2010).

II.4.1.1. K_{IR} subfamilies

Different physiological functions are assigned to K_{IR} channels. Aside from differential cellular expression the diverse subset of K_{IR} channel subtypes determines the pattern of electrical properties.

The $K_{IR}1$ subtype is a K_{IR} channel with weak inward rectifying properties, predominantly in the kidney, where it is important in homeostasis by conducting large amounts of K^+. $K_{IR}3$ channels are the predominant mediators of metabotropic inhibition and are activated directly by Gβγ subunits. They influence electrical activity in neuronal, cardiac and neurosecretory cells. $K_{IR}4$ is known to control K^+ homeostasis in glial cells and the inner ear. ATP-sensitive $K_{IR}6$ (K_{ATP}) channels play an important role in the pancreas. Moreover, this channel may contribute to the cell survival during anoxia or hypoglycemia in neuronal and cardiac structures (figure 2.4.4.) (Anumonwo & Lopatin, 2010; Lopatin & Nichols, 2001).

As said before, I_{K1} has been suggested to play as major role in the formation of the plateau phase of the cardiac action potential, since inward rectification results in a very low conductance at the action potential plateau (+20 to +50mV), and permits relatively small inward currents to maintain the plateau. As the cell repolarizes, the conductance recovers, allowing I_{K1} channels to contribute to repolarization and stabilization of the resting potential. Their unusual (anomalous) regulation by extracellular K^+ remained a puzzle for long time – figure 2.4.4. (Nerbonne & Kass, 2005).

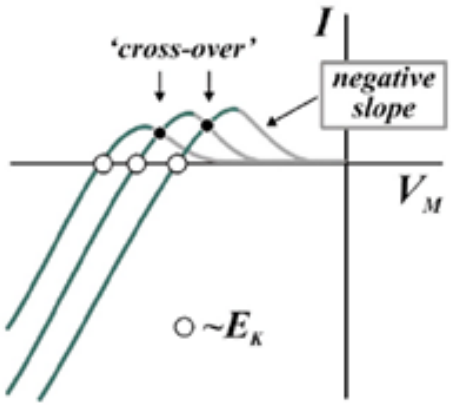

Figure 2.4.4. I/V relationship of I_{K1}. Upon membrane depolarization there is a block of the K_{IR} channel pore by intracellular polyamines and Mg^{2+} ions in response to membrane depolarization leads to a voltage-dependent decline of K^+ conductance producing a region of 'negative slope' conductance. Increase in the concentration of extracellular K^+ leads to a near parallel shift of current/voltage relationships and their 'crossover'. As a consequence, at more positive potentials K^+ conductance increases rather than decreases (adapted from Anumonwo & Lopatin, 2010).

A higher I_{K1} density is associated with faster late phase of action potential repolarization in ventricular myocytes and a reduced I_{K1} density with a slower repolarization in Purkinje cells. The rectification properties of I_{K1} are also different in ventricle and atrium: the region of 'negative slope conductance' at depolarized potentials is virtually absent in atrial cells but tends to be prominent in ventricular myocytes (De Boer et al., 2010a).

Around the resting potential, the ventricular I_{K1} conductance is much larger than that of any other K^+ current, with the exception of the K_{ATP} current which is normally not active. It is thus likely that physiological modulation of this current will have a significant effect on excitability. Unfortunately, lack of specific pharmacological tools or the inability to manipulate I_{K1} experimentally, have provided a significant impediment to further advance in the classical study of this conductance – figure 2.4.5. (Anumonwo & Lopatin, 2010).

The electrophysiological properties of K_{IR} channels include the conduction of more current in the inward than in the outward direction, owing to a reduced open probability in a depolarized membrane. Due to their peculiar properties in a particular cell, K_{IR} channels have a

key role in stabilizing the resting membrane potential. The K_{IR}-mediated outward flux prevents action potential firing by small electrical stimuli. In the case of strong electrical stimuli, however, these channels close and allow action potentials, thereby preventing massive K^+ loss. Furthermore, modulation of these channels can alter the membrane potential, cellular excitability and heart rate, and secretion of neurotransmitters or hormones (Anumonwo & Lopatin, 2010; Grunnet, 2010).

The rectification property of the "classical" $K_{IR}2.X$ includes the inwardly conductance (showing a linear behavior below the K^+ equilibrium potential), negligible outward currents during depolarization, preventing massive K^+ loss and allow prolonged depolarization (plateau of an action potential) (Lopatin & Nichols, 2001).

$K_{IR}2.X$ proteins constitute the pore forming subunits of I_{K1}. This K^+ channel family is fundamentally different from voltage-gated K^+ channels. These channels are recognized as inward rectifiers, but they are not regulated by changes in membrane potentials. The reason for this is obvious from a structural point of view. Human cardiac tissue expressed $K_{IR}2.1$, $K_{IR}2.2$ and $K_{IR}2.3$, but the first is the most abundant.

The main player of the inward rectifier K^+ current (I_{K1}) in the heart is thought to be $K_{IR}2.1$, which is encoded by the KCNJ2 gene and is widely expressed in both atrial and ventricular myocytes, but is absent from nodes. It contributes to maintaining the resting membrane potential and shaping the initial depolarization and the final repolarization of the cardiac action potential (that is about six times larger in ventricular cells). These channels allow the passage of K^+ inwardly at potentials negative to E_K or allow some efflux of K^+ in the terminal phase of repolarization following the action potential. Moreover, they can maintain the resting membrane potential after the action potential through their unusual ability to open at negative membrane potentials. Additionally, $K_{IR}2.2$ may co-assemble with $K_{IR}2.1$ into heteromultimeric channels (Anumonwo & Lopatin, 2010; Hibino *et al.*, 2010; Vaidyanathan, 2010; Persson, 2007; Hoger, 2002).

The ability of this current to affect the resting membrane potential is emphasized by the fact that in the locals with high I_{Kr} expression the resting membrane potential is around -80 mV while in the nodes lacking I_{K1} these values increase to about -50 mV.

The K_{IR} channels are inhibited by intracellular magnesium (Mg^{2+}) and polyamines at positive potentials. As a consequence, K^+ flux through these channels will be larger in amplitude in the inward direction compared to the outward direction, causing a strong rectification. As a consequence of strong rectification, K_{IR} channels will not conduct at positive potentials and therefore will only be active at phase 3 of the cardiac action potential at, approximately -40 mV, when they become released from intracellular inhibition. The

contribution from I_{K1} to action potential repolarization will thereby be important in the late part of phase 3 (Lopatin & Nichols, 2001).

Regarding I_{K1} regulation, opposite effects has been described. α- or ß-adrenergic stimulation on these channels may cause different effects such as up- or down-regulation of the channels, suggesting the existence of different types of adreno-receptor pathways in the heart. Anumonwo & Lopatin (2010) showed in a *Xenopus oocytes* expression system, that when stimulated, α_1- or $ß_3$-adrenoreceptors might cause antagonic effects on $K_{IR}2.1$ and $K_{IR}2.3$ channels. Up-regulation of $K_{IR}2.1$ and down-regulation of $K_{IR}2.3$ is observed by α_1- adrenergic stimulation whereas with $ß_3$-adrenoreceptors the opposite effect was noted.

Several studies (de Boer *et al.*, 2010b; Jansen *et al.*, 2008) referred phosphatidylinositol-4,5-bisphosphate (PIP_2) – a phospholipid component of the cell membrane – as an important component in second messenger signaling systems. Xie *et al.*, (2002) have demonstrated through biochemical experiments that PIP_2 binds directly to K_{IR} channels and activates them. The interaction of PIP_2 with a single $K_{IR}2.1$ subunit is enough for the channel to open to its full conductance state. As a consequence, if PIP_2 interacts with more subunits this effect will be stronger.

Since K_{IR} proteins function as ion channels, their accumulation in cell membrane and its location is an important event. One of the lipids of the plasma membrane, cholesterol, essential for cell function and growth, has been revealed as a crucial player in this process. Romanenko *et al.* (2004) demonstrated that $K_{IR}2.1$ channels are negatively sensitive to membrane cholesterol (when membrane cholesterol increases, the number of active $K_{IR}2.1$ channels decreases, without decreasing the total number of the channels in the plasma membrane). Interestedly, this may result from adjustments in the interactions of the channels with membrane protein or lipid components of lipid rafts, which are liquid-ordered membrane micro-domains with unique protein and lipid composition within the plasma membrane of most mammalian cells that are involved in multiple cellular processes, including signal transduction, regulating neurotransmission and receptor trafficking, and recycling of proteins to the membrane (Balijepalli & Kamp, 2008).

Finaly, Wischmeyer *et al.* (1998) demonstrated that these channels are also inhibited by phosphorylation at a single site, in the carboxyl terminus, by $K_{IR}2.1$ protein tyrosine kinase (PTK) and that this effect could be mimicked with growth factor receptor stimulation.

II.4.2. K_v – Voltage-gated K^+ channels

hERG (the human *Ether-à-go-go* Related Gene) is a gene (KCNH2) that codes for voltage gated K^+ ion channel, a protein, known as K_V11.1 hERG and it has an important role in the electrical activity of the heart – figure 2.4.5. This gene is responsible for the I_{Kr} and is critical for a correct repolarization.

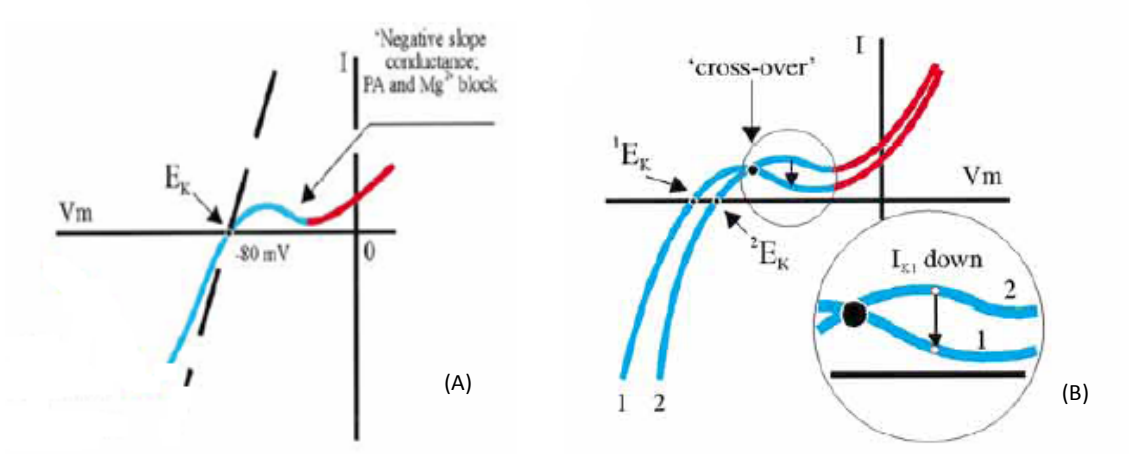

Figure 2.4.5. Molecular basis of I_{K1} activity: (A) cardiac I_{K1} conductance in K_{IR} channels (blue). Conductance declines upon depolarization, due primarily to channel block by Mg^{2+} ions and polyamines (PA), producing a ''negative slope'' region. Further depolarization activates voltage-gated K^+ conductances – K_v (red); (B) I_{K1} current–voltage relationships in solutions with different extracellular K^+ concentrations (1–normal [K^+], 2–elevated [K^+]) (adapted from Lopatin & Nichols, 2001).

Each major subunit from hERG homotetramer is composed of six TM domains. Figure 2.4.6. shows S5 and S6 TM segments and the pore helix between them. They form the pore domain, the main responsible for the high throughput ion conduction pathway. The remaining TM segments (S1-S4) regulate pore opening and closing and therefore form the voltage sensing domains (VSD) or the intrinsic voltage sensor of K_V channels. Especially the fourth transmembrane segment (S4), contains basic residues at every third position in an otherwise hydrophobic segment that spans the membrane (Akhavan *et al*., 2005; Yin *et al.*, 2001).

Torres *et al.* (2003) have shown that the 40 - amino acid extracellular domain between S5 and the pore helix (exclusive of these voltage K^+ channels) has an amphipathic helical region that interacts with the pore domain of the channel in a voltage-dependent manner.

Furthermore, hERG also has a particular C-terminal region with a putative cyclic nucleotide binding domain. Mutations in this region can cause loss of function of the channel by abnormal protein trafficking and are associated with long QT syndrome type 2 (Akhavan *et al*., 2005).

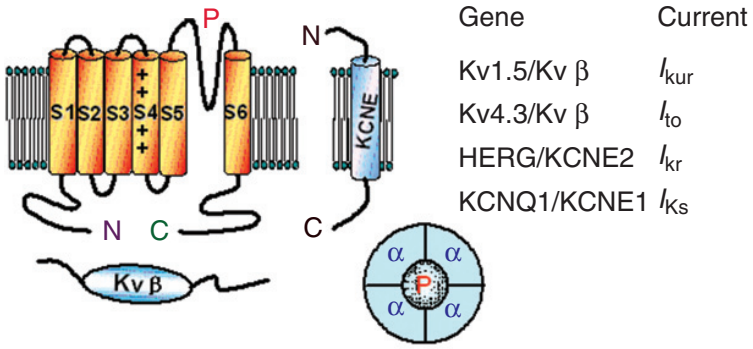

Figure 2.4.6. Schematic topology of K_V channels. Each subunit contains six transmembrane segments (S1-S6) and one pore forming loop (P) between S5 and S6 with the positive voltage sensor on the S4 segment. The currents carried by these channels are also represented (adapted from Li & Dong, 2010).

II.5. Internalization pathways

Protein degradation plays an important role in cell cycle, signal transduction, and maintains the integrity of proper folder state of proteins. Despite several pathways exist for this purpose, usually they are degraded by one of these two systems: lysosome or ubiquitin-proteasome system. The first contains proteases with optimal activity at an acidic pH and degrades membrane or endocytosed proteins. The second requires ATP, and generally is involved in the degradation of cytosolic and nuclear proteins and in the internalization of several plasma membrane proteins (Grant & Sato, 2006). In the heart, cardiomyocyte receptors and ion channels are regulated by these two systems combined (Willis & Patterson, 2006).

The cells internalize plasma membrane components and extracellular macromolecules by a vesicle-mediated process called endocytosis (figure 2.5.1.), which represents a hallmark of all eukaryotic cells. It is a basic cellular process that describes the uptake of membrane proteins and lipids, soluble molecules and extracellular ligands from the cell surface, allowing the control of the plasma membrane composition. Because these molecules can be quite diverse, understanding the different pathways that mediate their internalization and how these pathways are regulated is extremely important.

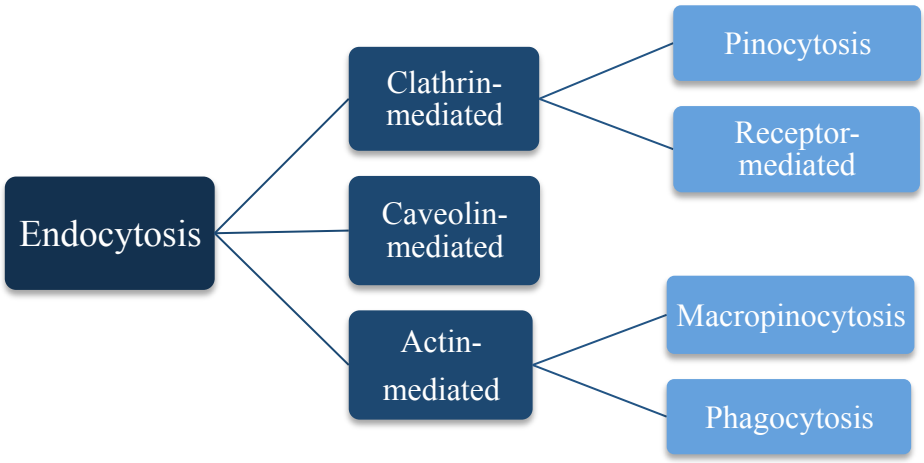

Figure 2.5.1. The three main endocytosis pathways of the internalization process (adapted from Doherty & McMahon, 2009)

A heterogeneous array of cellular processes involves endocytosis, including nutrient uptake, synaptic vesicle recycling, remodeling of the plasma membrane, regulation of cell-surface expression of signaling receptors and the generation of cell polarity. To meet the differing necessities of such processes, endocytic pathways must possess considerable diversity in terms of regulation, specificity for different cargoes, and ultimate destination within cells.

In any endocytic pathway, which mediates the transport of a specific cargo, the initiall step consists of recruiting and assembling, below the cell surface, specific adaptor and effector proteins to the forming vesicle (Doherty & McMahon, 2009; Leng *et al.*, 2007; Brodsky *et al.*, 2001) – figure 2.5.2.

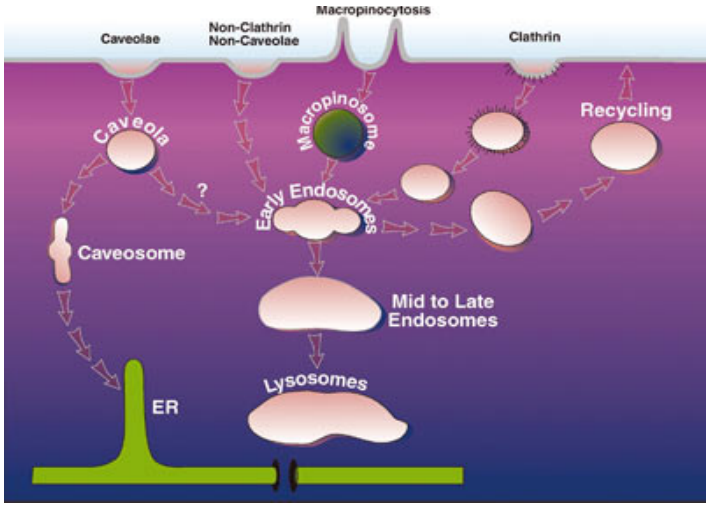

Figure 2.5.2. Endocytic pathways of nonviral delivery systems. This is the general model of the endocytic pathway. Cargo molecules are endocytosed and targeted to early endosomes. Some cargos are further transported to lysosomes through late endosomes. Others are recycled back to the plasma membrane via the recycling pathway (adapted from Leng *et al.*, 2007).

The mechanisms for vesicle formation differ among three main endocytic pathways, clathrin-mediated, caveolae-meadiated and actin-mediated, being the clathrin-mediated pathway the best studied in mammalian cells – figure 2.5.3. (Doherty & McMahon, 2009; Brodsky *et al.*, 2001).

In this pathway, clathrin-coated pits are formed by the association with their adaptor proteins, such as the four-subunit complex AP2, and a cluster of many receptors. Then, to pinch off the coated-pit, it needs GTPase dynamin to give rise to the final coated vesicle. Such vesicles are then uncoated by some proteins and then fuse with other intracellular vesicles forming early endosomes, in a reaction requiring some enzymes, such as the small GTPase Rab5. In early endosome acidic environment, ligands are dissociated from their receptors and protein sorting occurs to determine the subsequent intracellular route of the internalized vesicular contents. The proteins might be recycled back directly or indirectly to the cell surface or can be transported to lysosomes via the late endosomes (forming a "hybrid") for degradation Doherty & McMahon, 2009; Leng *et al.*, 2007).

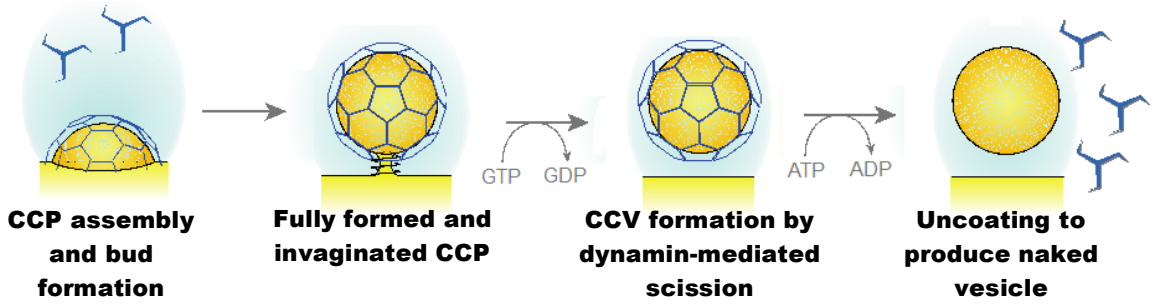

Figure 2.5.3. Steps of the clathrin coated pathway . Extracellular macromolecules (ligands) bind to cell surface receptors that are concentrated in clathrin-coated pits (CCP). These pits bud from the plasma membrane to form intracellular clathrin-coated vesicles (CCV). Electron micrographs showing four stages in the formation of a clathrin-coated vesicle from a clathrin-coated pit. (adapted from Perry *et al.*, 1979).

Alternatively, these molecules can be internalized by caveolin-coated vesicles (caveolae-mediated endocytosis pathway), called caveolae, at the cell surface. But before early endosome formation, they fuse with an intermediate compartment, a caveosome.

Caveolae (meaning "little cavities") are a type of lipid rafts, specialized membrane micro-domains enriched with cholesterol, which are present in multiple cell types including all the heart cells. They exhibit invaginated flask-shaped plasma membrane, marked by a structure protein – caveolin – that interacts with cholesterol. There are several types of this integral membrane protein with different functions in the cell beyond caveolae formation.

So, the availability of ion channels in the plasma membrane can be regulated by caveolae through their ability to control ion channel trafficking. However, although $K_{IR}2.1$ channels are sensitive to membrane cholesterol, studies suggest that they are not localized in caveolar lipid rafts due to the lack of interaction with caveolin-3, the most important caveolin in caveolae formation in cardiomyocytes (Balijepalli & Kamp, 2008). This pathway can be compared with clathrin-coated vesicle pathway regarding its delivery of molecular cargo to Golgi, endoplasmic reticulum (ER) and lysosomes.

Equally important, but less well characterized are non-clathrin endocytic pathways. These pathways do not use known coat complexes for cargo recruitment and budding of transport intermediates. Instead, they might exploit lateral heterogeneity in plasma membrane lipid and protein composition to select cargo as well as scaffolding or structural components into dynamic membrane micro-domains that bud into the cell – figure 2.5.4. (Doherty & McMahon, 2009; Balijepalli & Kamp, 2008).

The actin-mediated pathway is involved in the process of phagocytosis and/or macropinocytosis, referring to the uptake of vesicles much large in size than the other two pathways. Therefore, it is probably unlikely that ion channels, like $K_{IR}2.1$ channels, are specifically internalized into the cell by this route. Since internalization of $K_{IR}2.1$ channels was already associated with clathrin-mediated endocytosis (Steele *et al.*, 2007), we have chosen to study caveolae-mediated endocytosis to see if this pathway may be involved with $K_{IR}2.1$.

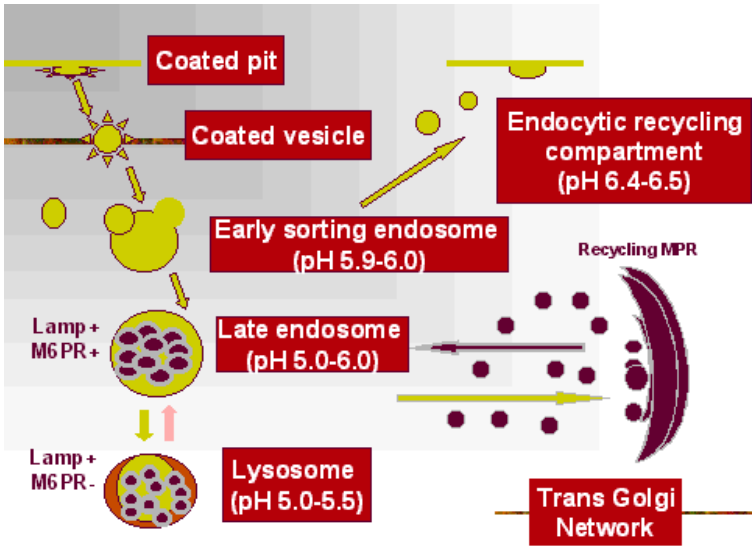

Figure 2.5.4. Internalization pathways, compartments and their pH (adapted from Perry *et al.*, 1979).

II.6. Trafficking

Many mutations that ion channels express may cause trafficking problems. That is the main reason for exploring the trafficking process. If the number of proteins that reach the sarcolemma decrease, the number of ion channel proteins is also diminished leading to less of the total current and hence to problems in repolarization (van der Heyden, Smits & Vos, 2008).

The voltage-gated ion channels expression and recycling at the plasma membrane of excitable cells is essential in maintaining cellular excitability and electrical impulse propagation. Trafficking of integral membrane proteins along the secretory pathway is a tightly regulated process, in particular the transition from the endoplasmic reticulum (ER) to the Golgi complex (Herfst *et al*., 2004). Shortly, the normal pathway implies that the protein is synthesized in the endoplasmic reticulum (ER), exported to the Golgi apparatus for complex glycosylation and eventually inserted into the cell surface membrane (Nanduri *et al.*, 2009).

The complexity of the intracellular trafficking of membrane proteins such as ion channels is still unknown, due to the lack of knowledge of many proteins involved in this process. These molecules start their life at the ER using the translocon complex for translocation and integration into the hydrophobic lipid bilayer. At this membrane they will be assembled, processed, trafficked and targeted to the cell membrane. At the cell surface, they will become effective in their function and eventually end up internalized for recycling or degradation. Involved are ER resident proteins like chaperones and glycosylases, microtubules and their associated motors, transport vesicle and Golgi apparatus components, actin cytoskeleton, myosins, anchoring proteins, etc. However, for many channels, the firsts trafficking steps, as for instance the sorting from ER to the Golgi apparatus, could be crucially affected by their oligomerization state and the binding of auxiliary subunits – figure 2.6.1. (Steele *et al.*, 2007; Heusser & Schwappach, 2005).

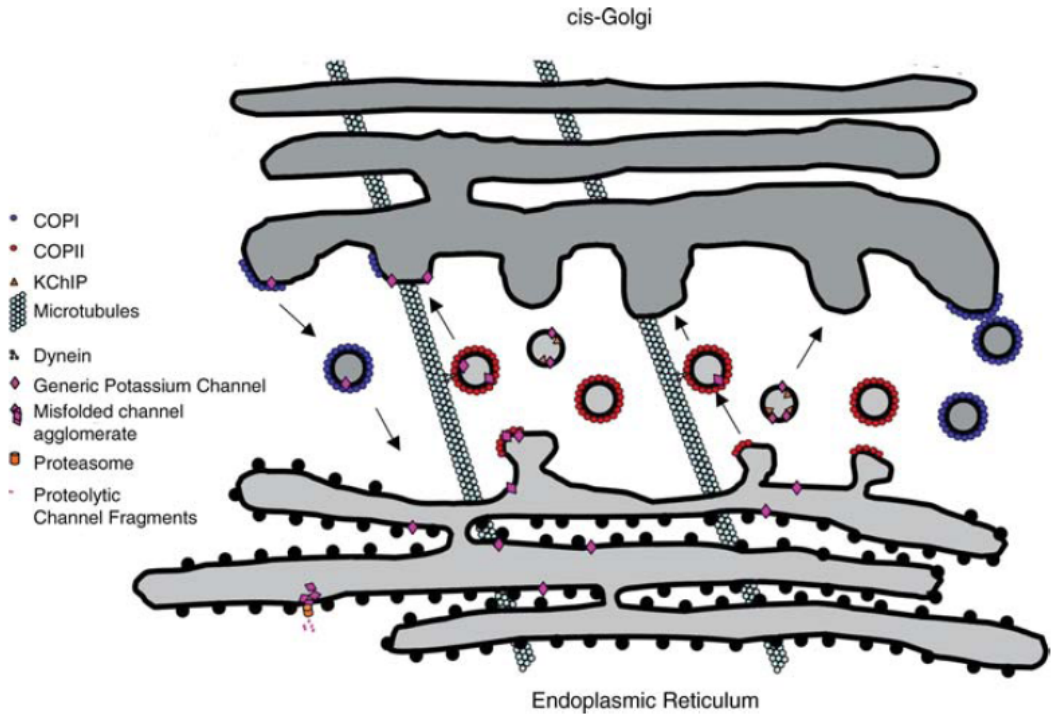

Figure 2.6.1. Trafficking of cardiac K^+ channels from ER to Golgi. After the synthesis on the rough ER, channels are assembled in COPII-coated vesicles (red) and then transported to the cis-Golgi in a dynein-dependent manner. Some channels are shown trafficking to the Golgi in vesicles lacking COPII. Some COPI coated vesicles are observed returning to ER through retrograde transport. Although sorting and targeting of cardiac K^+ channels has barely been explored, it is highly likely that this sorting, like that of other newly synthesized secretory and plasma membrane proteins, begins in the Golgi apparatus (Glycosylation) (adapted from Steele *et al*., 2007).

As it was mentioned before, these interactions involve the specific targeting of channels to distinct sub cellular locations, recruitment of signaling molecules, stabilization at the plasma membrane, and direct channel alteration by post-translational modification. The three most abundant channels inside the K_{IR} family ($K_{IR}2.1$, $K_{IR}2.2$, and $K_{IR}2.3$) contain a protein-protein interaction motif at their C terminus that can bind to scaffolding proteins that contain PDZ domains and proteins of the membrane-associated guanylate kinase (MAGUK) family as scaffolding components. Furthermore, it was shown that association of several proteins (namely synapse-associated protein 97 (SAP97), calmodulin-dependent serine protein kinase (CASK), Veli) with the $K_{IR}2.1$ channels are probably involved in ion channel targeting, trafficking and localization (Leonoudakis *et al.*, 2004).

Employing a promising strategy to selectively inactivate ion channels at the cell surface and following the replacement of functional copies (functional recovery after chemo-bleaching), some researchers (Sun *et al.*, 2003) discovered that several $K_{IR}2.1$ channels are non-functional when expressed heterologously at the cell surface of human embryonic kidney 293 (HEK293) cells (Heusser & Schwappach, 2005; Ma & Jan, 2002).

$K_{IR}2.1$ channel internalization involves dynamin-catalyzed scission of endocytotic vesicles and their transport from the cell surface to the cell interior. The dynein–dynactin complex responsible for retrograde trafficking of endocytotic vesicles is disrupted by overexpression of dynamitin (Pongs, 2009).

As $K_{IR}2.1$ channel, hERG biogenesis is in the ER and involves the synthesis of a core-glycosylated monomer with 135 kDa on western blot (immature hERG). It is followed by co-assembly of more three monomers (forming a tetramer) which are transported to the Golgi, where complex glycosylation occurs, resulting in the addition of sugar molecules to each subunit, now with 155 kDa on western blot (mature hERG). Finally, hERG tetramer is carried to the cell surface. As a result, in a western blot analysis of the wild type protein two bands should appear: one at 135 KDa (immature hERG) and other at 155 KDa (mature hERG) if the assemble and trafficking process were properly done. Otherwise, just the immature hERG band will appear.

hERG incorporation into vesicles exiting the ER involves an inclusion of a cytosolic ER exit signal into the protein. Additionally, if hERG subunits exit the ER without having the correct assembly two things can occur: they may be transport to the cell surface as non-functional monomers or they can be attached to vesicles that make the retrograde pathway to ER when RXR motifs they contain are exposed – figure 2.6.2. (Perrin *et al.*, 2008).

Walker *et al.* (2003) and Ficker *et al.* (2003) have noted numerous proteins, including chaperones, involved in hERG intracellular trafficking. hERG maturation and protein trafficking to the cell surface are regulated by Hsp90, a molecular chaperone that protects cytoplasmic proteins from misfolding and degradation (Nanduri *et al.*, 2009).

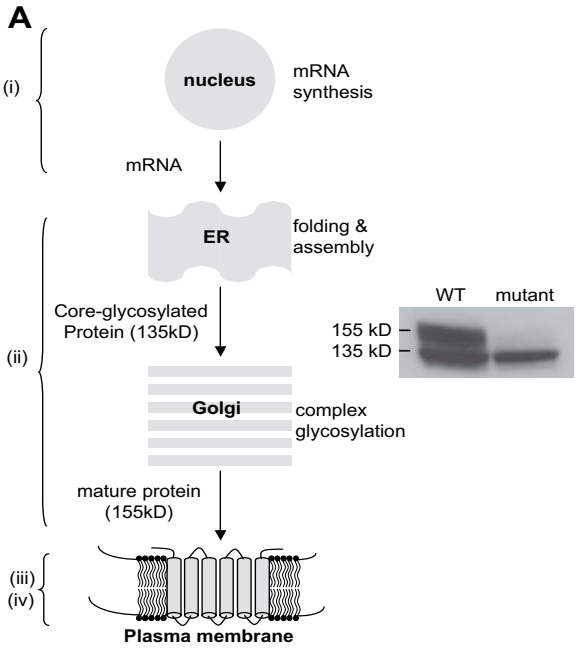

Figure 2.6.2. hERG K^+ channel biogenesis. (A) Functional hERG K^+ channels depend on mRNA synthesis (nucleus), protein synthesis, core-glycosylation, subunit assembly (endoplamatic reticulum, ER), complex glycosylation (Golgi) and sorting of the mature protein to the plasma membrane. hERG K^+ channel mutations are classified into four types as indicated: (i) defective synthesis, (ii) defective trafficking, (iii) defective gating and (iv) defective permeation. Trafficking defects (type 2) can be detected by western blotting, due to the absence of fully glycosylated – 155 kD band – and gating/permeation defects by patch clap analysis (adapted from Perrin *et al.*, 2008).

II.7. Gating mechanism of $K_{IR}2.x$ and K_V channels

Gating is the transitional process between a totally open and closed channel and is controlled by several physiological variables. The conformational changes in $K_{IR}2.1$ K^+ channel domains or the mechanical coupling of gating domain movements to pore to open and close makes possible to this channel to switch between two states: conducting and non-conducting (Pongs, 2009; Yu *et al*., 2005).

Voltage-gated K^+ channels opening are promoted by membrane depolarization whereas repolarization endorses closure. The protein composition of these channels contributes for this behavior. Bezanilla *et al.* (2000) has studied the nature of segment S4 movements (one segment of the TM subunits which contain repeated positive charges) by performing systematic mutagenesis experiments in combination with fluorescent tagging and voltage measurements (Loussouarn *et al*., 2002). The nature of the gating mechanism seems to involve the pore's block by the gating particle, however many details are still unclear. An example is the closure (N-type inactivation) that occurs in some channels, especially among K_{IR} family members, after voltage dependent opening involving the inner channel entrance blockage by the N terminus part (Hoshi *et al.*, 1990).

In their studies, Zhou *et al.* (2001) have shown that there were several amino acids located near the N-terminal that enter into the inner vestibule and cause this voltage-dependent blockage.

Lopatin & Nichols (1997) studies about the inward rectification showed that the pore was blocked, intracellularly, by Mg^{2+} and linear polyamines – figure 2.7.1. – with evidences that the polyamines also accumulates in the inner vestibule of the membrane.

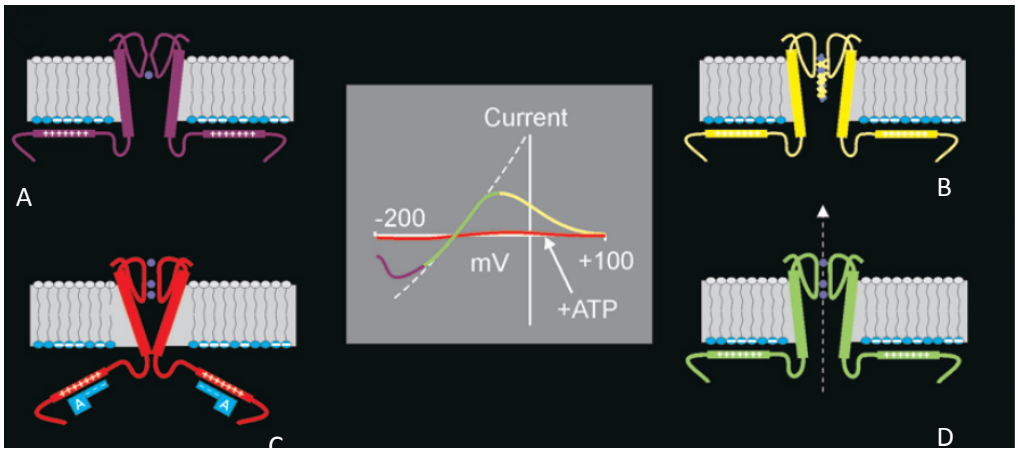

Figure 2.7.1. The current-voltage relationships of different gating modes. The P loop, M2, and C termini of two subunits are represented in each of the four diagrams. A. Closed conformation of the channel may arise from changes in the selectivity filter. B. Pore blockage by polyamine. C. Open channel stabilized by rearrangements of M2 (stabilized by, e.g., ATP). D. Open channel, stabilized by C-terminal interactions with PIP_2 (stabilized by PIP_2 of both the selectivity filter and M2 (adapted from Loussouarn *et al.*, 2002).

Important advances were made with the identification of the structural basis of pH-dependent gating of the *Streptomyces lividans* potassium K_{csA} channel. Transition conformational states (open/close) were accomplish by motions of the selective filter and/or transmembrane domains, where the movement of the M1 and M2 domains narrow the pore and prevent ion flow across this section – figure 2.7.1. (Loussouarn *et al.*, 2002; Yellen, 2002).

Proks *et al.* (2001) demonstrated that mutations in selectivity filter modify the burst kinetics. This phenomenon is a little different between channels, but Choe *et al*. (1999) research with $K_{IR}1.1/K_{IR}2.1$ chimeras suggests that the P loop region of the channel contributes for this difference allowing selectivity.

Like other ionic channels, the K_V channels can exist in at least three distinct conformational states, open if they are conducting, closed or inactivated if non-conducting. The C-type inactivation that causes constriction of the conduction pathway results from structural changes in the selectivity filter (between the pore helix and S6).

Despite there are many homologies among the K_V channels; hERG channels have very distinct kinetics, with a slow activation and a rapid and voltage-dependent inactivation (ranging from 1 to 10 ms). Therefore, these unusual characteristics of gating kinetics play an important role in suppressing the premature beats propagation as well as in normal repolarization (Lu *et al.*, 2001).

According to these properties, the outward current through hERG is large and the most important determinant of the termination of the plateau phase of the action potential, mainly due to faster inactivation.

Regarding hERG channels, S6 transmembrane helices regions near the intracellular membrane are the activator gates. The analysis of its crystal structures has shown that when the channel is closed, its helices form a crossing bundle narrowing the channel opening and consequently avoiding passage of K^+ ions (Doyle *et al.*, 1998). When the helices splay outwards (dilating the pore) there is the transition to the open state.

Hardman *et al.* (2007) demonstrates in their research that a glycine to proline mutation (at position 657) results in permanently open channels, similar to the observed in K_V1-K_V4 channels where the hinge, that cause the dilation, is formed by a conserved PVP motif.

Transitions between conducting and non-conducting states in these voltage-gated ion channels are regulated by S1-S4 VSDs. In hERG S4 domain, it can be observed that the positive charge required to sense membrane voltage is provided by amino acids positioned every three residues between positions 525 and 538. Moreover, Subbiah *et al.* (2005) has shown that contributing most for slow activation there were the aminoacids K525, R528 and K538.

Following slow activation it comes fast inactivation. This process may occur by

inactivating one of protein terminal regions, N- or C- region. N-type inactivation is called the "ball and chain" mechanism, in which the N-terminus of the protein behaves like a blocker tethered to the cytoplasmatic side of the channel and directly occludes the pore. In contrast, C-type inactivation is thought to occur by a structural collapse of the pore and selectivity filter.

Several groups have tried to find a relationship between these two gating processes, if they were coupled and if they have the same S4-derived VSD. Pipper *et al.* (2003) and Yellen (2002) have shown, in human hERG fusion protein with GFP tagged in the C-terminal region, that the inactivation event was coupled to the activation itself. Nevertheless, since neither pharmacological nor site-directed mutagenic disruption of the inactivation process affected the change in fluorescence, they were unable to establish a link between the fluorescence change and the inactivation event.

One possible explanation could arise from all these data suggesting that the voltage sensor for inactivation may be different from that for activation (Perrin *et al.*, 2008).

II.8. Potassium Channel Blockers

Most K^+ channels blockers prolong the cardiac action potential duration (APD) and refractoriness without slowing impulse conduction. In other words, they exhibit Class III anti-arrhythmic actions (according to the Vaughan-Williams classification). Increasing arrhythmia wavelength and developing a block within the reentrant circuit they can, effectively, prevent/suppress re-entrant arrhythmias. These drugs bind to and block K^+ channels responsible for phase 3 repolarization. Therefore, blocking these channels slows repolarization and increases the action potential duration and the effective refractory period (ERP). This means that the refractoriness of a ventricular myocyte increases at lower heart rates, decreasing the susceptibility of the myocardium to early after-depolarizations (EADs) – figure 2.8.1. (de Boer *et al*., 2010b; Roepke & Abbott, 2006; Tamargo *et al.*, 2004).

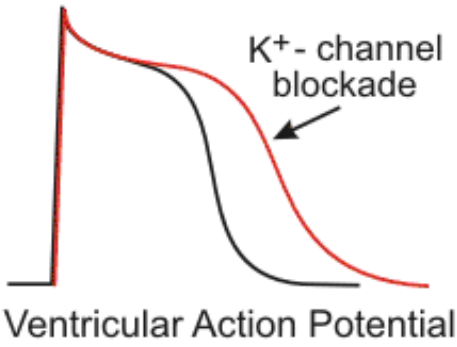

Figure 2.8.1. Delayed Repolarization by K^+-Channel Blockade (adapted from Mohrman, 2006).

Class III drug–induced action potential prolongation is usually caused by blockade of the rapidly activating component of the delayed rectifier K^+ current (I_{Kr}), although other currents are also being actively investigated as pharmacologic targets. For example, drug-induced inhibition of the slowly activating component of the delayed rectifier current (I_{Ks}), but not I_{Kr}, seems to lead to undiminished APD prolongation at short cycle lengths, commonly associated with arrhythmias. In contrast, pure block of I_{Kr} is associated with a diminution in drug-induced APD prolongation during fast heart rates. Available drugs and candidate blockers in advanced clinical trials, act by prolonging repolarization in both the atrium and ventricle. Clearly, agents that exert their electrophysiologic effects solely on atrial electrophysiology would be better for treating atrial arrhythmias because they would not have a risk of ventricular proarrhythmia (Roepke & Abbott, 2006; Mohrman, 2006; Sager, 2000; Petrich *et al*., 1992).

Common effects of class III anti-arrhythmics, on the electrocardiogram, are known: the increase of QT interval, and a prolongation of refractoriness. By increasing the ERP, these drugs are very useful in suppressing tachyarrhythmias caused by reentry mechanisms (Xie & Weiss, 2002).

Some drug examples of this therapeutic class are amiodarone, used in severe supraventricular and ventricular arrhythmias, dronedarone, bretylium, sotalol, etc.

All of these compounds like Class I compounds, are antiarrhythmic but may have also proarrhythmic effects. For example, the increase in action potential duration can produce a ventricular tachycardia called *torsades de pointes*, especially in patients with long-QT syndrome (Mohrman, 2006).

In recent years, a completely new class of compounds has been introduced. These are classified as hERG (or I_{Kr}) activators and their potential as anti- and proarrhythmic agents has been investigated. Initially, hERG channel activation was described for compounds otherwise accounted for as inhibitors to the channel. Under certain circumstances these inhibitors were observed to have transient activating effects. Common for these compounds is the ability to increase macroscopic hERG current in heterologous expression systems. Consequently, these compounds will result in a higher open probability of hERG channels at physiological potentials, which explain their activating properties. This implies that the hERG current is still primarily active in the repolarizing phase 3 of the action potential (Roepke & Abbott, 2006; Tamargo *et al.*, 2004).

II.8.1. Cardiac I_{K1} blockers

The importance of I_{K1} for proper cardiac function is emphasized by the fact that monogenetic diseases have been associated with mutations in all three respective channel complexes. So, specific I_{K1} blockers will have strong clinical benefits in gain-of-function mutations of $K_{IR}2.1$ channels, however, information about inhibitors and activators of $K_{IR}2.X$ channels is very sparse. A specific and exclusive ion channel blocker is difficult to find because ion channels can be inhibited by different mechanisms, such as direct interaction with the channel pore region, regulating lipid interaction and/or phosphorylation or by indirect interference with protein trafficking (de Boer *et al.*, 2010a). This is the reason why several drugs that inhibit I_{K1} by interfering with the channel pore morphology, also inhibit others channels.

Actually the only known blocker of these channels that has some specificity is Barium (Ba^{2+}), at low concentrations. However, its administration can cause severe poisoning problems related to skeletal, cardiac and smooth muscle dysfunctions (de Boer *et al.*, 2010a). Ba^{2+} ions are more potent blockers of I_{K1} than any other divalent ion and block the channel, extra-cellularly, at hyperpolarized potentials (Lopatin & Nichols, 2001). Imoto *et al.* (2007) have studied the mechanism underlying Ba^{2+}-induced automaticity in ventricular myocytes and showed that this effect, the blockage, was voltage- and time-dependent and quicker at more negative potentials.

In Liu *et al.* (2010) studies with Tenidap, an anti-rheumatoid drug, it was shown that this drug can, effectively, act as a specific activator of human $K_{IR}2.3$ channels without affecting the other $K_{IR}2.x$ members.

Celastrol is an anti-inflammatory and antioxidant drug that causes QT prolongation and reduction of ion channel density at the plasma membrane. Nevertheless, it is a non-specific drug since also inhibits I_{Kr} (Sun *et al.*, 2006).

Treatment with pentamidine, an antiprotozoal drug, has been associated with QT prolongation and U wave alterations. It inhibits I_{K1} by interacting with $K_{IR}2.1$ pore region from the cytoplasmic side and can also promote traffick defects by decreasing $K_{IR}2.1$ expression levels after a relatively long-term application.

Chloroquine, an antimalarial drug, also inhibits both $K_{IR}2.1$ and hERG channels. The inhibition of I_{K1} is due to blocking of the ion channel pore region from the cytoplasmic side (Rodriguez-Menchaca *et al.*, 2008). Besides the fact that this drug also inhibits another ion channel, it was demonstrated that chloroquine may also inhibit channel degradation (Jansen *et al.*, 2008).

Another drug that might inhibit I_{K1} is methoxamine (a α_1-adrenergic receptor agonist), through direct interaction with $K_{IR}2.1$. Stimulation of these receptors results in increase contractility through Ca^{2+} release from the sarcoplasmic reticulum and in a positive inotropic effect in the heart (Fedida *et al.*, 1991).

II.8.2. Pentamidine analogs

The antiprotozoal agent pentamidine belongs to the diamine family, and is a drug with antimicrobial properties given for prophylaxis and treatment of *Pneumocystis carinii* pneumonia and Leishmaniasis, a common opportunistic infection in patients who have contracted the human immunodeficiency virus or in patients' immunosuppressed during chemotherapy. The exact mechanism of its anti-protozoal action is unknown (it may involve reactions with ubiquitin) (de Boer *et al*., 2010a; Grunnet, 2010; Jansen *et al.*, 2008; Roepke & Abbott, 2006).

This drug can cause allergic and toxic side effects, most commonly having effects on the pancreas. Regarding the cardiovascular system, these effects are, usually, hypotension (which may be severe), malignant arrhythmias associated with a correct QT (QTc) prolongation and U-wave alterations and heart failure.

Most drugs known to have negative side effects (*e.g.* acquired long QT syndrome, short QT syndrome, and ventricular tachycardia) do so by direct trafficking blockade of the cardiac K^+ channel $K_v11.1$. This channel underlies the rapid component of the delayed rectifier K^+ current, I_{Kr}, in the human heart.

Pentamidine reduces I_{Kr} and prolongs the action potential, by acting on hERG ion channel protein transportation towards the sarcolemma. On the other hand, some studies (De Boer *et al.*, 2010a) demonstrate that, in isolated ventricular myocytes, pentamidine inhibits I_{K1} at clinical concentrations; it produces an acute block of $K_{IR}2.x$-based I_{K1} by plugging the pore region. Moreover, some acidic amino residues seem to be involved in the process of blockage of the outward I_{K1} (De Boer *et al*., 2010a; Jansen *et al.*, 2008).

Some cardiac arrhythmias associated with QTc prolongation and U wave alterations appear in treatments with pentamidine. This proarrhythmic effect was linked to inhibition of $K_v11.1$; however, since the U wave may be link to I_{K1}, de Boer *et al.* (2010a) studied the mechanism of pentamidine-mediated I_{K1} block. They observe an I_{K1} inhibition in $K_{IR}2.1$-HEK293 cells 10 min after drug application. Some molecular models suggest the interaction of three negatively charged amino acids E224, D259 and E299 in the cytoplasmic pore region of $K_{IR}2.1$ with pentamidine as responsible for cardiac inhibition of I_{K1}.

To determine a drug-related cardiac toxicity, hERG block is usually detected directly, by patch-clamp electrophysiology on the previously cloned hERG channel. Although $K_v11.1/I_{Kr}$ is most extensively studied, other cardiac K^+ currents may provide additional plausible substrates for acquired long QT syndrome, such as the slow component of the I_{Ks}, the ultra-rapidly activating delayed rectifier current I_{Kur}, or the transient outward current I_{to} (De Boer *et al*., 2010a).

II.8.3. Chloroquine

Like pentamidine, chloroquine (an antimalarial drug) blocks several currents, including Na^+, L-type Ca^+ and two K^+ currents. It inhibits both, I_{K1} and I_{Kr}, by blocking the ion channel pore region form the cytoplasmic side (Rodriguez-Rodriguez-Menchaca *et al.*, 2008). As a consequence, the cardiac action potential duration is longer, the maximum velocity diminished and automaticity enhanced. Moreover, these data indicate that the cardiovascular toxicity of this drug has a narrowed therapeutic ratio. In the micromolar range significant electrophysiological effects occur (Wagner *et al*., 2010; White, 2007; Ravens *et al*., 2004).

A special attention should be devoted to chloroquine. The LD_{50} value for $K_{IR}2.1$ channels seems to drop within the clinical range and chloroquine is associated with serious problems that include lethal ventricular arrhythmias (El Harchi *et al.*, 2009; Lopez-Izquierdo, 2009). Besides inhibiting two ion channels, it was demonstrated that chloroquine also interferes with channel degradation (de Boer *et al.*, 2010a; Jansen *et al.*, 2008).

The major responsible for these cellular and electrocardiographic side-effects is I_{K1} blockage and in a lesser degree, the block of the rapidly activating delayed rectifier, I_{Kr} (Lopez-Izquierdo, 2009).

El Harchi *et al.* (2009) searched for specific sites in the cytoplasmic domain responsible for $K_{IR}2.1$ inhibition and found out that the residues E224, D259 and E299 have strong binding determinants, whilst an alanine mutant D172A produced a comparatively small attenuation of chloroquine potency (Menchaca *et al.*, 2008). Nevertheless, chloroquine might still be an effective inhibitor of D172N-$K_{IR}2.1$ channels.

Nalos *et al.* (2011) studied several lysosomal inhibitors drugs, like chloroquine and have shown an intracellular granular accumulation of $K_{IR}2.1$ in what they presumed to be lysosomes.

II.9. Antidepressant drugs

Central nervous system is the major target of most of the drugs of this class, however, there are some studies referring some antidepressant-related cardiac side effects. Among those we may highlight, for instance, QT prolongation, associated with pharmacological inhibition of I_{Kr} current, carried by hERG channels, which is vital for normal ventricular repolarization (Hancox & Mitcheson, 2006; Pacher *et al.*, 2000). This characteristic is associated with an increased risk of potentially fatal polymorphic ventricular tachycardia and TdP.

To understand the mechanism of action of these drugs, it's important to recall "the main" neurotransmitter where they act, serotonin or 5-hydroxytryptamine (5-HT). It is produced mainly in the digestive tract (80%) and in the brain where it performs its primary function, helping to modulate synapses in serotonergic neurons. However, it can be found throughout the body, especially in blood platelets (Zhou *et al.*, 2007).

Once post-synaptic neuron activation has occurred, it releases neurotransmitter molecules back into the synapse where they are reuptaken by the pre-synaptic neuron for reuse in a future message.

Many researchers believe that an imbalance in serotonin levels, caused by problems in its production by brain cells, a lack of receptor sites or the inability of serotonin to reach them, or even a shortage in tryptophan (serotonin precursor), may influence memory, learning and mood leading to depression, as well as obsessive-compulsive disorders, anxiety and panic (Thomas *et al.*, 2002; Rang *et al.*, 2001).

There are three other major classes of antidepressant drugs besides selective serotonin reuptake inhibitors (SSRIs). They are: monoamine oxidase inhibitors, tricyclic compounds, and atypical antidepressant drugs with different primary molecular targets (Briley & Moret, 1993).

II.9. 1. Selective serotonin reuptake inhibitors – Fluoxetine (Prozac)

Depression is associated with reduced levels of some monoamines in the brain including 5-HT. A presynaptic neuron action potential stimulates Ca^{2+}-dependent exocytic release of serotonin from presynaptic vesicles into the synaptic cleft, interacting with both post- and presynaptic receptors. At the presynaptic side, 5-HT activates receptors (HTR1A, HTR1B and HTR1D), and attenuate its exocytosis. This feedback loop regulates the 5-HT concentration in the synaptic cleft and therefore, the extent of stimulation of various HT receptor subclasses at the postsynaptic membrane (Boadle-Biber, 1993). Prolonged administration of SSRIs desensitizes these feedback loops. Thus, their inhibitory effects on the serotonergic neurotransmission are weakened (Briley & Moret, 1993).

So, the selective 5-HT re-uptake inhibitors (SSRIs) are thought to restore the levels of

5-HT in the synaptic cleft by binding at the 5-HT reuptake transporter preventing the reuptake and subsequent degradation of 5-HT. This reuptake blockade leads to the accumulation of 5-HT in the synaptic cleft and the concentration of 5-HT stabilizes within the normal range. This action of SSRIs is thought to contribute to the alleviation of the symptoms of depression. In the presence of the SSRI, small amounts of 5-HT continue to be degraded in the synaptic cleft (Rang *et al.*, 2001).

Chronic administration of antidepressant treatments have been reported to commonly increase the expression of brain-derived neurotrophic factor (BDNF), an activity-dependent secreted protein that is critical to organization of neuronal networks and synaptic plasticity (Drago *et al.*, 2009; Balu *et al.*, 2008).

The molecular target for SSRI is SLC6A4, resulting in an inhibition of 5-HT reuptake in the presynapse from the synaptic cleft (Cipriani *et al.*, 2009; Demyttenaere *et al.*, 2008; Donati & Rasenick, 2003; Hiemke & Hartter, 2000). SSRIs have high affinity for 5-HT reuptake transporters, low affinity for noradrenaline reuptake transporters, and very low affinity for neurotransmitter receptors – figure 2.9.1..

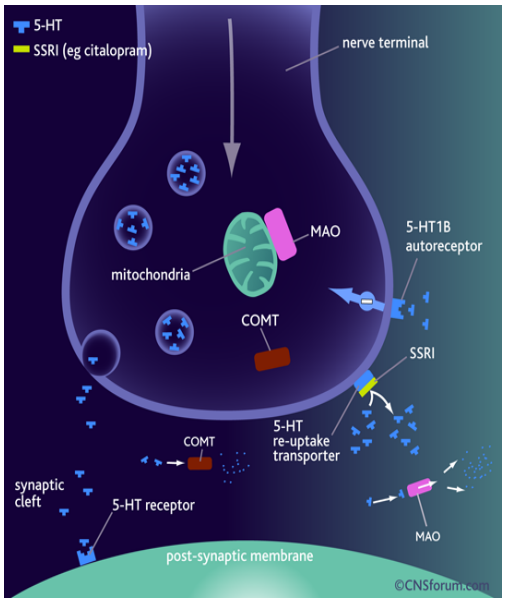

Figure 2.9.1. The SSRIs normal mechanism in combination with MAOIs (Rang *et al.*, 2001).

Since the reuptake of 5-HT is prevented, these molecules end up staying in the synapse longer than they normally would, and get more of a chance to activate the post-synaptic neuron.

Fluoxetine is widely used for the treatment of depression, but should not be used in combination with monoamine oxidase inhibitors (MAOIs), another type of antidepressants, since they increase serotonin amount in the synaptic cleft leading to serotonin syndrome (Qu *et*

al., 2009). Although flouxetine pharmacological profile has been studied extensively in animals and human, the mechanism of drug action related to its clinical efficacy remains poorly understood. In mammals, the cytochrome P450 activity biotransforms fluoxetine to norfluoxetine – figure 2.9.2. – (Hiemke and Härtter, 2000), increasing the half-life of this drug from 1-4 days to 4-16 days, respectively. It is generally believed that a significant part of the therapeutic activity of fluoxetine is attributable to its most important active metabolite norfluoxetine (Qu *et al.*, 2009; Paterson & Metcalfe, 2008).

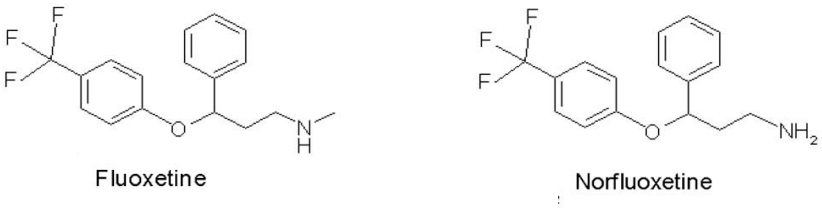

Figure 2.9.2. Structure of fluoxetine and its metabolite norfluoxetine (adapted from Hiemke and Härtter, 2000).

Antidepressant drugs such as clomipramine (Jo *et al.*, 2008), doxepin (Duncan *et al.*, 2007), norfluoxetine (Choi *et al.*, 2000) and fluoxetine (Hancox & Mitcheson, 2006) are known for their inhibitory activity on hERG potassium channel and affect its intracellular trafficking – figure 2.9.3.

Studies by Rajamani *et al.*, 2006, Kobayashi *et al.*, 2003 and Thomas *et al.*, 2002 have noted a straight relationship between K_{IR} and hERG channels during phase 3 and 4 of the cardiac repolarization curve suggesting that their functional similarity probably explains why drugs that affect one of these channels frequently also disturb the others.

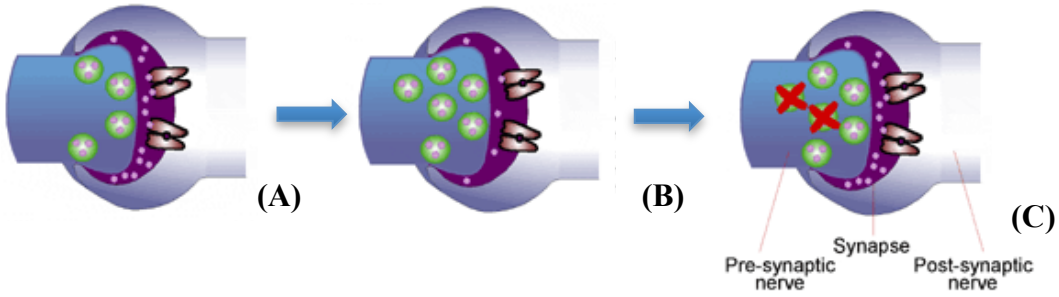

Figure 2.9.3. Mechanism of action of fluoxetine. (A) Usually serotonin is released into the synapse to activate the postsynaptic neuron. (B) In a depressed status, serotonin is reabsorbed too quickly by the pre-synaptic neuron. The low-concentration of serotonin in the synapse prevents activation of the post-synaptic neuron and neuronal electrical impulse propagation. (C) Under fluoxetine administration (Prozac), serotonin reuptake receptors are inhibited, keeping the concentration of serotonin in the synapse high enough to carry nerve impulses across the synapse (adapted from Rang *et al.*, 2001).

Jo *et al.* (2008) has shown that the tricyclic antidepressant clomipramine induces a concentration-dependent decrease in the current amplitude at the end of the voltage steps and inhibit the channels with an IC_{50} value of 0.13 μM. The hERG channels were mainly affected in the open and inactivated states but not in the closed state.

The increased heart rate by tricyclic antidepressants is due primarily to the anticholinergic action, whereas the EKG changes and conduction disturbances induced by the drugs are largely the result of direct membrane effects.

Blockage of the pore by extracellular Ba^{2+} and Cs^{+}, both open K_{IR} channel typical blockers, shows a concentration-, strong voltage- and time-dependent behavior. Regarding fluoxetine, although channels inhibition has shown to be concentration-dependent, it is only slightly dependent on voltage and time with a predominant effect on the instantaneous current. It was suggested that fluoxetine probably causes a conformational change in the channels, but does not act as typical blockers, although all these molecules act at the channels from the extracellular side (Barbey & Roose, 1998).

Recent studies have also shown that fluoxetine inhibits the function of several receptors and ion channels, such as 5-HT2C and 5-HT3 receptors, nicotinic acetylcholine receptors, voltage-gated Ca^{2+}, Na^{+} and K^{+} channels and Cl^{-} channels. These effects might be involved in the molecular and cellular mechanisms underlying the multiple therapeutic effects and side effects of fluoxetine (reviewed in Kobayashi *et al.*, 2003).

In an attempt to understand fluoxetine effects on the heart, and more specifically in K^{+} channels, Pacher *et al.* (2000) analyzed guinea pig, rabbit and canine ventricular myocytes and papillary muscles using conventional and whole cell voltage clamp techniques and noted a significant shortness in APD with low concentrations of this drug (< 10 μM) with no effect on resting membrane potential curve, an event occurring with higher fluoxetine concentrations (100 μM) – figure 2.9.4. Since they were using cardiomyocytes, they were able to measure and analyze several ionic channels and their currents. They observed a concentration-dependent block of Ca^{2+} current. However, these effects were quickly and also fully reversible. As they didn't verify any changings in the amplitude of K^{+} currents (I_{K1}, I_{to}), they assumed that proarrhythmic effects of antiarrhythmic (classes I and IV) and fluoxetine in other clinical studies may be due to the inhibition of cardiac Ca^{2+} and Na^{+} (Pancrazio *et al.*, 1998).

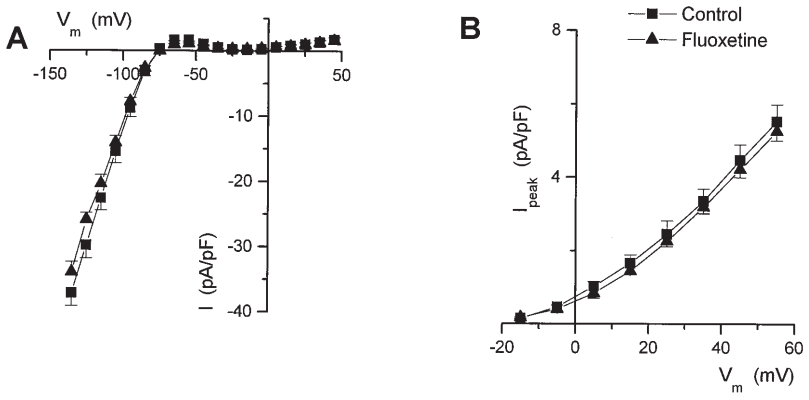

Figure 2.9.4. Effect of fluoxetine (10μM) on I_{K1} current voltage. A. Steady-state current voltage/intensity curve obtained in five cells with test pulses of 400 ms. Small differences are observed between control and fluoxetine. B. Transient outward current densities measured as a function of the test potential. (adapted from Pancrazio *et al.* 1998).

In outer hair cells isolated from guinea pig cochlea this drug potently inhibited leak K^+ currents, showing a reversible and voltage-independent behavior. At concentrations higher than plasma levels, fluoxetine reversibly blocked voltage-activated K^+ currents (Traboulsie *et al.*, 2006).

Although fluoxetine and realated antidepressant drugs are generally believed to cause fewer proarrhythmic side effects compared with tricyclic antidepressants – TCAs block Na^+ channels in cardiac tissues, an effect that may contribute to their alteration in cardiac conduction and dysrhythmogenic actions (Bian *et al.*, 2002) – serious concerns have been raised by case reports of tachycardia and syncopes associated with fluoxetine treatment.

II.10. hERG blockers

Rajamani *et al.* (2006) and Kennard *et al.* (2005) studying hERG expressing cells treated with fluoxetine and its major metabolite norfluoxetine (via the cytochrome P450 metabolization) showed a dual effect. One by direct channel block, where the drugs binds to a unique structurally unique receptor domain in the pore region and other by selective disruption of hERG channel protein trafficking to the cell surface where fewer mature hERG channels reach the cell membrane, thus reducing I_{Kr}.

Another compound that reduces I_{Kr} by interfering with channel trafficking, is geldanamycin, a benzoquinoid antibiotic that specifically inhibits the cytosolic chaperone Hsp90. Drug derivatives are being tested in clinical trials for the treatment of various forms of

cancer with no reports of adverse cardiac events currently available – figure 2.10.1. (Caballero *et al.*, 2010; Persson, 2007; Kuryshev *et al*., 2005; Ravens *et al*., 2004).

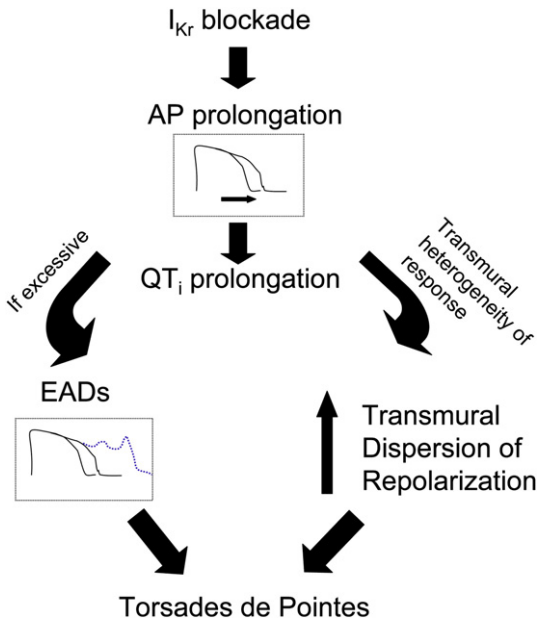

Figure 2.10.1. Schematic diagram of Torsades de Pointes (TdP) arrhythmia caused by I_{Kr} inhibition. The consequence of I_{Kr} blockage may lead to AP prolongation at the cellular level, reflected by QT interval (QT_i) prolongation in the EKG. If excessive, it could lead to early after-depolarization (EAD's). In the heart, the transmural dispersion of repolarization (TDR) is heterogeneous and related with the differential effect of I_{Kr} blockage across the ventricular wall, forming a substrate for re-entrant arrhythmia. EAD's and enhanced TDR may combine to produce sustain TdP (adapted from Hancox *et al.*, 2008).

Due to the lack of data, at atomic resolution, for the TM complex structure, the molecular basis of hERG blockade is not well understood. Several groups (Chen, *et al.*, 2002; Mitcheson *et al.*, 2000) studied S6 segment of the TM domain, since it seems to be critical in forming the ligand-binding cavity and showed that there were two residues (Y652 and F656) primarily responsible for the high affinity interaction of these channels with a number of known ligands, such as cisapride, terfenadine, quinidine, dofetilide, and MK499.

II.11. Clinical disorders

After studying the I_{K1}, it's also important to highlight its role in channel disorders. Various genetic approaches confirmed that $K_{IR}2.x$ channels underlie cardiac I_{K1}, whereas $K_{IR}2.2$ contributes less than $K_{IR}2.1$ and that this current should be a 'first responder' during hypoxia and cardiac ischemia (Piao *et al*., 2007).

Disorders of these channels or channelopathies include several clinical issues that started to be study fifteen years ago, with the identification of a defective ion channel gene that could cause abnormal prolongation of the QT interval on the EKG (Anumonwo & Lopatin, 2010).

Currently, there are many channelopathies associated with the up-regulation and down-regulation of I_{K1}, associated with gain-of-function or loss-of-function mutations in KCNJ2 gene (encodes $K_{IR}2.1$ subunits). They include short QT syndrome (SQTS), catecholaminergic polymorphic ventricular tachycardia (CPVT), familiar atrial fibrillation (FAF), chronic atrial fibrillation and Andersen-Tawil syndrome (ATS) also known as long QT syndrome type 7 (LQTS) and heart failure (HF), respectively – figure 2.11.1. (Anumonwo & Lopatin, 2010; Knollmann & Roden, 2008; Seemann *et al.*, 2007; Deslile *et al*., 2004).

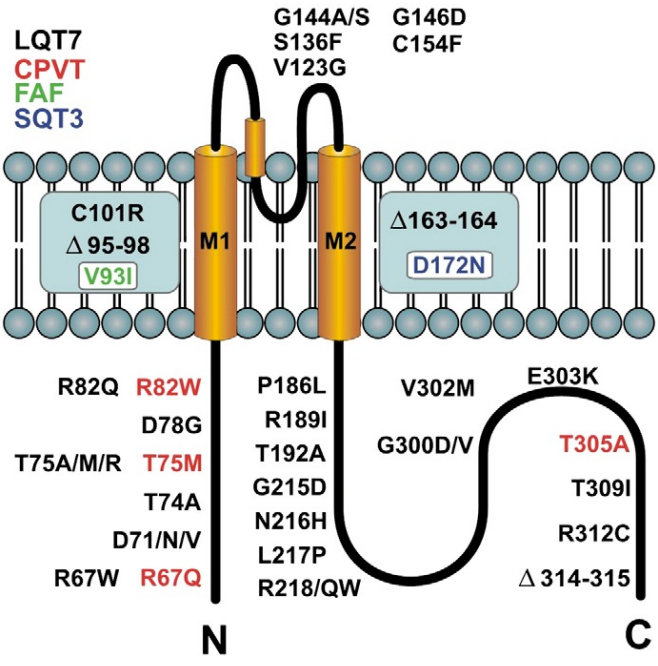

Figure 2.11.1. Channelopathies of the inward rectifier channel. The mutant residue of LQT7 is colored black; CPVT is red, FAF green and SQT3 in blue (adapted from Anumonwo & Lopatin, 2010).

II.11.1. Short QT Syndrome

SQTS is an inherited disorder that predisposes patients to life-threatening arrhythmias, most likely to ventricular fibrillation, characterized by a remarkable accelerated repolarization. To date, three genes (KCNH2, KCNQ1 and KCNJ2) encoding different K^+ ion channels involved in repolarization have been linked to the syndrome. Mutations-dependent effects on the action potential are shown in figure 2.11.2. (Brugada *et al*., 2005; Priori *et al*., 2005).

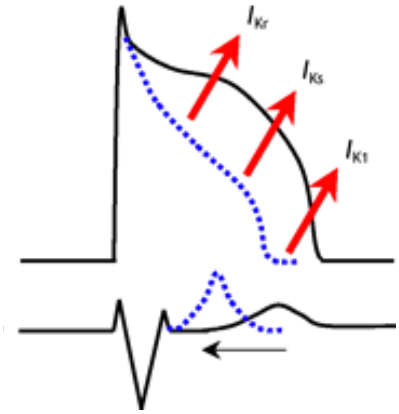

Figure 2.11.2. A. Schematic representation of the action potential in gain-of-function mutations in three different K^+ channels, where the cardiac action potential shortens and the QT interval decreases (adapted from Brugada *et al*., 2005).

The KCNH2 gene is often referred to as hERG, the human ether-a-go-go–related gene, which expresses a protein that makes up the K^+ channel responsible for the rapidly activating rectifier outward current (I_{Kr}) (Shagufta *et al.*, 2009). Brugada *et al.* (2005) has identified that an asparagine is substituted for a lysine at codon 588, an area at the outer mouth of the channel pore, leading to a larger outward K^+ current in the ventricles (Brugada *et al.*, 2005).

The KCNQ1 gene encodes a subunit of the channel responsible for the slowly activating delayed outward K^+ current (I_{Ks}) and a relevant mutation in this protein was first identified by Bellocq *et al*. (2004) in a 70-year-old man with ventricular fibrillation and a shorter QT interval after resuscitation. The amplitude obtained with this mutation is similar to that of a normal channel. Nevertheless, the short QT syndrome mutated phenotype may be explained by three facts: i) the pronounced shift of the half-activation potential, ii) channel activation at more negative potentials and iii) accelerated activation kinetics, which led to the gain of function in the outward current (Bellocq *et al*., 2004; Jongsma & Wilders, 2001; Tristani-Firouzi *et al*., 2001).

SQT3 is associated with a gain-of-function mutation in KCNJ2 gene and is characterized by asymmetrical T waves associated with an abnormally rapid terminal phase and a. This causes a significant increase in the outward current leading to an accelerated

repolarization. The mutation in this gene resulted from a change at position 172 of an aspartic acid to an asparagine (D172N) in the M2 transmembrane region of the $K_{IR}2.1$ channel – figure 2.11.1. a position highly conserved that plays a critical role for inward rectification in these channels (Priori *et al.*, 2005; Tristani-Firouzi *et al*., 2001; Boyden, 1996).

Computer simulations in a human ventricular myocyte model showed that mutations in KCNJ2 gene result in an up-regulation of I_{K1}, which greatly accelerates the final phase of repolarization and shortens the cardiac APD (Xu *et al.*, 2007).

Recently, Rodriguez-Menchaca *et al*. (2008) have demonstrated that chloroquine can block $K_{IR}2.1$ ion channels by interacting with the cytoplasmic domain of the channel, instead the transmembrane pore. A year after, El Harchi *et al*. (2009) have shown that $K_{IR}2.1$ channels with D172N mutation results in a greater outward current. Furthermore, since chloroquine is an effective pharmacological inhibitor of I_{K1}, compounds with chemical similarity to chloroquine could assuage the condition caused by the increase of $K_{IR}2.1$ function.

The heterogeneity of the disease or the three forms of short QT syndrome is linked to three different K^+ channels and respective currents at different voltages.

II.11.2. Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

CPVT was first recognized in 1975 and is a heritable electrophysiological disorder in which patients frequently present ventricular arrhythmia and, consequently, sudden cardiac death triggered by exercise and/or acute emotional stress. Ekhard *et al.* (2007) reported four mutations in $K_{IR}2.1$ channels (R67Q, R85W, T305A, T75M) – figure 2.11.1., which revealed reduced outward currents. It was also shown that T305A mutation selectively reduced this outward current but increased inward rectification, therefore affecting channel rectification properties. Another loss-of-function mutation, V227F, was shown to be relating to CPVT by adrenergic stimulation through protein kinase A (PKA)-dependent $K_{IR}2.1$ phosphorylation (Vega *et al.*, 2009).

II.11.3. Familiar Atrial Fibrillation

Xia *et al.* (2005) identified another gain-of-function mutation in KCNJ2 gene in FAF, which is a heart rhythm disturbance characterized by an irregular and rapid activation of the atrium. It is the most common sustained cardiac arrhythmia and a major cause of morbi-mortality in developed countries (Go *et al.*, 2008). These researchers demonstrated the association of this disorder with a single valine-to-isoleucine substitution at position 93 (V93I) of $K_{IR}2.1$ channels – figure 2.11.1., with a significant increase in both inward and outward

current without affecting the kinetics and rectification properties of the channels. This valine is a highly conserved amino acid in $K_{IR}2.1$ proteins among different species.

Discovery of mutations in families with AF has provided compelling evidence that genetic factors can have a role in the development of AF.

For study the effect of overexpression of $K_{IR}2.1$, Li *et al.* (2004) using a transgenic mouse model, observed that this phenomenon results in the up-regulation of I_{K1} leading to multiple abnormalities of cardiac excitability, including marked shortening in APD and ERP, and near-complete elimination of the slow T wave, with a short QT interval.

II.11.4. Chronic Atrial fibrillation (CAF)

In clinical practice, this is the most frequently encountered and sustained cardiac arrhythmia, characterized by non-coordinated atrial activation and at a long term deteriorating it mechanical function and where up-regulation of I_{K1} is a consistent finding. CAF is most commonly classified according to its temporal pattern and it is persistent when exists for a period longer than a year and the treatment (cardioversion) is unsuccessful.

Zhang *et* al. (2005) have performed computer models of electrical activity of human atrial cells to screen the remodeling of I_{K1}, contributing to APD shortening.

II.11.5. Andersen-Tawil Syndrome (ATS)

ATS or LQTS is a congenital disorder manifested by prolongation of the QT interval and a large U wave on EKG, cardiac arrhythmias, periodic paralysis, and dysmorphic features (Perez Riera *et al.*, 2008). In this subtype, K^+ ion channels are blocked, or they open for a shorter period than the regular one, or they are open with a delay, resulting in a decrease of the K^+ outward current and prolong repolarization.

Plaster *et al*. (2001) have documented several missense mutations in KCNJ2 gene in these patients. In this research, nine mutations were identified, all in highly conserved amino acids. It was shown that two of these mutations outcome from amino acid changes that resulted in loss-of-function and a dominant effect in I_{K1}. This dominant negative effect means that the mutated subunit can suppress completely tetramer channel function. Nowadays, more than 30 mutations in this gene are associated with ATS1 – figure 2.11.1. (Anumonwo & Lopatin, 2010).

Most of the ATS1 mutations result in loss-of-function or suppression in the $K_{IR}2.1$ channels either through a trafficking dysfunction or by preventing the molecule phospholipid phosphatidylinositol 4,5-bisphosphate – PIP_2 – from binding to the channel and effectively regulating their activity (Lopes *et al.*, 2002).

Miake *et al.* (2003), using a $K_{IR}2.1$ mutant dominant-negative expression, demonstrated that suppression of I_{K1} decelerates the AP repolarization, prolongs APD, and depolarizes the resting membrane potential, where significant alterations on the EKG were observed in transgenic mice with suppressed I_{K1}. Electrophysiologic studies in these patients are characterized by a development of delayed after depolarization (DAD) and ventricular arrhythmias suggesting for the contribution of the reduced $K_{IR}2.1$ current.

Studies from Ma *et al*. (2007) on the valine-to-methionine (V302M) ATS1 mutation revealed that this position is critical to maintain the $K_{IR}2.1$ K^+ conduction pathway in an open state through the interaction with PIP_2.

Bendahhou *et al.* (2007) demonstrated that corticosteroid intake can triggered ATS's symptoms in some patients, and despite the mechanism underlying this phenomenon is still unclear, the use of corticosteroid in these patients' treatments should be taken carefully.

II.11.6. Heart Failure (HF)

HF is a condition characterized by an inability of the heart to supply sufficient blood flow to the body's needs and a down-regulation of I_{K1} is often observed. The prolongation of APs is a characteristic that is believed to predispose the heart to a delay depolarization and reentrant arrhythmias where the remodeling of I_{K1} contributes for this. However, this mechanism is still unclear. Elevated levels of Ca^{2+} in the diastole may block the channel by reducing I_{K1} or through PKC-dependent mechanisms (Fauconnier *et al*., 2005, Nattel, 2003).

The understanding of the molecular mechanism of I_{K1} in several pathological conditions like this, might offer new and potential therapeutic targets to prevent life-threatening arrhythmias (Fauconnier *et al*., 2005).

These clinical phenotypes of the above-inherited cardiac diseases, taken together implicate an important role and explain the significance of I_{K1} in cardiac excitability.

II.11.7. Mechanisms of hERG channel dysfunction

According to its function it is natural to expect that mutations that affect hERG channels would have deleterious effects on cardiac electrical activity – figure 2.11.3. So, the understanding of activation/inhibition mechanisms will definitely contribute to improve the physiologic knowledge of hERG channels and development of new therapies (Perrin *et al.*, 2008).

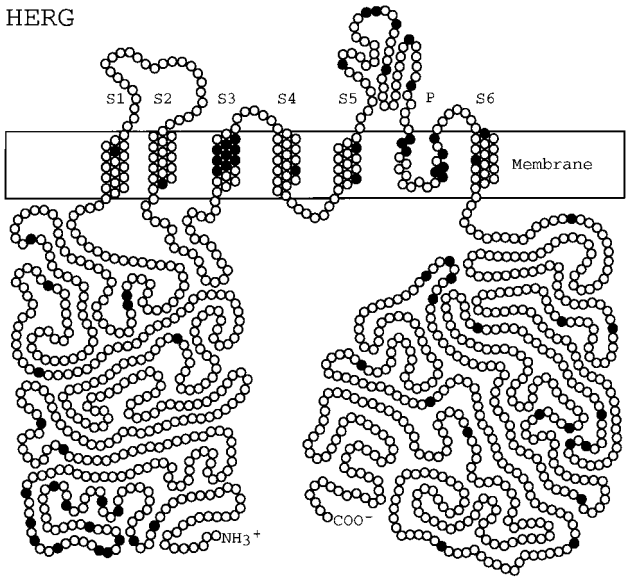

Figure 2.11.3. hERG mutations. The black dots represent mutations associated with LQT syndrome (adapted from Splawski *et al.*, 2000).

Berthet *et al.* (1999) studied two LQT syndrome-related mutations and used low levels of K^+ (hypokalemia) as a trigger factor of TdP. They showed that these mutations, in *HERG* C-terminus, imply that this region plays a significant role in cardiac repolarization. Nevertheless, they may be less malignant than mutations occurring in the pore region.

III. Materials and methods

III.1. Cell culture

All cell lines were regularly cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with FBS 10% fetal calf serum, 2 mM L-glutamine, 50 µg/ml streptomycin, and 50U/ml penicillin (all from Lonza, Verviers, Belgium) and incubated in a 95% humidified air and 5% CO_2 atmosphere at 37ºC.

HEK-KWGF cells are human embryonic kidney (HEK-293) cells that stably express murine wild type (WT) $K_{IR}2.1$ with green fluorescence protein (GFP) tagged in the C-terminal.

HEK-HERG cells are also human embryonic kidney cells that stably express hERG channels.

III.2. Pharmacological treatment

The cells were treated with several drugs in order to perform molecular and electrophysiological analysis of hERG and K_{IR} proteins and I_{K1}, respectively.

Fistly, all drugs were dissolved in water to provide a stock solution for future uses, sterilized by filtration (0.22 mm), aliquoted and stored at -20°C until use. They have been prepared stocks of: Pentamidine-isethionate (Pentacarinat® 300, Sanofi Aventis, Gouda, The Netherlands) – 0.1 M; Chloroquine (Sigma®, St. Louis, MO, USA) – 0.1 M; 2EVK, a pentamidine analog, (Abbott ®, USA, dissolved in 4%DMSO) – 1 mM; Fluoxetine (Abbott®, USA) – 30 µM; Tunicamycin (AppliChem mbH®, Germany) – 10 µg/mL; As_2O_3 (Sigma®, St. Louis, MO, USA) – 0.1 M.

III.3. Immunofluorescence microscopy

HEK-KWGF cells were cultured on 12-well cell culture plate containing one circle laminin-coated glass coverslip (⦸12mm) (Menzel-Gläser, Braunscweig, Germany) and stimulated for 24 and 48 hours, individually, with several drugs. After this period, the cells were washed with phosphate buffered saline (PBS) (Lonza ®), fixed using 3% paraformaldehyde in PBS for 25 minutes and then washed with PBS. Afterwards, cells were quenched twice with PBS containing 50 mM glycine for 10 minutes. Non-specific binding was blocked by further incubation in NET-gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.05% Igepal). HEK-KWGF cells were left overnight at room temperature with anti-GFP (Santa Cruz Biotechnology®) primary antibody diluted in NET-gel. Non-bound antibodies were removed by washing 5 times with NET-gel, followed by incubation of 2 hours with anti-rabbit secondary

antibody (GAM-HRP cat. No. 711-075-152, Jackson Imunoresearch®) alone or with 4',6-diamino-2-phenylindole, dihydrochloride (DAPI) (cat. no. D-1306; Molecular Probes®, Leiden, The Netherlands) to stain cell nuclei. Non-bound antibodies were removed again by 5 washes with NET-gel, coverslips were mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and sealed with nail polish. Cells were examined using a Nikon Optiphot-2 epifluorescence-equipped microscope and photographs were taking at 400x and 600x amplification using a Nikon DS-Fil digital camera. Images were adjusted for brightness and contrast using Adobe Photoshop CS5.

III.4. Western Blot

HEK-HERG and HEK-KWGF cells were harvested with cell scrapers (Corning-Costar®) from cell culture dishes, washed with PBS and dissolved in lysis buffer D consisting of 1% Triton X-100, pH 7.6, 125 mM NaCl, 20 mM HEPES, 10% glycerol, 1 mM EDTA, and 1mM EGTA. Lysates were incubated on ice for 15 minutes and clarified by centrifugation at 14,000 g for 5 minutes at 4ºC.

After centrifugation, the total level of proteins in the solution (quantification) was assessed in supernatant using the bicinchoninic acid assay (BCA method) in a spectrophotometer with the transmission set at 562 nm.

Sample concentration was then uniformly prepared, adding a certain amount of water and 20 µL of loading buffer to a total volume of 100 µL. The remaining sample was stored at -20ºC.

Before electrophoresis, HEK-KWGF samples were heated to 60-65ºC for 5 minutes and HEK-hERG samples were heated at body temperature (37ºC) for the same time.

Proteins (25 µL) were separated by sodium dodecyl sulfate-10% polyacrylamide gel (10% SDS-PAGE) and subsequently electro-blotted onto nitrocelulose membrane (Bio-Rad®, Veenendaal, The Netherlands). Protein's transfer was assessed by Ponceau S staining (Sigma®). Blots were blocked with 5% non-fat milk in TBST (20 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 60 minutes, washed 4 times for 5 minutes in TBST and incubated with the primary antibodies anti-hERG (cat. no. Sc-18708; Santa Cruz Biotechnology®) or anti-GFP (cat. no. Sc-9996; Santa Cruz Biotechnology®) and GAPDH (Milipore®, California) diluted in TBST, respectively, overnight. For secondary antibody incubations, the blots were washed 5 minutes 4 times in TBST, incubated with anti-mouse and anti-rabbit DaG-HRP (cat. no. 711-057-152; Jackson ImmunoResearch®) secondary antibodies, correspondingly, for 2 hours and washed again 5 times 5 minutes in TBST. Secondary antibodies were finally detected using a

standard ECL procedure (Amersham Bioscience, Roosendaal, The Netherlands).

III.5. Statistical analysis

All data was statistical analysed. Group averages are presented as mean ± standard error of the mean. Differences between group averages were tested using a one-way ANOVA with a post-hoc test (Dunnett's Multiple Comparison and Bonferroni's All Pairs Comparison) in the case of more than two groups. For these treatment it was used Kaleidograph 4.1 software (Synergy Software, Reading, PA, USA) and the results would be consider significant for $P < 0.05$.

IV. Results and Discussion

IV.1. Pharmacological treatment

In order to figure out the effect of several drugs in $K_{IR}2.1$ or hERG intracellular trafficking, HEK-KWGF or HEK-HERG cells were incubated with a particular drug for 48 hours, respectively.

To overcome the problem of channel interference and to obtain much higher specific I_{K1} or I_{Kr} currents we have chosen to use these two cell lines instead of cardiomyocytes.

As said before, the drugs tested were pentamidine and its analog, 2EVK, tunicamycin, a glycosylation inhibitor, fluoxetine and its biometabolite norfluoxetine, a widely used anti-depressive, arsenic trioxide – As_2O_3, a drug used in some types of leukemia treatments and chloroquine as a negative control – table 4.1.1.

Until now, all the studies that have analysed both channels and their currents showed that when one current is affected the other is also altered. Kuryshev *et al.* (2005) studied the effects of pentamidine in hERG expressing cells and concluded that it reduces hERG current and inhibits trafficking and hERG maturation. With a concentration of 10μM it also prolonged the APD_{90} from 374,3 ± 57,1 to 893,9 ± 86,2 ms. De Boer *et al*. (2010a) have found that this drug also blocks the inward rectifier current I_{K1} carried by most $K_{IR}2.x$ family members and partially mimic polyamines, plugging the cytoplasmatic pore. Moreover, some acidic amino residues seem to be involved in the process of blockage of the outward I_{K1}. Nalos *et al.* (2011) has shown that pentamidine is a pro-arrhythmic drug that acts on $K_{IR}2.1$, decrease its current and the amount of total protein.

Studies with tunicamycin related with the heart (Buckley & Whorton, 1997; Friedman *et al.*, 1988) referred that it increases the permeability of intracellular Ca^{2+}, leading to depletion of this store and activation of Ca^{2+} influx across the plasma membrane. Treatment with 5 μg/ml caused almost the complete loss of cells.

Thomas *et al.* (2002) tried to determine the electrophysiological basis for the arrhythmogenic potential of fluoxetine and discovered that this drug blocked hERG channels with an IC_{50} value of 3.1 μM.

Ficker *et al.* (2004) demonstrated that long exposure to As_2O_3 neither block cardiac I_{Ks} nor I_{K1} currents, however increased cardiac Ca^{2+} currents and reduces surface expression of hERG at clinically concentrations

Jansen *et al.* (2008) studied chloroquine and demonstrated that this drug can increase the amount of intracellular K_{IR} protein within hours and found the presence of a degradation product, probably by N-terminal cleavage.

Meanwhile during these treatments it was observed that cellular growth rate was extremely affected by increasing chloroquine concentration. . However, with pentamidine it was observed always a decreased in cell number of K_{IR} and hERG expressing cells and lower growth rates as comproved by other groups (Nalos *et al.*, 2011; de Boer *et al.*, 2010a).

With drugs like hexamidine, arsenic trioxide or 2EVK no decrease in cell number was observed and cellular growth presented a normal rate.

The only drug with a markedly augmentation in both cells was fluoxetine, where after 24 hours it was clearly the increase of the number of cells.

When tunicamycin was used, an agent that blocks all N-linked glycoprotein synthesis and causes a G1 cell arrest, in a concentration of 5µg/mL for 24 hours all the cells were dead. With 1µg/mL tunicamycin the number of K_{IR}2.1 and hERG expressing cells was reduced after 24 h and there were no cells after 48 hours.

Table 4.1.1.
Cell growth after 48h drug incubation (not by direct counting of the number of cells)

Drug	Concentration	Growth rate	
		K_{IR} cells	hERG cells
Pentamidine	10 µM	Diminish	Diminish
Tunicamycin	5µg/mL	Cells died	Cells died
	1µg/mL	Diminish	Diminish
2 EVK	0,2 µM	Normal	Normal
Fluoxetine	30 µM	Increase	Increase
As_2O_3	1 µM	Normal	Normal
Chloroquine	10 µM	Diminish	Diminish

IV.2. Molecular analysis – Western Blot

IV.2.1. hERG cells

As said before, if a drug affects I_{K1} it is expected to affect also I_{Kr}. To see if that drug activity is felt at the level of protein traffick and maturation, a western blot was done. In HEK-HERG cells each hERG subunit is presented in two forms: as a N-glycosylated immature form (~ 135 kDa) and as a fully N-glycosylated mature form (~155 kDa) (Zhou et al., 1998) – figure 4.2.1.

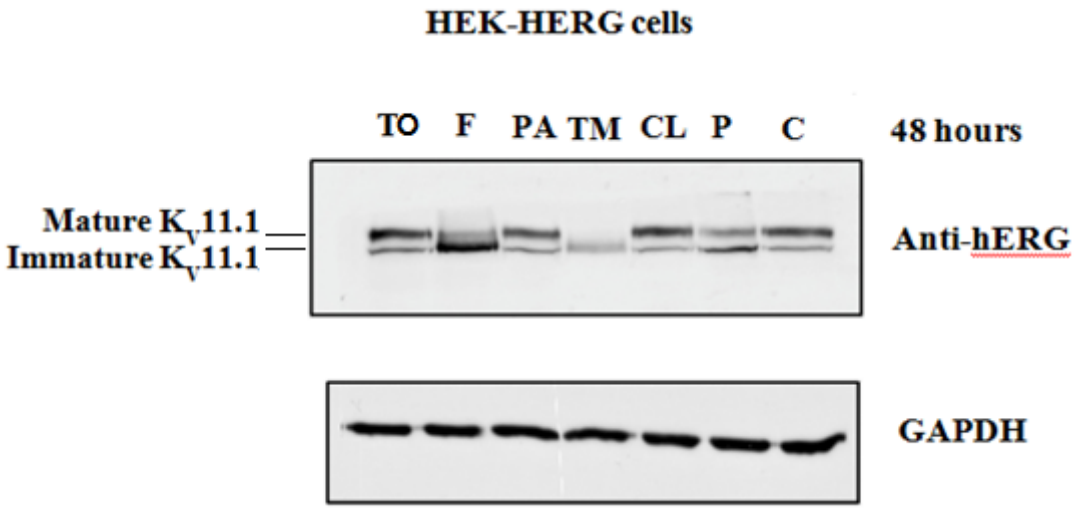

Figure 4.2.1. Western blot for hERG. HEK-HERG cells were incubated for 48 hours with the respective drug and processed for western blot. It can be observed a decrease in the mature hERG subunit with pentamidine (P), its nonappearance with tunicamycin (TM) and an a increase in the immature hERG subunit with fluoxetine (F). The other samples, treated with As_2O_3 (TO), 2EVK (PA) and chloroquine (CL) had the same band pattern as non-treated cells (C).

When mature and immature hERG form appear, it means that we have the normal trafficking pathway and therefore the correct protein folding and as a consequence the appropriate function of the channel at the cell surface. In the figure above, as expected, both bands can be observed in untreated cells, nevertheless, the mature band appears stronger because more mature hERG forms are localized at the membrane to play their role. The same is true for As_2O_3, 2EVK and chloroquine. With pentamidine, as predictable, the immature band has a stronger signal and less proteins reaches cell surface, explaining why APD gets longer leading to prolongation of QT interval after pentamidine administration (Robinson *et al.*, 2008; Cordes *et al.*, 2005; Kuryshiev *et al.*, 2005). With fluoxetine this event is even stronger suggesting that the protein is core-glycosylated but doesn't complete the glycosylation process (absence of fully glycosylated band – 155 kD) (Perrin *et al.*, 2008). This explains hERG defective gating or defective permeation as side effects of fluoxetine, a fact already seen by

Thomas *et al.*, 2002. These data are indicative of fluoxetine interference in channel N-glycosylation, disturbed ER to Golgi transport, or both.

With tunicamycin (TM) since it was very toxic to cells, we barely had a hERG protein signal. The immature band is very weak and the absence of mature band indicates that hERG isn't enable to touch the membrane in its mature form.

In general, when a weak signal of the mature hERG form appears in a western blot when compared to the immature form it suggests a glycosylation problem, a trafficking disturbance or, eventually, a particular transporter blockage.

IV.2.2. K_{IR} cells

We were expecting to obtain in HEK-KWGF assays the same response as in HEK-HERG drug experiments, *i.e.*, a strong interference by pentamidine and fluoxetine in $K_{IR}2.1$ cell surface levels. To test that hypothesis a western blot was made.

HEK-KWGF cells stably express GFP-tagged $K_{IR}2.1$ and the channel protein is presented as a single band in the western blot– figure 4.2.2.

In contrast to hERG experiments, and despite not showed in the figure below, tunicamycin treatment for up to 24 h did not decrease the expression levels of $K_{IR}2.1$, confirming in mammalian cells what had been seen in insect cells (Nalos et al., 2011).

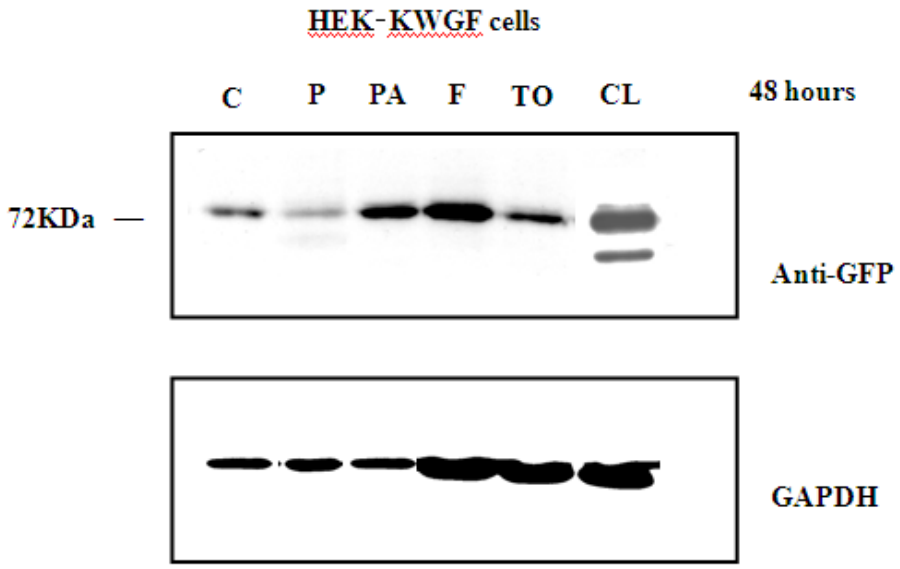

Figure 4.2.2. Western blot for $K_{IR}2.1$. HEK-KWGF cells were incubated for 48 hours with the respective drug and processed for western blot. It can be observed an increase in the $K_{IR}2.1$ band with 2EVK (PA) and fluoxetine (F) and a decrease of $K_{IR}2.1$ levels with pentamidine (P) and As_2O_3 (TO). An increase with chloroquine (CL) with the characteristic $K_{IR}2.1$ degradation band (lower band) can be observed. (C) Untreated cells.

Furthermore, upon chloroquine treatment, HEK-KWGF cells yielded an additional $K_{IR}2.1$ band at aproximatly 65 KDa. Since GFP is tagged in the C-terminus of $K_{IR}2.1$, this product is, most likely, a protein product that results from N-terminal $K_{IR}2.1$ cleavage, as reported by others (Jansen *et al.*, 2008). In contrast, hERG is predominantly degraded via the proteasomal pathway under normal culture conditions and therefore no extra lower band is observed (Gong *et al.*, 2005).

With the pentamidine analog and fluoxetine it was detected an increase in total protein content in the same way as with chloroquine, a well-known inhibitor of $K_{IR}2.1$traffic usually used as positive control (Lopez-Izquierdo *et al.*, 2009; Rodríguez-Menchaca *et al.*, 2008). Nevertheless, $K_{IR}2.1$ degradation band observed with chloroquine was absent after 2EVK or fluoxetine treatment, suggesting that different inhibitory mechanisms may be involved.

Additionally, $K_{IR}2.1$ immunofluorescence experiments were performed in order to verify if there were any differences in intracellular $K_{IR}2.1$ channels localization after a 48-hour incubation with each drug, revealing different mechanisms of action among them. In HEK-KWGF cells, $K_{IR}2.1$ channels can be seen, under the epifluorescence microscope through GFP (green) – figure 4.2.3.

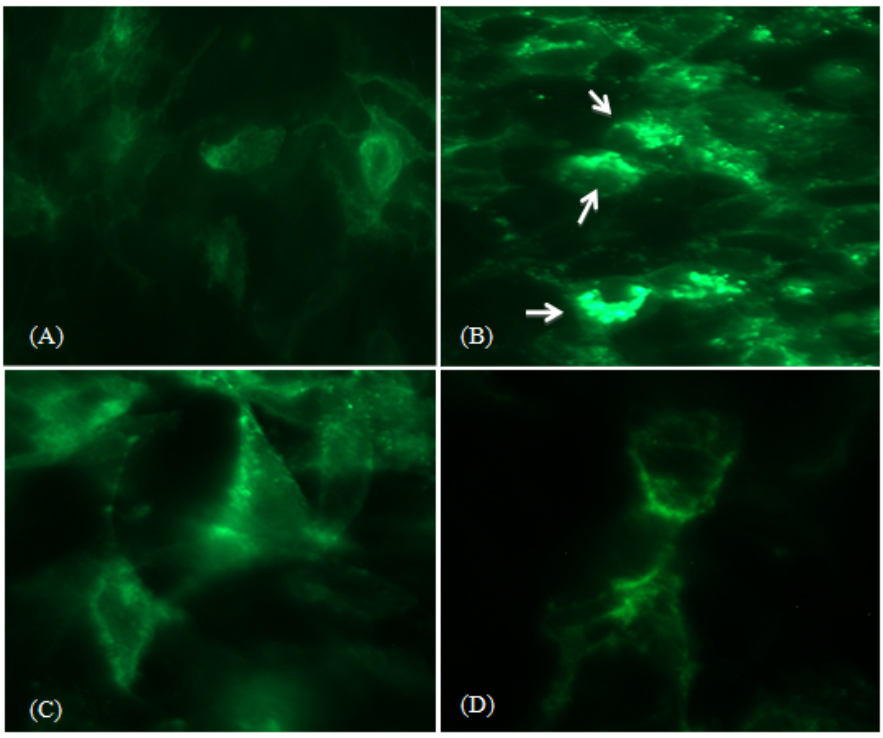

Figure 4.2.3. $K_{IR}2.1$ immunofluorescence microscopy (green) in non-treated HEK-KWGF cells (A) or after a 48-hour incubation of the same cells with different drugs: fluoxetine (30µM) (B), tunicamycin (1µg/mL) (C) and As_2O_3 (1 µM) (D). In all samples, an accumulation of $K_{IR}2.1$-GFP proteins at the membrane can be seen, except in B, where this accumulation is also observed around the nucleus, probably in ER or Golgi apparatus (white arrows). Images were taken with a 400x amplification and 1,5 seconds exposure time.

From immunofluorescence experiments an accumulation of $K_{IR}2.1$ at plasma membrane could be noted in HEK-KWGF cells in all drugs tested, fluoxetine, tunicamycin and As_2O_3, suggesting a clear interference in channels traffic and recycling pathways. However, with fluoxetine a strong signal appeared around the nucleus, reflecting posssibly $K_{IR}2.1$ channels held in ER or Golgi apparatus (white arrows).

IV.3. 2EVK (PA) and pentamidine (P)

Curiously, in all experiments, the pentamidine analog 2EVK has shown an activity that was different of pentamidine activity. Accordingly, this fact was further investigated with a time course was experiment and $K_{IR}2.1$ quantification – figure 4.3.1.

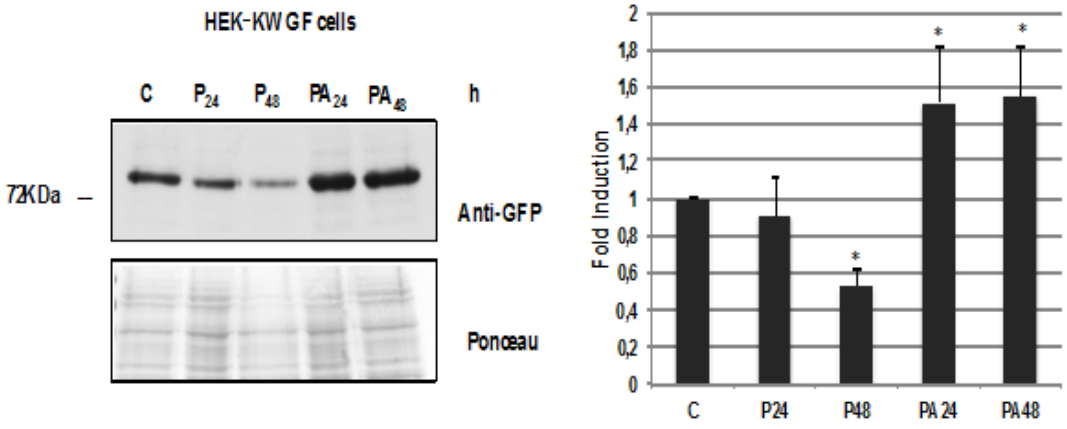

Figure 4.3.1. Western blot for $K_{IR}2.1$. HEK-KWGF cells were treated with pentamidine (P) and its analog 2EVK (PA) for 24 and 48 hours and processed for western blot. For $K_{IR}2.1$ quantification a Ponceau staining was performed. Quantification of the bands was made with ImageQuant TL v2002.01 and results for $K_{IR}2.1$ are shown as an average of six individual experiments. After 48 hours a decrease in the levels of $K_{IR}2.1$ protein with P and a slight increase in the protein levels with PA could be observed ("*" p value < 0,05).

After 48 hours of HEK-KWGF treatment with P there is a statistical significant decrease in $K_{IR}2.1$ protein (0,52 ± 0,09), which clearly contrasts with the slight increase with PA after 24 and 48 hours (1,52 ± 0,29 and 1,55 ± 0,26, respectively). Additionally, in Bonferroni analyses between 24 and 48 hours of pentamidine treatment, $K_{IR}2.1$ protein decrease was also statistical significant (from 0,90.± 0,20 to 0,52 ± 0,09).

After these preliminary results an immunofluorescence microscopy analysis was made in order to figure out if intracellularly P and PA had also different effects figure 4.3.2. Also, it would be important to address this issue at an electrophysiologic level, an analysis presently being done in our lab.

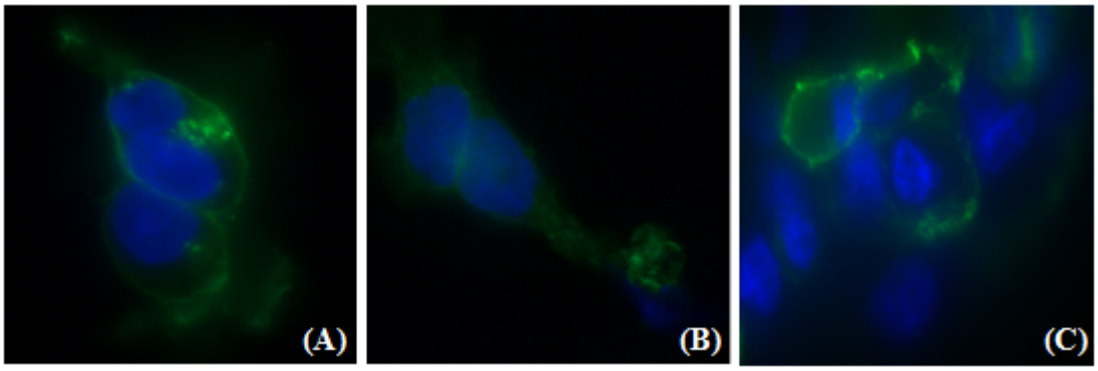

Figure 4.3.2. $K_{IR}2.1$ Immunofluorescence microscopy (green). (A) Non-treated HEK-KWGF cells and (B) the same cells treated with pentamidine (10 μM) and (C) its analog 2EVK. (200 nM) Images were taken with a 400x amplification and 1,5 seconds exposure time. Nuclei were stained with DAPI (blue).

It can be perceived an increase of $K_{IR}2.1$-GFP proteins in the membrane of HEK-KWGF cells treated with 2EVK. (figure 4.3.2.c.). By contrast, pentamidine treatment revealed less $K_{IR}2.1$ at the membrane of HEK-KWGF cells, in concordance with time-course experiments shown above (figure 4.3.1). Taken together these results suggest, surprisingly for the first time, different mechanisms of action between pentamidine and its analog 2EVK.

Additionally, although 2EVK and fluoxetine seem to increase $K_{IR}2.1$ protein content (figure 4.2.2.), $K_{IR}2.1$ localization inside the cells is completely different between those drugs.

It would be interesting to see, at electrophysiological level, how I_{K1} current is influenced by 2EVK and therefore if it can cause QT prolongation symptoms, usually observed with its precursor pentamidine. However, as 2EVK is a very potent acute inhibitor that is difficult to wash out, it will be rather demanding to study this.

IV.4. Fluoxetine as an inhibitor of intracellular trafficking

Fluoxetine (Prozac®), a lipophilic antidepressant, is a selective serotonin reuptake inhibitor (SSRI) and its side effects don't refer any cardiac problems. However, after stimulating HEK-KGWF cells with fluoxetine it was observed an accumulation of K_{IR} proteins around cell nucleus and not only at the plasma membrane, as expected. This accumulation may reveal a lysosomal trapping of the proteins (Daniel *et al.*, 2001).

Daniel & Woájcikowski (1997) showed that an important mechanism of distribution among basic lipophilic psychotropics was lysosomal trapping. However, they suggested that for drugs like fluoxetine, its extensive tissue distribution should depend more on phospholipid binding than on lysosomal trapping, an event more pronounced in lysosome rich organs such as liver, lungs and kidneys and less in the brain and heart.

Additionally, fluoxetine was further investigated with a time course was experiment and $K_{IR}2.1$ quantification – figure 4.4.1.

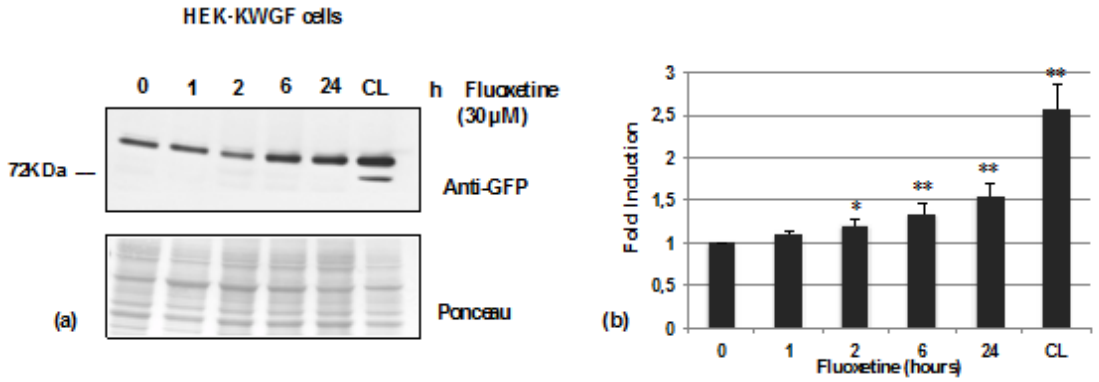

Figure 4.4.1. Western blot for $K_{IR}2.1$. HEK-KWGF cells treated with fluoxetine (F) at several time points between 1 and 24 hours and with chloroquine (CL) as positive control. For $K_{IR}2.1$ quantification a Ponceau staining was performed. Quantification of the bands was made with ImageQuant TL v2002.01 and results for $K_{IR}2.1$ are shown as an average of six individual experiments. An increase in $K_{IR}2.1$ protein can be observed along time ("*" p value < 0,05; "**" p<0,01).

These results have suggested that despite having neurotransmitter serotonin as the major physiological target and a half-life of 1-4 days, fluoxetine it is also a candidate to alter $K_{IR}2.1$ protein levels throughout 24 hours (1,09±0,045; 1,20±0,058; 1,34±0,13; 1,54±0,156; 2,54±0,275, for 1hour, 2 hours, 6 hours, 24 hours and chloroquine, respectively, n=7).

Rajamani *et al.* (2006) studied the effects of fluoxetine on hERG K^+ channels in cardiomyocytes from different species and reached the same results we obtained for $K_{IR}2.1$.

It is difficult to compare our results with other groups because to our knowledge all the reports in this context only refer electrophysiologic measurements. Pacher *et al.* (2000) has shown that fluoxetine in a concentration of 10 µM had no effect on the shape of steady-state current-voltage curve and therefore, they suggested that neither I_{K1}, nor other K^+ currents are likely to be affected by the drug. However, other studies had opposite results (Rajamani *et al.*, 2006) much more in line with the molecular analysis we obtained in this study.

Since the range of therapeutic fluoxetine plasma concentrations was reported to vary between 0.15 and 1.5 µmol/L in humans (Blardi *et al.*, 2002; Brunswick *et al.*, 2002; Koran *et al.*, 1996) a concentration effect experiment was also developed – figure 4.4.2.

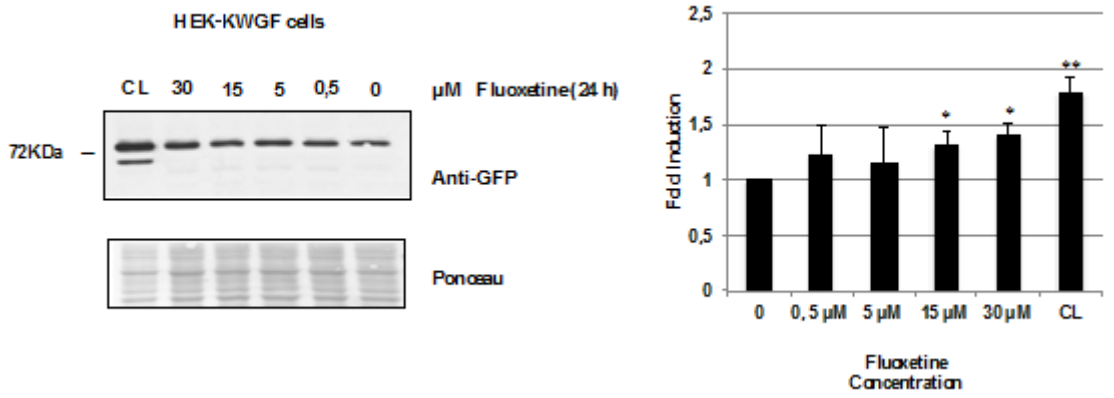

Figure 4.4.2. Western blot for $K_{IR}2.1$. HEK-KWGF cells treated with fluoxetine (F) at several concentrations ranging from 0 to 30 µM and with chloroquine (CL) as positive control. For $K_{IR}2.1$ quantification a Ponceau staining was performed. Quantification of the bands was made with ImageQuant TL v2002.01 and results for $K_{IR}2.1$ are shown as an average of six individual experiments. A successive increase in $K_{IR}2.1$ protein can be observed with higher concentrations of fluoxetine ("*" p value < 0,05; "**" p<0,01).

As expected, our results showed that total amount of $K_{IR}2.1$ protein was increasing with fluoxetine concentration, however, this increase wasn't so pronounced as in the time course experiment, *i.e.*, the values were closer and even slight decrease could be noted from 0,5 µM to 5 µM (1,24±0,235; 1,15±0,285; 1,30±0,112; 1,41±0,095; 1,78±0,121 for 0,5 µM; 5 µM; 15 µM; 30 µM and chloroquine, respectively, n=7). As note, this increase was statistical significant above 15 µM.

Moreover, $K_{IR}2.1$ proteins that have as final spot the plasmatic membrane, where they are able to work as an ionic channel, may accumulate with fluoxetine exposure and fluoxetine concentration.

Frequently, patients under fluoxetine treatment are also under extreme conditions such as decreased metabolism in elderly, acute overdose and many drug interactions, circumstances where plasma concentrations of fluoxetine can reach even higher levels (Hale 1993) highlighting the need of further investigation.

It must be emphasized, however, that the fluoxetine-induced electrophysiological alterations are not necessarily always proarrhythmic. Obviously, in patients with electric disorders (e.g.: deficient impulse conduction) they are, but could be beneficial in cases with short QT syndrome. Therefore, in depressed patients with cardiac disorders, EKG control may be suggested during fluoxetine therapy (Pacher *et al.*, 2000).

In an attempt to further understand where the increase in $K_{IR}2.1$ protein localizes intracellularly along time, an immunofluorescence microscopy experiment was made with cells treated with 30µM fluoxetine in two time points, 24 and 48 hours – figure 4.4.3.

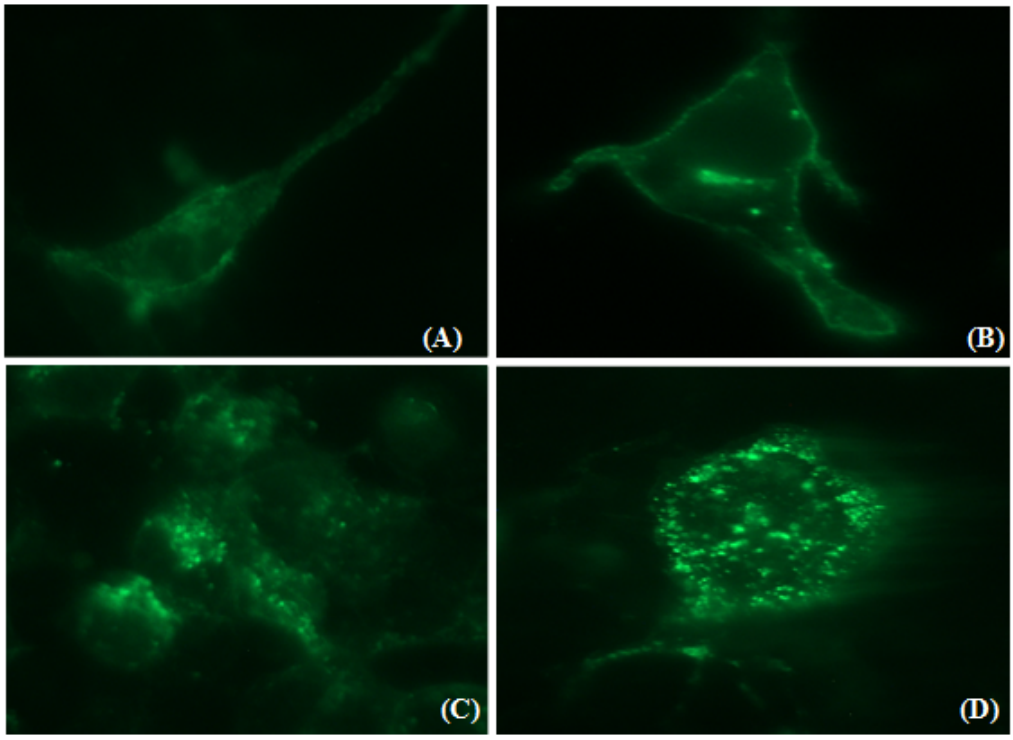

Figure 4.4.3. $K_{IR}2.1$ Immunofluorescence microscopy (green). (A) Non-treated HEK-KWGF cells and (B) the same cells treated with fluoxetine (30μM) for 24 hours and (C) 48 hours. (D) Chloroquine treatment for 24 hours was used as positive control. Images were taken with a 400x amplification and 1,5 seconds exposure time.

Under the microscope, it could be observed that $K_{IR}2.1$ pattern of fluorescence is not identical between untreated and treated cells or between drugs. In untreated cells $K_{IR}2.1$ tends to give rise to a slightly staining in the cytoplasm and to form a thin line at cell membrane (figure 4.4.3.A.). Although with fluoxetine treatment for 24 hours we can see $K_{IR}2.1$ at the membrane, curiously, it starts to accumulate in a well defined region inside the cell (figure 4.4.3.B.). After 48 hours it disappears completely from cell surface and, interestingly, it accumulates in clusters at one side of the nucleus (figure 4.4.3.C.). Although this pattern of staining does not seem to be due to lysosome accumulation and we suspect of ER or Golgi apparatus involvement, further investigation would be required to clarify that fact. Nevertheless, fluoxetine is robustly affecting $K_{IR}2.1$ traffic and accordingly, we anticipate strong interference in I_{K1} currents. Despite fluoxetine has been generally accepted to cause few side effects, our results clearly indicate that fluoxetine may have various unwanted effects, especially on $K_{IR}2.1$ ion channels.

As expected, upon chloroquine treatment an uniform spread of green fluorescence indicating $K_{IR}2.1$ diffusion inside the cells and concordant with channel accumulation inside lysosomes, was observed (figure 4.4.3.D.) (Jansen *et al.*, 2008).

Fluoxetine is metabolized via the cytochrome P450 enzyme system into multiple metabolites, including norfluoxetine, a N- demethylated compound. Although norfluoxetine, has similar pharmacological properties as its precursor, it presents a five-time longer half-life (Hiemke & Härtter, 2000). Since it was observed a relevant interference in $K_{IR}2.1$ traffic upon fluoxetine treatment, we decided to make a time-course experiment with norfluoxetine. Unexpectedly, we did not find any kind of interference of norfluoxetine on $K_{IR}2.1$ channels (results not shown), however more research should be done to address that point.

V. Conclusions

Among the causes of $K_{IR}2.1$ and hERG malfunctions we were specifically interested in drug side effects. Nowadays, the number of persons under drug therapy is huge, and the percentage of patients addicted to drugs is elevated. However, many cardiac-related side effects remain to be elucidated at the cellular level.

There are many drugs with known negative effects in the heart, especially on the channels referred above. Accordingly, we went to study several drugs in HEK-HERG and HEK-KWGF cell systems, namely, pentamidine and its analogue 2EVK (antiprotozoal drugs), fluoxetine (antidepressant), tunicamycin (glycosilation inhibitor), arsenic trioxide (cytostatic/anticancer) and chloroquine (antimalarial).

In all experiments, tunicamycin revealed to be very toxic for the cells and consequently no relevant observations were performed. On the other hand, arsenic trioxide presented a protein pattern identical to untreated cells in hERG western blot and showed little or no effect in $K_{IR}2.1$ western blot. For that reason we do not anticipate any kind of cardiac-related side effects at the clinical level for arsenic trioxide.

Chloroquine strongly inhibited $K_{IR}2.1$ channel recycling and produced the characteristic $K_{IR}2.1$ degradation band, as expected. Surprisingly it presented no effect on hERG western blot.

We were expecting similar results for pentamidine and its analog, 2EVK, however they revealed totally opposite effects in HEK-HERG and HEK-KWGF cells. Molecular analysis demonstrated less hERG maturation and reduced $K_{IR}2.1$ levels (0,90 ± 0,20 times less) when compared to control after pentamidine treatment. By contrast, 2EVK had no effect in hERG maturation and produced increased $K_{IR}2.1$ protein levels (1,52 ± 0,29). Upon fluoxetine treatment, the core-glycosylated protein was not frequently added and only the band of ER folding and assembly was observed, presumably due to trafficking defects. In $K_{IR}2.1$ analysis, once again, the protein levels accumulate (1,54 ± 0,16) but unlike chloroquine the degradation band did not appear.

With fluorescence microscopy, in HEK-KWGF cells after fluoxetine incubation we demonstrated for the first time that increased protein levels observed in western blots are an internal polarized accumulation of $K_{IR}2.1$ around the nucleus, probably in ER or Golgi, since this is a fluorescence pattern totally new and different from the one seen with chloroquine after 24 hours.

Despite there is no information about the role of fluoxetine on cardiac ion channels such as hERG and $K_{IR}2.1$, our results clearly demonstrate a strong influence on maturation and intracellular channel protein traffic, respectively and strongly indicate that this drug may

possible interfere with I_{Kr} and I_{K1}. Unexpectedly, at the clinical level this drug has almost no reported side effects at the cardiac level. This discrepancy remains to be elucidated in further studies. Although it is still a matter of debate the identification of which mechanism fully explains the antidepressant action of fluoxetine, it is clear that it interferes with several types of receptors and ion channels whereas the viability of target cells is not affected or may even increase.

One of the limitations of this thesis was probably the obvious limitation of HEK-HERG and HEK-KGWF cell systems in the lack of ancillary subunit expression for most of the ion channels, which can result in temporal differences in proteins synthesis, subcellular trafficking and degradation when comparing with native cardiomyocytes. Nevertheless, many findings in native cells can most often be reproduced in the HEK293 expression systems, although kinetics and modulation might be slightly different.

There are no perfect drugs with just one action on its physiological target, so it is our mission trying to diminish at maximum the number of related side effects. On this line of thought, our results may be very important for the pharmacological industry that continuously chases that task.

VI. Future work

As a suggestion for further studies, we think it would be important to measure I_{K1} currents and correlate them with altered protein traffic.

Additionally, more immunofluorescence studies are needed to identify, precisely, the intracellular spot where ions channels accumulate under the influence of particular drugs.

Regarding chloroquine treatment of HEK-KWGF cells, it would be interesting to understand if degradation band of $K_{IR}2.1$ is in fact a N-terminal protein cleavage or not, and if it is the case when did it happen along the recycling pathway.

Another important point would be to understand the mechanism of action of fluoxetine in the heart. Since its main target is a 5-HT reuptake transporter in the brain and fluoxetine is clearly interfering in $K_{IR}2.1$ traffic, a protein transporter important for $K_{IR}2.1$ vesicular transfer might also be involved.

To exclude late endosome accumulation of $K_{IR}2.1$ proteins upon drug treatment, experiments, in the presence bafilomycin A1, could be done to see if the channel co-localizes with this late endosome maturation inhibitor.

References

Akhavan, A., Atanasiu, R., Noguchi, T., Han, W. *et al.* (2005). Identification of the cyclic-nucleotide-binding domain as a conserved determinant of ion-channel cell-surface localization. *Journal Cell. Sci.*, 118: 2803-2812.

Anumonwo, J. & Lopatin, A. (2010). Cardiac strong inward rectifier potassium channels. *Journal of Molecular and Cellular Cardiology*, 48: 45-54.

Balijepalli, R. & Kamp, T. (2008). Caveolae, ion channels and cardiac arrhythmias. *Progress in Biophysics and Molecular Biology*, 98 (2): 149-160.

Balu, D., Hodes, G., Hills, T., Lucki, I. *et al.* (2009). Flow cytometric analyzes of BrdU incorporation as a high-throughput method for measuring adult neurogenesis in the mouse. *Journal of Pharmacological and Toxicological Methods*, 59(2): 100-107.

Barbey, JT. & Roose, S. (1998). SSRI safety in overdose. *Journal of Clinical Psychiatry*, 59(15): 42-48.

Barford, E., Moore, A., Roe, M. & Lidofsky, S. (2007). Ca^{2+}-activated I_{K1} Channels Associate with Lipid Rafts upon Cell Swelling and Mediate Volume Recovery. *The Journal of Biological Chemistry*, 282 (12): 8984-8993.

Bellocq, C., van Ginneken, AC., Bezzina, CR., *et al.* (2004). Mutation in the KCNQ1 gene leading to the short QT-interval syndrome. *Circulation,* 109: 2394-2397.

Berthet, M., Denjoy, I., Donger, C., Demay, L., Guicheney, P. *et al.* (1999). C-terminal hERG mutations. The role of hypokalemia and a KCNQ1-associated mutation in cardiac event occurrence. *Circulation*, 99: 1464-1470.

Bezanilla, F. (2000). The Voltage Sensor in Voltage-Dependent Ion Channels. *Physiological Reviews*, 89(2): 555-592.

Bian, JT., Yeh, J., Aistrup, G., Narahashi, T. & Moore, E. (2002). Inhibition of K^+ currents of outer hair cells in guinea pig cochlea by fluoxetine. *European Journal of Pharmacology*, 453: 159-166.

Boadle-Biber, M. (1993). Regulation of serotonin synthesis. *Prog. Biophysical Mollecular Biology*, 60(1): 1-15.

Boyden, P. (1996). Cellular Electrophysiologic Basis of Cardiac Arrhythmias. *American Journal of Cardiology*, 78: 4-11.

Briley, M.& Moret, C. (1993). Neurobiological mechanisms involved in antidepressant therapies. *Clinical Neuropharmacology*, 16(5): 387-400.

Brodsky, F., Chen, C., Knuehl, C., Towler, M. & Wakehan, D. (2001). Bioogical basket weaving: Formation and function of clathrin-coated vesicles. *Annual Review of Cell and Developmental Biology*, 17: 517-568.

Brugada, R., Hong, K., Dumaine, R. *et al.* (2005). Sudden death associated with short-QT syndrome linked to mutations in HERG. *Circulation*, 109: 30-35.

Caballero, R., Dolz-Gaitón, P., Delpón, E. *et al.* (2010). Flecainide increases $K_{IR}2.1$ currents by interacting with cysteine 311, decreasing the polyamine induced rectification. *Proceedings of the National Academy of Science*, 107 (35): 15631-15636.

Carmeliet, E. (1999). Cardiac Ionic Currents and Acute Ischemia: From Channels to Arrhythmias. *Physiological Reviews*, 79 (3): 917-1017.

Chen, J., Seebohm, G. & Sanguinetti, MC. (2002). Position of aromatic residues in the S6 domain, not inactivation, dictates cisapride sensitivity of HERG and eag potassium channels. *Proc. Natl. Acad. Sci.*, 99, 12461–12466.

Cheng, CF., Kuo, HC. & Chien, K. (2003). Genetic modifiers of cardiac arrhythmias. *TRENDS in Molecular Medicine*, 9 (2): 59-66.

Choe, H., Palmer, LG., & Sackin, H. (1999). Structural determinants of gating in inward-rectifier K^+ channels. *Biophysical Journal*, 76: 1988-2003.

Choi, B., Choi, JS., Yoon, S., Rhie, DJ., Hahn, S. *et al.* (2001). Effects of norfluoxetine, the major metabolite of fluoxetine, on the cloned neuronal potassium channel $K_V3.1$. *Neuropharmacology*, 41: 443-453.

Cipriani, A., Furukawa, T., Salanti, G., Geddes, J., Barbui, C. *et al.* (2009). Comparative efficacy and acceptability of 12 new-generation antidepressants: a multiple treatment meta-analysis. *The Lancet*, 373(9665): 746-758.

Cohen, A., Ben-Abu, Y. & Zilberberg, N. (2009). Gating the pore of potassium leak channels. *European Biophysics Journal*, 1: 61-73.

D'Avanzo, N., Cho, HC., Tolokh, I., Backx, P. *et al.* (2005). Conduction through the Inward Rectifier Potassium Channel, $K_{IR}2.1$, is increased by negatively charged extracellular residues. *Journal of General Physiology*, 125: 493-503.

Daniel, W., Wójcikowski, J. & Palucha, A. (2001). Intracellular distribution of psychotropic drugs in the grey and white matter of the brain: the role of lysosomal trapping. *British Journal of Pharmacology*, 134: 807-814.

Daniel, W. & Wójcikowski, J. (1997). Contribution of Lysosomal Trapping to the Total Tissue Uptake of Psychotropic Drugs. *Pharmacology & Toxicology*, 80: 62-68.

De Boer, T., Nalos, L., Stary, a., Kok, B., Houtman, MJC, van der Heyden, MAG. *et al.*, (2010)a. The anti-protozoal drug pentamidine blocks $K_{IR}2.x$-mediated inward rectifier current by entering the cytoplasmic pore region of the channel. *British Journal of Pharmacology*, 159: 1532-1541.

De Boer, T., Houtman, MJC., Compier, M. & van der Heyden, MAG. (2010)b. The mammalian $K_{IR}2.x$ inward rectifier ion channel family: expression pattern and pathophysiology. *Acta Physiologica*, 199: 243-255.

De Boer, T., Van Veen, T., Van der Heyden, MAG. *et al.* (2006). Inhibition of cardiomyocyte automaticity by electronic application of inward rectifier current from $K_{IR}2.1$ expressing cells. *Medical and Biological Engineering Computer*, 44: 537-542.

Delisle, B., Anson, B., Rajamani, S. & January, C. (2004). Biology of Cardiac Arrhythmias: Ion Channel Protein Trafficking. *Circulation Research*, 94: 1418-1428.

Demyttenaere, K., Adelin, A., Patrick, M., Walthère, D., Katrien, B. & Michèle, S. (2008). Six-month compliance with antidepressant medication in the treatment of major depressive disorder. *International Clinical Psychopharmacology*, 23 (1): 36-42.

Doherty, G.J. & McMahon, H.T. (2009). Mechanisms of endocytosis. *Annual Rev Biochemistry*, 78: 857-902.

Donati, R. & Rasenick, M. (2003). G protein signaling and the molecular basis of antidepressant action. *Life Sciences*, 73(1): 1-17.

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A. *et al*. (1998). The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science*, 280: 69-77.

Drago, F., Tamburella, A., Micale, V. & Navarria, A. (2009). Antidepressant properties of the 5-HT_4 receptor partial agonist, SL65.0155: Behavioral and neurochemical studies in rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 33(7): 1205-1210.

Duncan, R., McPate, M., Ridley, J., Gao, Z., Hancox, J., *et al.* (2007). Inhibition of the HERG potassium channel by the tricyclic antidepressant doxepin. *Biochemistry Pharmacology,* 74(3): 425-437.

El Harchi, A., McPate, M., Zhang, Y., Zhang, H. & Hancox, J. (2009). Action potential clamp and chloroquine sensitivity of mutant KIR2.1 channels responsible for variant 3 short QT syndrome. *Journal Molecular Cell Cardiology*, 47: 743-747.

Fauconnier, J., Lacampagne, A., Rauzier, JM., Vassort, G. & Richard, S. (2005). Ca^{2+}-dependent reduction of I_{K1} in rat ventricular cells: a novel paradigm for arrhythmia in heart failure? *Cardiovascular Research*, 68: 204–12.

Fedida, D., Braun, AP. & Giles, WR. (1991). Alpha 1-adrenoreceptors reduce background K^+ current in rabbit ventricular myocytes. *Journal of Physiology*, 441: 673-684.

Ficker, E., Kuryshev, Y., Dennis, A., Brown, A. *et al.* (2004). Mechanisms of arsenic-induced prolongation of cardiac repolarization. *Molecular Pharmacology*, 66(1): 33-44.

Ficker, E., Dennis, A., Wang, L. & Brown, A. (2003). Role of the Cytosolic Chaperones Hsp70 and Hsp90 in Maturation of the Cardiac Potassium Channel hERG. *Circulation Research*, 92: 87-100.

Grant, BD. & Sato, M. (2006). Intracellular trafficking. *WormBook*, ed. The C. Elegans Research Community. Retrieved July, 27th 2011 from http://www.wormbook.org

Grunnet, M. (2010). Repolarization of the cardiac action potential: Does an increase in repolarization capacity constitute a new anti-arrhythmic principle? *Acta Physiologica*, 198 (Supplement 676): 1-48.

Hancox, J., McPate, M., El Harchi, A. & Zhang, Y. (2008). The hERG potassium channel and hERG screening for drug-induced *torsade de pointes*. *Pharmacological & Therapeutics*, 119: 118-132.

Hardman, R., Stansfeld, P.J., Dalibalta, S., Sutcliffe, M.J., Mitcheson, J.S. (2007). Activation gating of hERG potassium channels: S6 glycines are not required as gating hinges. *Journal of Biology and Chemistry*: 282, 31972–31981.

Herfst, L., Rook, M. & Jongsma, H. (2004). Trafficking and functional expression of cardiac Na^+ channels. *Journal of Molecular and Cellular Cardiology*, 36: 185-193.

Heusser, K. & Schwappach, B. (2005). Trafficking of potassium channels. *Current Opinion in Neurobiology*, 15: 364-369.

Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I. & Kurachi, Y. (2010). Inwardly rectifying potassium channels: their structure, function and physiological roles. *Physiological Reviews*, 90: 291-366.

Hoger, J., Ilyin, V., Forsyth, S. & Hoger A. (2002). Shear stress regulates the endothelial $K_{IR}2.1$ ion channel. *Proceedings of the National Academy of Science*, 99 (11): 7780-7785.

Hoshi, T., Zagotta, WN. & Aldrich, RW. (1990). Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science*, 250: 533-538.

Imoto, Y., Ehara, T. & Matsuura H. (1987). Voltage- and time-dependent block of I_{K1} underlying Ba^{2+}-induced ventricular automaticity. *American Journal Physiology*, 252: H325-33.

Jansen, A., de Boer, T., Wolswinkel, R., van Veen, T., Vos, M., van Rijen, H. & van der Heyden, MAG. (2008). Lysosome mediated $K_{IR}2.1$ breakdown directly influences inward rectifier current density. *Biochemical and Biophysical Research Communications*, 367: 687-692.

Jo, SH., Hong, HK., Chong, SH., Jung, SJ. & Choe, H. (2008). Clomipramine block of the hERG K^+ channel: Accessibility to F656 and Y652. *European Journal of Pharmacology*, 592(1-3): 19-25.

Jongsma, H. & Wilders, R. (2001). Channelopathies: $K_{IR}2.1$ mutations jeopardize many cell functions. *Current Biology*, 11: R47-R50.

Klabunde, R. (2005). *Cardiovascular Physiology Concepts*. Lippincott Williams and Wilkins. 2nd Edition: United States of America.

Kobayashi, T., Washiyama, K. & Ikeda, K. (2006). Inhibition of G Protein-Activated Inwardly Rectifying K^+ Channels by Ifenprodil. *Neuropsychopharmacology*, 31: 516-524.

Knollmann, B. & Roden, D. (2008). A genetic framework for improving arrhythmia therapy. *Nature*, 451: 929-936.

Kuryshev, Y., *et al.* (2005). Pentamidine-Induced Long QT Syndrome and Block of hERG Trafficking. *The Journal of Pharmacology and Experimental Therapeutics*, 312: 316-323.

Lees-Miller, JP., Duan, Y., Teng, G. & Duff, H. (2000). Molecular determinant of high-affinity dofetilide binding to HERG1 expressed in Xenopus oocytes: involvement of S6 sites. *Molecular Pharmacology*, 57, 367–374.

Leng, Q., Goldgeier, L., Zhu, J., Cambell, P., Ambulos, N. & Mixson, J. (2007). Histidine-Lysine peptides as carriers of nucleic acids. *Drug New & Perspectives*, 20(2): 77-82.

Leonoudakis, D., Conti, L., Anderson, S., Vandenberg, C. *et al.* (2004). Protein Trafficking and Anchoring Complexes Revealed by Proteomic Analysis of Inward Rectifier Potassium Channel ($K_{IR}2.x$)-associated Proteins. *The Journal of Biological Chemistry*, 21:22331-22346.

Lesage, F., Guillemare, E., Fink, M., Duprat, F., Barhanin, J. *et al.* (1995). TWIK-1, a ubiquitous human weakly inward rectifying K+ channel with a novel structure. *The EMBO Journal*, 15(5): 1004-1011.

Li, J., McLerie, M. & Lopatin, AN. (2004). Transgenic upregulation of I_{K1} in the mouse heart leads to multiple abnormalities of cardiac excitability. *American Journal of Physiology: Heart Circ Physiol*, 287: H2790–802.

Lin, G. & Dong, M. (2010). Pharmacology of cardiac potassium channels. *Advances in Pharmacology*, 59: 93-134.

Liu, A., Tang, M., Xi, J., Liang, H. *et al.* (2010). Functional Characterization of Inward Rectifier Potassium Ion Channel in Murine Fetal Ventricular Cardiomyocytes. *Cellular Physiology Biochemistry*, 26: 413-420.

Lopatin, A. & Nichols, C. (2001). Inward Rectifiers in the Heart: An Update on I_{K1}. *Journal of Molecular and Cellular Cardiology*, 33: 625-638.

Loussouarn, G., Rose, T. & Nichols, CG. (2002). Structural basis of inward rectifying potassium channel gatting. *Trends Cardiovascular Medicine,* 12 (6): 253-258.

Lu, Y., Mahsut-Smith. M.P., Varghese, A., Huang, C.L., Kemp, P.R., Vandenbrg, J. (2001). Effects of premature stimulation on HERG K^+ channels. *Journal of Physiology*, 537: 843-851.

Ma, D. & Jan, L.Y. (2002). ER transport signals and trafficking of potassium channels and receptors. *Current Opinion in Neurobiology*, 12: 287-292.

Miake, J., Marban, E. & Nuss, HB. (2003). Functional role of inward rectifier current in heart probed by $K_{IR}2.1$ overexpression and dominant-negative suppression. *J Clin Invest*, 111: 1529-1536.

Mitcheson, J S., Chen, J., Lin, M., Culberson, C. & Sanguinetti, MCA. (2000). Structural basis for drug-induced long QT syndrome. *Proc. Natl. Acad. Sci.*, 97, 12329–12333.

Mohrman, D. (2006). *Cardiovascular Physiology (Lange Physiology Series)*. McGraw-Hill Medical Editions. 3rd edition. United States of America.

Nalos, L., de Boer, TP., Hutman, JC., Houtman, M., Rook, M., Vos, M. & van der Heyden, M. (2011). Inhibition of lysosomal degradation rescues pentamidine-mediated decreases of $K_{IR}2.1$ ion channel expression but not that of $K_v11.1$. *European Journal of Pharmacology*, 652: 96-103.

Nanduri, J., Bergson, P., Wang, N., Ficker, E. & Prabhakar N. (2009). Hypoxia inhibits maturation and trafficking of the hERG K+ channel protein: Role of Hsp90 and ROS. *Biochemical and Biophysyical Research Communications*, 388: 212-216.

Nattel, S. (2003). Remodeling of cardiac inward-rectifier currents: an often-overlooked contributor to arrhythmogenic states. *Journal of Molecular Cellular Cardiology*, 35: 1395–1398.

Nerbonne, J. & Kass, R. (2005). Molecular Physiology of Cardiac Repolarization. *Physiological Reviews*, 85: 1205-1253.

Nichols, C. & Lopatin, A. (1997). Inward Rectifier Potassium Channels. *Annual Reviews Physiology*, 59: 171-191.

Pacher, P., Magyar, J., Szigligeti, P., Bányász, T., Nánási, P. *et al.* (2000). Electrophysiological effects of fluoxetine in mammalian cardiac tissues. *Naunyn-Schmiedeberg's Archives Pharmacology*, 361: 67-73.

Pancrazio, J., Kamatchi, G., Roscoe, A. & Lynch III, C. (1998). Inhibition of neuronal Na^+ channels by antidepressant drugs. *The Journal of Pharmacology and Experimental Therapeutics*, 284: 208-214.

Paterson, G. & Metcalfe, C. (2008). Uptake and depuration of the anti-depressant fluoxetine by the japanese medaka (Oryzias latipes). *Chemosphere*, 74: 125-130.

Perrin, M., Subbiah, R., Vandenberg, J. & Hill, A. (2008). Human ether-a-go-go related gene (hERG) K^+ channels: Function and dysfunction. *Progress in Biophysics and Molecular Biology*, 98: 137-148.

Persson, F. (2007). Mechanism of Action of the Antiarrhythmic Agent AZD7009. *Department of Medical Biophysics Institute of Neuroscience and Physiology*.

Petrich, E., Leblanc, N., Schanne, O. *et al.* (1992). Effects of K^+ channel blockers on the action potential of hypoxic rabbit myocardium. *British Journal Pharmacology*, 106: 924-930.

Piao, L., Li, J., McLerie, M. & Lopatin, AN. (2007). Cardiac I_{K1} underlies early action potential shortening during hypoxia in the mouse heart. *Journal of Molecular Cellular Cardiology*, 43(1): 15-17.

Pongs, O. (2009). Ins and outs of cardiac voltage-gated potassium channels. *Current Opinion in Pharmacology*, 9: 311-315.

Proks, P., Capener, CE., Jones, P. *et al.* (2001). Mutations within the P-loop of $K_{IR}6.2$ modulate the intraburst kinetics of the ATP-sensitive potassium channel. *Journal General Physiology*, 118: 341-353.

Prüβ, H. (2004). $K_{IR}2$ potassium channels in rat striatum are strategically localized to control basal ganglia function. Medizinischen Fakultät Berlin.

Qu, Y., Aluisio, L., Lord, B., Lovenberg, T. *Et al.* (2009). Pharmacokinetics and pharmacodynamics of norfluoxetine in rats: increasing extracelular serotonin level in the frontal córtex. *Pharmacology, Biochemistry and Behavior*, 92: 469-473.

Rajamani, S., Eckhardt, L., Valdivia, C., Klemens, C., Gillman, B., January, C. *et al.* (2006). Drug-induced long QT syndrome: hERG K^+ channel block and disruption of protein trafficking by fluoxetine and norfluoxetine. *British Journal of Pharmacology*, 149: 481-489.

Rang, HP., Dale, MM. & Ritter, JM. (2001). Other peripheral mediators: 5-hydroxytryptamine and purines. In *Pharmacology*, 4th Edition. Edonburgh, UK: Harcourt Publishers Ltd: 165-176

Ravens, U. & Cerbai, E. (2008). Role of potassium currents in cardiac arrhythmias. *Europace* 10: 1133-1137.

Ravens, U., Wettwer, E. & Hála, O. (2004). Pharmacological modulation of ion channels and transporters. *Cell Calcium*, 35: 575-582.

Rodriguez-Menchaca, A., Sánchez-Chapula, J. *et al.* (2008). The molecular basis of chloroquine block of the inward rectifier $K_{IR}2.1$ channel. *Proceedings of the National Academy of Science*, 105 (4): 1364-1368.

Roepke, T. & Abbott, G. (2006). Pharmacogenetics and cardiac ion channels. *Vascular Pharmacology*, 44: 90-106.

Romanenko, V., Fang, Y., Byfield, F., Travis, A., Vandenberg, C., Rothblat, G. & Levitan, I. (2004). Cholesterol Sensitivity and Lipid Raft Targeting of $K_{IR}2.1$ Channels. *Biophysical Journal*, 87(6): 3850-3861.

Rourke, R., Alexander, R. & Fuster, V. (2002). *Hurst's – The heart*. McGrawHill Medical Publishers: 18th Edition, United States of America.

Roth, B. (1991). Action propagation in a thick strand of cardiac muscle. *Circulation Research*, 68: 162-173.

Sager, P. (2000). New advances in class III antiarrhythmic drug therapy. *Opinion Cardiology*, 15: 41-53.

Seemann, G., Sachse, F., Weiss, D. *et al.* (2007). Modeling of I_{K1} mutations in human left ventricular myocytes tissue. *American Journal Physiology Heart Circulation*, 292: H549-H559.

Shagufta, Guo, D., Klaasse, E., Van der Heyden, MAG, Uzerman, A. *et al.* (2009). Exploring chemical substructures essential for hERG K^+ channel blockage by synthesis and biological evaluation of Dofetilide Analogues. *CheMedChe*, 1-12.

Spawski, I., Shen, J., Timothy, K., Lehmann, M., Keating, M. *et al.* (2000). Spectrum of mutations in long QT syndrome genes KVLQT1, hERG, SCN5A, KCNE1 and KCNE2. *Circulation*, 102: 1178-1185.

Steele, D., Eldstrom, J. & Fedida D. (2007). Mechanisms of cardiac potassium channel trafficking. *Journal of Physiology*, 582.1: 17-26.

Sun, H., et al. (2006). Chronic inhibition of cardiac $K_{IR}2.1$ and hERG potassium channels by celastrol with dual effects on both ion conductivity and protein trafficking. *Journal of Biological Chemistry*, 281(9): 5877-5884.

Tamargo. J., Delpón, E. *et al.*, (2004). Pharmacology of cardiac potassium channels. *Cardiovascular Research*, 62: 9-33.

Thomas, D., Gut, B., Wendt-Nordahl, G. & Kiehn, J. (2002). The antidepressant drug fluoxetine is an inhibitor of human ether-a-go-go-related gene (HERG) potassium channels. The *Journal of Pharmacology and Experimental Therapeutics*, 300: 543-548.

Torres, A., Bansal, P., Sunde, M., Clarke, E. *et al.* (2003). Sructure of the HERG K^+ Channel S5P Extracellular Linker. *The Journal of Biological Chemistry*, 278 (43): 42136-42148.

Traboulsie, A., Chemin, J., Kupfer, E., Nargeot, J. & Lory, P. (2006). T-type calcium channels are inhibited by fluoxetine and its metabolite norfluoxetine. *Molecular Pharmacology*, 69: 1963-1968.

Tristani-Firouzi, M., Chen, J., Mitcheson, J. & Sanguinetti, M. (2001). Molecular Biology of K^+ Channels and their role in cardiac arrhythmias. *American Journal of Medicine*, 110: 50-59.

Vaidyanathan, R., Taffet, SM., Vikstrom, KL & Anumonwo, JM. (2010). Regulation of cardiac inward rectifier potassium current (I_{K1}) by sinapse associated protein-97. *Journal of Biological Chemistry*, 285: 28000-28009.

Van der Heyden, M., Smits, M. & Vos, M. (2008). Drugs and trafficking of ion channels: a new pro-arrhytmic threat on the horizon? *British Journal of Pharmacology*, 153: 406-409.

Wagner, M., Riepe, K., Eberhardt, E. & Volk, T. (2010). Open channel block of the fast transient outward K^+ current by primaquine and chloroquine in rat left ventricular cardiomyocytes. *European Journal of Pharmacology*, 647: 13-20.

Widmaier, E., Raff, H., Strang, K. (2006). *Vander's Human Physiology: The Mechanisms of Body Function*. McGraw-Hill: 11th edition, United States of America.

White, N. (2007). Cardiotoxicity of antimalarial drugs. *Lancer Infection* Disease, 7: 549-558.

Xia, M., Jin, Q., Bendahhou, S., He, Y., Larroque, MM., Chen, Y., *et al.* (2005). A $K_{IR}2.1$ gain-of-function mutation underlies familial atrial fibrillation. *Biochem Biophys Res Commun*, 332: 1012–9.

Xie, L. John S. & Weiss J. (2002). Spermine Block of the Strong Inward Rectifier Potassium Channel $K_{IR}2.1$: dual role of surface charge screening and pore block. *Journal of General Physiology*, 120: 53-66.

Yellen, G. (2002). The voltage-gated potassium channels and their relatives. *Nature*, 419: 35-42.

Yi, BA., Minor, D., Lin, YF., Jan, Y. & Jan L. (2001). Controlling potassium channel activities: interplay between the membrane and intracellular factors. *Proceedinds of the National Academy of Sciences*, 98 (20): 11016-11023.

Yu, F., Yarov-Yarovov, V., Gutman, G. & Catterall, W. (2005). Overview of Molecular Relationships in the Voltage-Gated Ion Channel Superfamily. *Pharmacological Reviews*, 57: 387-395.

Zaza, A. (2010). Control of the cardiac action potential: The role of repolarization dynamics. *Journal of Molecular and Cellular Cardiology*, 48: 106-111.

Zhang, H., Garratt, CJ., Zhu, J. & Holden, AV. (2005). Role of up-regulation of I_{K1} in action potential shortening associated with atrial fibrillation in humans. *Cardiovascular Research*, 66: 493–502.

Zhang, Y., Robertson, J., Gray, D. & Palmer, L. (2004). Carboxy-terminal determinats of conductance in Inward-rectifier K^+ channels. *Journal of General Physiology*, 214: 729-739.

Zhou, D., Harrison, B., Shah, U., Mewshaw, R. *et al.* (2006). Studies toward the discovery of the next generation of antidepressants. *Bioorganic & Medicinal Chemistry Letters*, 16(5): 1338-1341.

Zhou, M., Morais-Cabral, JH., Mann, S. *et al.* (2001). Potassium channel receptor site for the inactivation gate and quartenary amine inhibitors. *Nature*, 411: 657-661.