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Direct Regulation of Inward Rectifier K^+ (Kir) Channel by Endocannabinoids

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Résumé

La famille des canaux potassiques à rectification entrante (Kir), exprimée de manière ubiquitaire, repolarise et maintient le gradient de tension à travers les membranes des cellules excitables et non-excitables. Les canaux Kir sont fortement régulés par divers lipides membranaires, tels que les phosphoinositides, les phospholipides anioniques secondaires, le cholestérol, le Coenzyme A (CoA) à longue chaîne et l'acide arachidonique. Kir2.1 est fortement exprimé dans le tissu musculaire strié des cellules cardiaques auriculaires et ventriculaires. Il joue un rôle essentiel dans la régulation du potentiel de membrane au repos et de la contraction des cellules musculaires cardiaques et lisses en générant le courant K^+ à rectification entrante (I_{K1}). (I_{K1}). Les mutations de Kir2.1 avec perte de fonction sont à l'origine du syndrome d'Andersen-Tawil (ATS). Par conséquent, l'altération de la fonction de Kir2.1 est un déterminant essentiel au bon fonctionnement du cœur. Les endocannabinoïdes sont une classe spéciale de lipides naturellement exprimés dans une variété de cellules, y compris les cellules cardiaques, neuronales et immunitaires. Le système endocannabinoïde, y compris les récepteurs cannabinoïdes (CBR), agit comme un système de réponse au stress qui s'active. Des études menées chez l'animal et chez l'homme suggèrent que la modulation pharmacologique de ce système pourrait représenter une nouvelle approche thérapeutique. Cependant, ces dernières années, il est devenu clair que si les endocannabinoïdes peuvent déclencher des changements de signalisation en aval par l'intermédiaire des CBR, ils peuvent également interagir directement avec les canaux ioniques indépendamment des CBR pour moduler la fonction cellulaire.

Dans cette étude, nous avons utilisé la technique de double électrode en voltage imposé pour examiner les effets d'un panel d'endocannabinoïdes sur la fonction de Kir2.1. Nous avons montré qu'un sous-ensemble d'endocannabinoïdes, mais pas tous, peut réguler la fonction de Kir2.1 à des degrés divers, indépendamment des CBR. Nous avons également démontré que les endocannabinoïdes peuvent également réguler les protéines mutées menant à l'ATS (G144S et V302M). Nous avons également observé que l'effet des endocannabinoïdes n'est pas conservé parmi les membres de la famille Kir, avec des différences observées entre les canaux Kir2.1, Kir4.1 et Kir7.1. Ces résultats pourraient avoir des implications plus larges pour les fonctions des cellules cardiaques, neuronales et immunitaires.

Mots clés : Kir2.1, Endocannabinoïdes, LQT7, Rectification entrante, G144S, Kir7.1, Kir4.1

Abstract

The ubiquitously expressed family of inward rectifier potassium (Kir) channels repolarizes and maintains the voltage gradient across excitable and non-excitable cell membranes. Kir channels are highly regulated by various membrane lipids, such as phosphoinositides, secondary anionic phospholipids, cholesterol, long chain acyl- Coenzyme A (CoA), and arachidonic acid. Kir2.1 is highly expressed in striated muscle tissue of atrial and ventricular heart cells. It is critically involved in regulating the resting membrane potential and contraction of cardiac and smooth muscle cells through the generation of the current I_{K1}. Loss-of-function mutations in Kir2.1 cause Andersen-Tawil syndrome (ATS). Therefore, altered Kir2.1 function is a critical determinant of proper heart function. Endocannabinoids are a special class of lipids that are naturally expressed in a variety of cells, including cardiac, neuronal, and immune cells. The endocannabinoid system, including cannabinoid receptors (CBRs), acts as a stress response system that is activated. Studies in both animals and humans suggest that pharmacological modulation of this system might represent a novel approach to treatment. However, in recent years, it is becoming clear that while endocannabinoids can trigger downstream signaling changes through CBRs, they can also directly interact with ion channels independently of CBRs to modulate cellular function.

In this study, we used the electrophysiology technique called two-electrode-voltage-clamp (TEVC) in combination with mutagenesis studies to examine the effects of a panel of endocannabinoids on the function of Kir2.1. We showed that a subset of endocannabinoids, but not all, can regulate the Kir2.1 function to varying degrees, independent of CBRs. We also demonstrated that endocannabinoids can also regulate mutants linked with ATS (G144S and V302M). We also observed that the effect of endocannabinoids is not conserved among Kir family members, with differences observed between Kir2.1, Kir4.1 and Kir7.1 channels. These findings could have broader implications for cardiac, neuronal, and immune cell functions.

Key words: Kir2.1, Endocannabinoids, LQT7, Inward rectification, G144S, Kir7.1, Kir4.1

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Abbreviations

2-AG: 2-Arachidonoylglycerol

AC: Adenylyl cyclase

AEA: Anandamide

ATS: Anderson Tawil Syndrome

BiVT: Bidirectional Ventricular Tachycardia

cAMP: Cyclic Adenosine Monophosphate

CBR1: Cannabinoid receptor type 1

CBR2: Cannabinoid receptor type 2

CBD: Cannabidiol

CBN: Cannabinol

CD: Cytoplasmic domain

CoA: Coenzyme A

PKA: Protein kinase A

cKO: Conditional knockout

CHO: Chinese Hamster Ovary cells

cGMP: Cyclic guanine monophosphate

cCMP: Cyclicytidine monophosphate

CNBD: Cyclic-nucleotide binding domain PD: Pore domain

CNS: central nervous system

CPVT: Catecholaminergic polymorphic ventricular tachycardia

CTD: Cytoplasmic C-terminal domain

DADs: Delayed after depolarizations

DDs: Depressive Disorders

DGS-NTA:1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]

EADs: Early After Depolarizations

EAST/ SESAME: Epilepsy, Ataxia, Sensorineural Deafness, and Tubulopathy

ECS: Endocannabinoid system

ESC: Endocannabinoid system

ELISA: Enzyme-linked immunoassay

FAAH: Fatty acid amide hydrolase

FAEs: Fatty acid ethanolamides

FAF: Familial atrial fibrillation

GABA: Gamma-aminobutyric acid

GIRKs: G-protein-coupled inwardly rectifying K^+ channels

GPCR: G protein-coupled receptor

G_{max}: Slope of the I-V curve

GYG: Glycine-tyrosine-glycine selectivity filter

Cryo-EM: Cryogenic Electron Microscopy

5-HT: 5-hydroxytryptamine

HEK293: Human Embryonic Kidney

I_{Ca-L}: ionic currents of L-type calcium current

***I_{Kr}*:** Rapid component of the delayed rectifier potassium current

KCNE1: Potassium voltage-gated channel subfamily E member 1

KCNE2: Potassium voltage-gated channel subfamily E regulatory subunit 2

KCNJ2: Potassium inwardly rectifying channel subfamily J member 2

KCNJ10: Potassium inwardly rectifying channel subfamily J member 10

KCNJ11: Potassium inwardly rectifying channel subfamily J member 11

KCNJ13: Potassium inwardly rectifying channel subfamily J member 13

TRIP8b: Tetratricopeptide repeat–containing Rab8b-interacting protein

TMD: Transmembrane domain

Kv: Voltage-gated potassium channel

LCA: Leber Congenital Amaurosis

LQT7 : Long QT syndrome type 7

2-MG: 2-Monoacylglycerols

MAPK: Mitogen-activated protein kinase

MbCD: Methyl-b-cyclodextrin

E_{max}: Maximal effect

NEA: Nervonoyl Ethanolamide

PA: Phosphatidic acid

PG: Phosphatidylglycerol

PI: Phosphoinositides

PIP_2: Phosophatidylinositol-4,5-bisphophate

PNS: Peripheral nervous system

POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol

PS: Phosphatidylserine

RPE: Retinal pigment epithelium

SQT3: Short QT syndrome type 3

SSRIs: Selective serotonin reuptake inhibitors

SVD: Snowflake Vitreoretinal Degeneration

TCAs: Tricyclic antidepressants

THC: Δ^9-tetrahydrocannabinol

THCV: Tetrahydrocannabivarin

TRPV: Transient receptor potential vanilloid

VGCC: Voltage gated calcium channel

VSD: Voltage sensor domain

WIN 55, 212: Potent aminoalkylindole synthetic cannabinoid

1. Introduction

Many known methods of cell communication exist such as gap junction, hormonal signaling, neurotransmission, as well as electrical activity. The function of excitable cells heavily relies on electrical activity through transmitting information between or within the cells. Protein complexes embedded in the membrane which regulate ionic current across the lipid membranes are known as ion channels and respond to electrical stimuli (Hille, 2001). Ion channels are key proteins that are essential for many cellular mechanisms such as signal transduction across lipid membranes. There are different mutations that encode ion channel genes that are associated with many human diseases. Genetic data have shown that there is a close relationship between the mis-regulation or aberrant behavior of ion channels and many diseases. These misbehaving channels lie within channelopathies that delve into all diseases linked to ion channels defects. The presence of ion channels makes them key targets for numerous drugs; additional efforts are being made to understand the role of ion channels in pathophysiological processes in the development of various diseases. In neurons, cardiac cells and muscle cells ion current are responsible for the excitable activity and is very important in the regulation of physiological signals in all eukaryotic cells (Amin et al., 2010; Niemeyer et al., 2001).

Among the many families of membrane proteins, K^+ channels play a crucial role in regulating the transportation of the K^+ ions across the cell membrane in all organisms. There are more than 90 genes reported to encode subunits of K^+ channels. Structural and functional analyses have led to a deep understanding of K^+ channel selectivity, gating, and conductance. These include key aspects such as regulation of K^+ channels by different ligands, and trafficking of proteins and polymers through lipid membrane interaction (Weingarth et al., 2013). One of the key features of K^+ channels is that they carry K^+ ions better than Na^+ ions (Hille, 2001). Selective transport of K^+ ions is essential for many physiological functions such as homeostasis and repolarization of action potential in excitable cells. Therefore, the requirement for selecting K^+ current across the lipid membrane leads to K^+ channels present in all plasma membrane of cells (animals and plants). The dysregulation of each member or subfamily of these K^+ ion channels might cause serious disorders such as Alzheimer's, epilepsy, and Parkinson's disease. A vast amount of information in molecular biology has provided evidence for the role of ion channel function in various diseases (Gui et al., 2012; Kasianowicz, 2012; Li et al., 2013; Wulff et al., 2009). K^+ channel modulators have been produced by chemical synthesis, assessed by virtual screening, and combined in order to provide improvement

of many pathophysiological diseases (Bodendiek et al., 2009; Chen et al., 2012; Jenkins et al., 2011; Kroigaard et al., 2012).

K^+ channels are principally formed by arrangements of subunits called α and β. Subunits α define the structural properties of K^+ channels while the auxiliary β subunits are involved in modifying the properties of the pore-forming α subunits. K^+ channels α-subunits can be clustered into at least 8 families based on structural and functional features (Wei et al., 1996). Three of these families including Kv, ether-a-go-go-related gene (EAG), and KCNQT are gated by voltage and have shown a common motif of six transmembrane domains (TM). While two other families (CNG and SK/IK) share a common motif, they are not voltage-gated and are respectively, dependent on cyclic nucleotides and calcium-dependant. The other three potassium channel α-subunits share different TM domain patterns. For instance, BK channels or Slo family potassium channels possess 7 TM domains (Meera et al., 1997) and they are primarily controlled by voltage, Ca^{2+}, and pH (Schreiber et al., 1998). In addition, the inward rectifier K^+ channel (Kir) comprises two transmembrane domains. One last family called K2P contains the inward-rectifier motif in tandem conferring it a 2-pore structure. In general, K^+ channels are categorized into four subgroups based on the number of TM domains to create a pathway for ionic permeation across the membrane. Two transmembrane helices which are linked by a short loop known commonly as a “P loop” are trademark features of the K^+ channels. Thus, the K^+ channels share a common feature: they all have a canonical architecture consisting of two inner helices and a loop (2TM/P). However, each subfamily of channels also exhibits unique characteristics. For instance, Kir channels (2TM/P) have two transmembrane helices surrounded by a P loop, regulating the influx of potassium ions into the cell. The second subfamily represents the predominant type of K^+ channels, including both voltage-gated and ligand-gated types that play a crucial role in cellular processes and communication. The hybrid K^+ channel, consisting of six TM/P and two TM/P, was first discovered in yeast. Lastly, the 4TM/2P channels, known as "leakage," channels, play an essential role in maintaining ionic homeostasis (Figure 1) (Choe, 2002).

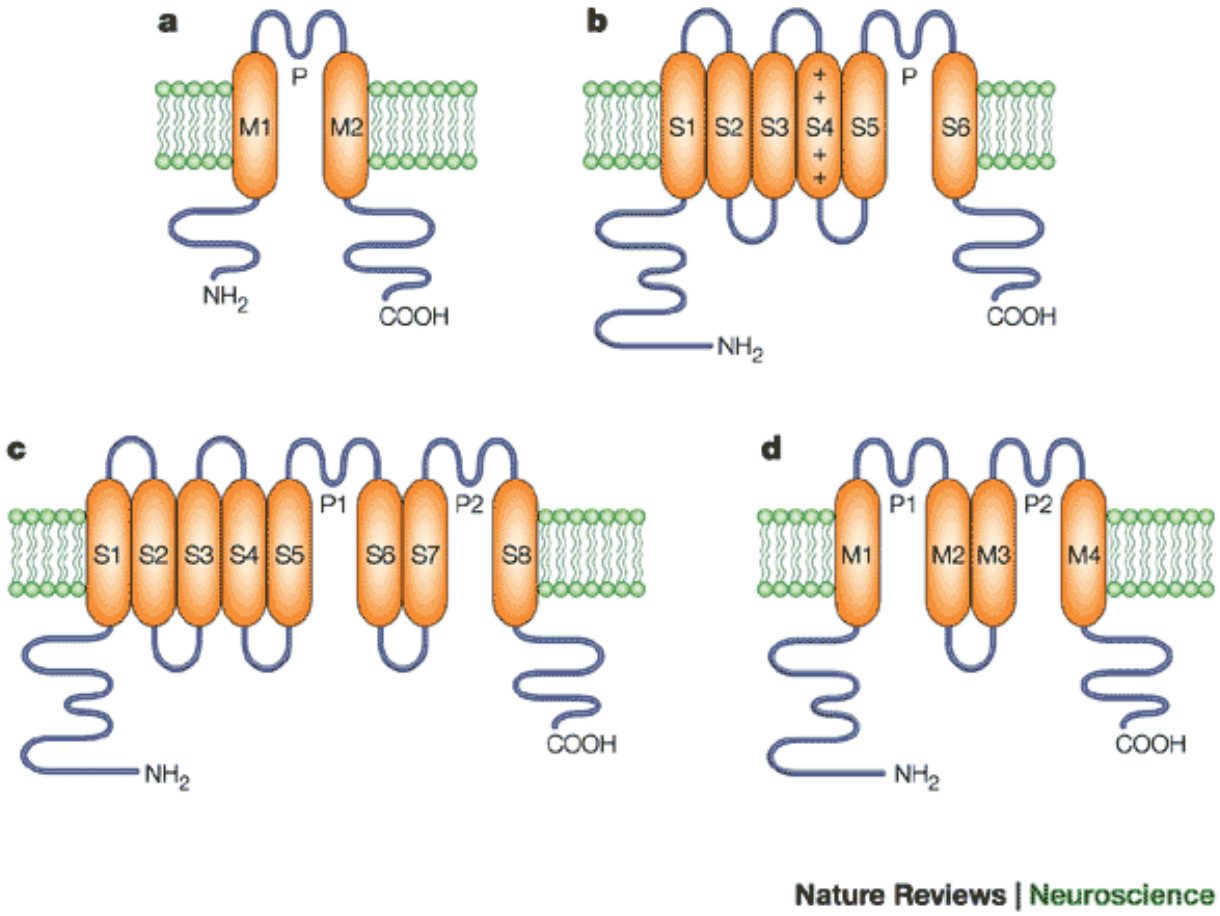


Figure 1. Four main classes of K^+ ion channels. a) It represents an inward-rectifying K^+ channel (2TM/P) containing two transmembrane helices with a P loop located between them. b) It is a predominant class of voltage-gated and ligand-gated K^+ channels. c) The hybrid K channel described here contains both 6TM/P and 2TM/P. d) 4TM/2P is a channel with four transmembrane and two P loops. These channels are referred to as "leakage" channels. From (Choe, 2002).

1.1. Two transmembrane potassium channels or inward rectifier K^+ channels

Inward rectifier K^+ channels present in both eukaryotic and prokaryotic cells (Durell & Guy, 2001). To date, 15 well-known Kir channels are discovered in mammalian categorized into seven different families and can be further divided into four functional groups: The first group is the classical Kir channels also called Kir2.x which are constitutively active; the second group are G-protein-gated Kir channels or Kir3.x and regulated through G-protein-coupled receptors; the third group is ATP-sensitive K^+ channels often referred to as K_{ATP} channels, are indeed the same as the Kir6.x channels.

These are tightly related to cellular metabolism; and the last functional group of Kir channels is, K^+ transport channels including Kir1.x, Kir4.x, Kir5.x, and Kir7.x, each having their own specific expression pattern and functional characteristics (Dahal et al., 2012) (Figure 2.A). Besides strong homology, the simplicity of the Kir channels subunits facilitates the combination of both homomeric and heteromeric to shape a functional Kir channel.

In 1993, for the first time, using the expression-cloning technique, two cDNAs of Kir channels, Kir1.1 (Ho et al., 1993) and Kir2.1 (Kubo et al., 1993) were isolated from a rat's outer medulla of the kidney and a mouse macrophage cell line, respectively. In their main structure, they hold a common motif of two recognized membrane-spanning domains (TM_1 & TM_2). They are linked by an extracellular pore-forming region (H_5), a cytoplasmic amino (NH_2)- and a carboxy (COOH)-terminal domains (Figure 2.B). This topology is now known as a basic building block among all types of Kir channels. The pore-forming region acts as an "ion selectivity filter" (Heginbotham et al., 1994). This region has a common motif sequence T-X-G-Y(F)-G- as a signature in all K-selective ion channels. Kir channels do not possess the S4 which is a voltage sensor region, while it is expressed in all voltage-gated channels (Na^+, Ca^{2+}, K^+). Thus, Kir channels are insensitive to the voltage of the membrane. According to their definition, inward rectification is not an intrinsic characteristic of inward rectifying K^+ channels, while it is a result of blocking of K^+ current to the outside of the cell by intracellular substances including Mg^{2+} and polyamines. Regarding structure, two transmembrane strands are insufficient to form a complete Kir channel; Therefore, Kir channels are composed of four such subunits (Figure 2.B).

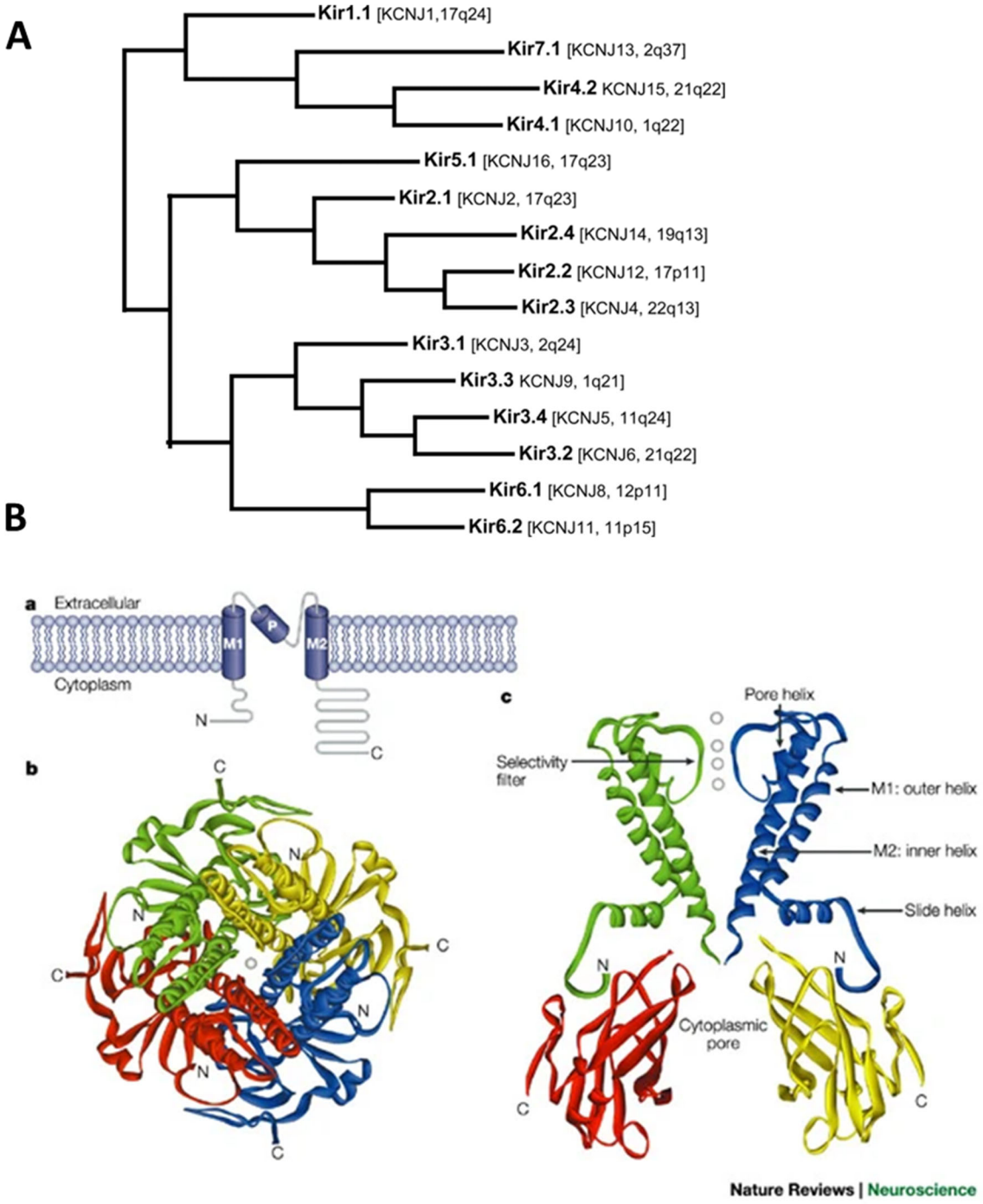


Figure 2. Phylogenetic tree and molecular structure of Kir channels. A) Amino acid sequencing and phylogenetic analysis for 15 known members of the human Kir family. The gene names used by the International Pharmacological Union and the Gene Hugo Nomenclature Committee are provided. The subunits have been grouped into four functional categories. Adapted (Kubo et al., 2005), B) a) graphic of a Kir channel subunit. Each subunit consists of two transmembrane helices (M1 and M2), a hole-forming region containing the pore helix (P) and a cytoplasmic domain formed by amino (N) and carboxy (C)-terminals. b) See the tetrameric structure of the KirBac1.1 channel 4 (PDB ID:1P7B) on the extracellular side. The monomers are individually red, green, yellow and blue. The K^+ ion (white) indicates the transmission path. c) The side view of the KirBac1.1 structure shows the transmembrane domain of the two subunits (green and blue) and the C-terminal domain of their neighbouring subunits (red and yellow). The white spheres represent K^+ ions in the selective filter. From (Bichet et al., 2003).

An inward rectifying K^+ channel was discovered for the first time in skeletal muscle (Katz, 1949). Despite what the Nernst equation predicts, Kir channels actually have a higher inward flow of K^+ ions instead of outward. This means that more potassium ions enter the cell than leave it (Figure 3.A). Inward rectifying K^+ channels seem not to follow the Hodgkin-Huxley kinetic relationship in terms of voltage membrane, even though they are highly dependent on the electrochemical gradient for K^+ ion [E_m (membrane potential) – E_k (Equilibrium potential)] (Figure 3.A) (Hagiwara et al., 1976). The specific features of Kir currents do not arise from a Kir channel divergence from the basic laws of biological chemistry. Instead, they arise from the obstructive effects of intracellular divalent cations and other molecules on the open channel pore, causing asymmetry (Hibino, et al 2009). Except for Kir7.1, the conductance of nearly all Kir channels follows the square root of the extracellular potassium concentration ($[K^+]o$) (Hagiwara & Takahashi, 1974; Kubo et al., 1993). Instead of the standard permeability theory, this behaviour confirms the multi-ion pore concept (Hille & Schwarz, 1978). The degree to which variations in $[K^+]o$ modify Cs-induced inhibition of the Kir current in starfish eggs. Kir channels are thought to have at least two K^+ binding sites (Ciani et al., 1980). Even without Mg^{2+} and polyamines, Kir2.1 conductance exhibits a square-root dependency on $[K^+]o$, suggesting that this is a characteristic of the open-channel pore (Lopatin & Nichols, 2001).

I_{K1} (inward rectifier potassium current) is regulated by alterations in $[K^+]o$, which is the relationship between $[K^+]o$ and I_{K1}. In most cells, I_{K1} conductance increases along with the extracellular potassium concentration. This square-root dependence on $[K^+]o$ controls the relationship between $[K^+]_o$ and I_{K1} conductance. The magnitude of I_{K1} ,therefore, increases together with $[K^+]o$, but not linearly (Bouchard et al., 2004).

Thus, Kir channels generate large inward K^+ currents at potentials that are negative to Ek but allow less K^+ conductance at potentials positive to Ek under physiological conditions (Miyazaki et al., 1974; Sakmann & Trube, 1984). Therefore, cells with great Kir conductance show a resting membrane potential (Eres) close to Ek. Thus, this feature plus their direct link to cellular potential leads Kir channels to play a very important role in the maintenance of resting membrane potential and the duration of action potential in excitable cells (Cardiac and neurons) (Hagiwara & Takahashi, 1974; Miyazaki et al., 1974; Sakmann & Trube, 1984) (Figure 3.B).

Kir channels are expressed in a wide variety of excitable and non-excitable cells including cardiac myocytes (Beeler Jr & Reuter, 1970; McAllister & Noble, 1966; Rougier et al., 1968), neural cells (Gähwiler & Brown, 1985; North et al., 1987; Williams et al., 1988), immune cells (Kurata et al., 2007; Lewis et al., 1991), endothelial cells (Silver & DeCoursey, 1990), epithelial cells (Hebert et al., 2005; Lu et al., 2002), glial cells (Kuffler & Nicholls, 1966) and oocytes (Hagiwara & Takahashi, 1974). In addition, G protein-gated K (KG) (Wulff et al., 2009) and ATP-sensitive K (K_{ATP}) channels are also shown to have inward rectification properties (Kurachi, 1995; Sakmann et al., 1983). Therefore, Kir channels are not only involved in the regulation of electrical properties of cells, but they also interact with G-protein coupled receptors (GPCR), and may be involved in linking metabolic activity state and membrane excitability in vivo (Hibino et al., 2010).

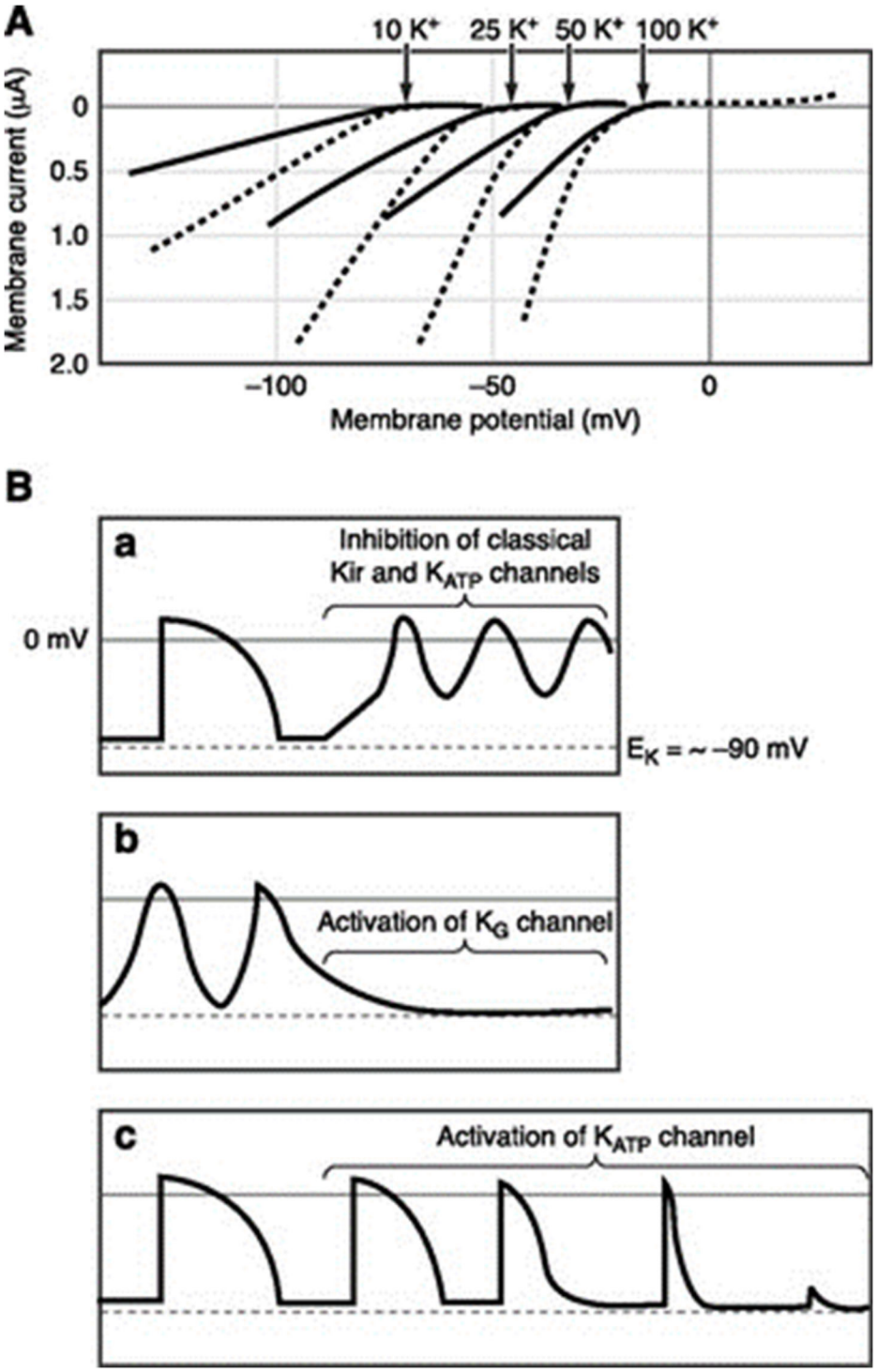


Figure 3. Functional characteristics of Kir channels. A) The relationship between potassium's extracellular concentrations and Kir channels' conductance. Continuous and broken lines show instantaneous and steady-state currents, respectively. From (Hagiwara et al., 1976). B: Kir channels control cardiac cell excitability. Schematically, action potentials in the sinoatrial node (b) and ventricular myocytes (a and c) under different conditions can be seen. Eres depolarizes, and Em can vary in ventricular myocytes when classical Kir and KATP channels are inhibited (a). On the other hand, activation of KATP channels causes Em to become hyperpolarized, shortens the time between action potentials, and may prevent action potentials from forming (c). Many KG channels are expressed by sinoatrial node cells, and when these channels are activated, Em may become hyperpolarized or experience bradycardia (b). From (Hibino et al., 2010).

1.1.1. Kir2.x subfamily with focus on Kir2.1

One of the classically large superfamilies of potassium inward rectifiers is Kir2.x channels which include five subfamilies: 1) Kir2.1, 2) Kir2.2, 3) Kir2.3, 4) Kir2.4 and 5) Kir2.6 (Table 1). All 4 different Kir2.x subunits have a length between 427 and 445 amino acids (Shealy et al., 2003). Subunits are stabilized through the salt bridges and electrostatic interactions (Preisig-Müller et al., 2002). The disulfide bonds, which presented between the subunits, have a crucial role in the folding process and the assembly of ion channels protein (Nichols & Lopatin, 1997). Kir2.x subunits functionally form homotetramers and in some cases heterotetramers (Preisig-Müller et al., 2002; Zobel et al., 2003). When looking at heterotetramers, subunits which include Kir2.2, and also Kir2.6 show the greatest level of conductance, Kir2.1, and Kir2.4 display lower levels of conductance (two to three times less) (Lopatin & Nichols, 2001). From the perspective of the strength of its conductivity, these channels follow this order Kir2.4> Kir2.2 > Kir2.3 > Kir2.1> Kir2.6.

Key features of Kir2.x channels include, but are not limited to: 1) always being active and having a strong rectification which results in the holding membrane staying in the negative potentials (close to the resting membrane potential) in excitable cells such as cardiomyocytes (review in (Dhamoon & Jalife, 2005)). When the potential of the membrane is more positive than the equilibrium potential (E_K), polyamines and intracellular Mg^{2+} lead to channel plugging, affecting the efflux of K^+ by an electrostatic and steric block. The role of Kir2.x channels in establishing and holding the resting membrane potential creates latency in consecutive action potential firing and contributes an appropriate QT interval (the required time for the contraction and recovery of heart muscle).

Generally, Kir2.x channels are expressed in the brain, cardiac, smooth, and skeletal muscle cells. Kir2.1 and Kir2.3 subunits demonstrate a high pattern of expression in the brain; Kir2.2 in the cerebellum and brain and Kir2.4 has a very limited expression pattern and mostly it displays in cranial nerve motor nuclei (Anumonwo & Lopatin, 2010; Hibino et al., 2010). Kir2.1, Kir2.2, and Kir2.3 channels are co-expressed and likely to co-assemble in cardiac muscle cells (Preisig-Müller et al., 2002).

In the heart, Kir2.1 channels are the molecular proteins that tightly correlate with the I_{K1} current (Figure 4), through the controlling of the resting membrane potential and contraction of cardiac and smooth muscle cells (final phase of ventricular repolarization). Some of the Kir channels play crucial roles in cardiac excitation-contraction coupling (Hibino et al., 2010). Atrial and ventricular chamber contractions must be precisely coordinated for the heart to function as a pump. The action potentials (AP) that originate in the sinoatrial node (SAN) and spread to the atria and ventricles start these contractions (Swale et al., 2014). I_{K1} is playing a key role in ventricular myocytes and Purkinje fibers but is notably smaller in atrial myocytes unless in mouse atria.

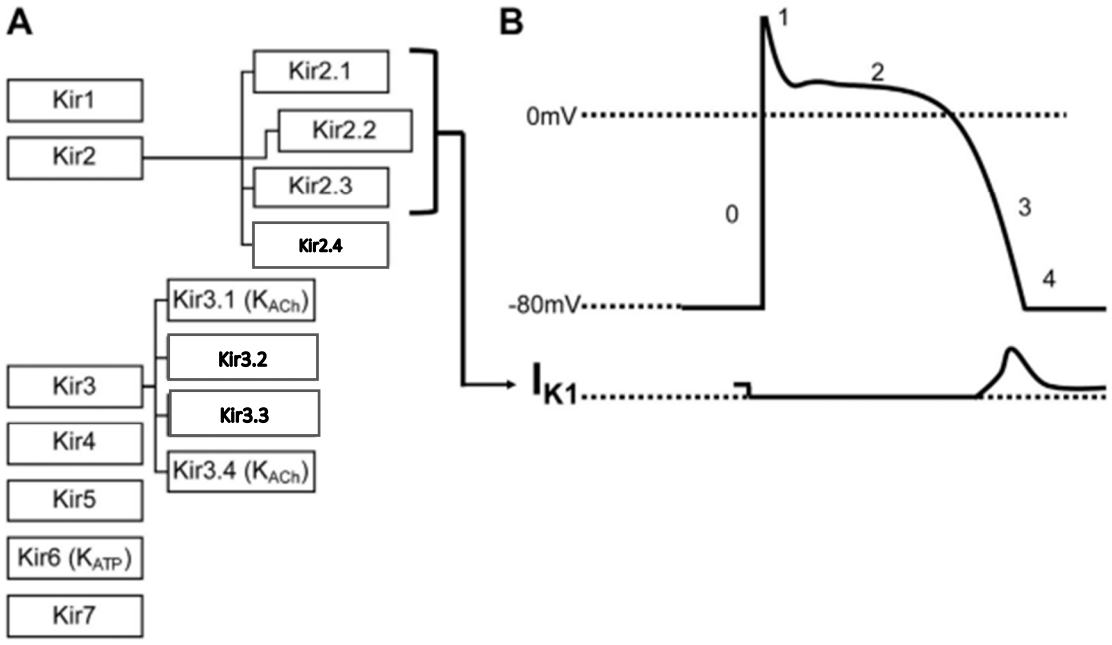


Figure 4. Schematic diagram of Kir superfamily and their function in the action potential of heart cells. From (Reilly & Eckhardt, 2021).

Although Kir channels lack voltage sensors, they display voltage-dependent because cations like Mg^{2+} and polyamines, which are typically present in all cells, block these channels from the inside (Yang et al., 2003). Thus, the voltage dependency results in a decrease in K^+ conductance with depolarization of the membrane. At physiological voltages, the channels conduct outward K^+ current with a peak between −60 to −40 mV, which reduces at more positive voltages (Zhou et al., 1994). When Ek is close to −80 mV, currents start to reverse to inward. The original definition of inward

rectification emphasizes the large inward current portion (Mascher & Peper, 1969) and has been highlighted as the significant characteristic of Kir2.x channels historically (Anumonwo & Lopatin, 2010). In normal physiological conditions, voltages below −80 mV are not reached in the heart. Therefore, the predominant component of I_{K1} is the outward current, which contributes to cardiac electrical stability. On the other hand, the outward current plays a crucial role in phase 3 repolarization in order to regulate the cardiac membrane potential and allow Na^+ channel recovery from inactivation (Vaidyanathan et al., 2016).

1.1.2. Kir2.x pharmacology

There are several pharmacological factors that affect Kir2.1 currents. As the importance of I_{K1} current is better understood, thus the agents that modulate I_{K1} are critical for both proarrhythmic and antiarrhythmic effects.

1.1.3. Pore blockers of Kir2.x channels

It is well known that Kir2.1 channels conduct the K^+ ions inside of the cells rather than outside (Hille, 1978). In a variety of native tissues and transfected cells, Kir2.1 channels have been seen to be blocked by divalent cations (Dart et al., 1998; Shioya et al., 1993). There are two different channel sites reported to interact with these cations: the first one is the site close to the surface which does not sense any electrical voltage of the membrane. The second one is deeper within the membrane, halfway through the electrical field (Shioya et al., 1993). Only one single ion can block the channel at both mentioned sites. The studies have shown that Mg^{2+} demonstrates an antagonist effect to K^+, meaning that a competition exists between K^+ and Mg^{2+} to block the external activation site. Extracellular Mg^{2+} reduces the inward current of Kir2.2 channel. Computational studies reported that Mg^{2+} blocks the channel by staying close to the selectivity filter and leading to the reduction in Kir2.x channels current. Mutations to the negatively charged residues on the outer side of the selectivity pore of the Kir2.2 led to the reduction of voltage-dependent blockage current in the presence of Mg^{2+} through electrostatic repulsion (Li et al., 2014).

Other divalent cations that induce extracellular block include Ca^{2+} and Ba^{2+} (Matsuda & Cruz, 1993). Transient increases in Ca^{2+} during the recording of AP resulted in an inhibition of the I_{K1} (Zaza et al., 1998). The reduction of the current might be due to the decrease in the outward current through decreasing the open probability of the channel in the open state (Mazzanti & DiFrancesco, 1989). Approximately 50% of Kir2.1 current will be blocked via concentrations of 3-10 μM of Ba^{2+} if the membrane potential nears -80 mV. Barium disrupts the rectification of Kir2.1 through a non-competitive mechanism. Moreover, loss-of-function mutations (E125N and T141A) within the pore region disrupt Ba^{2+} entry and impair binding within the Kir2.x channel (Standen & Stanfield, 1978). Studies have shown that Ba^{2+} binding site is in the deeper site of the pore specifically, within the innermost cation-binding site of the selectivity filter (Sabirov et al., 1997; Shioya et al., 1993). Both results of homomeric Kir2.1 and Kir2.3 channels expressed in *Xenopus oocytes* demonstrate similar Ba^{2+} sensitivities, however, their sensitivity was less than native cardiac I_{K1} (Schram et al., 2003). The Kir2.2 channel demonstrated a similar sensitivity to I_{K1} of cardiac tissue, but with different blocking kinetics. Ba^{2+} is also known as an inhibitor for other types of K^+ channels such as delayed rectifier potassium channels, Ca^{2+}-activated K^+ channels and hyperpolarization- and cyclic nucleotide-activated channels (Shieh et al., 1998). Moreover, Ba^{2+} is a strong blocker of the BK channel pore (Zhou et al., 2012). Ba^{2+} also plays the role of charge carrier for L-type Ca^{2+} channels (Ferreira et al., 1997). Therefore, although Ba^{2+} has been used widely as a useful pharmacological tool for studying I_{K1}, it can also affect other ion channels and may influence the results in certain cell systems.

Studies have reported that there are some critical residues in the channel structure of Kir2.1, including D172 (Lu & MacKinnon, 1994), E299, E224 (Yang et al., 1995), D259, and D255, which play a crucial role in the determination of how polyamines block the channel. According to structural crystallographic studies, D255 impacts the kinetics of the blocking (Kurata et al., 2007). In another study, it has been shown that modifying specific residues by methanethiosulfonate (MTS) reagents in the channel at position C169 (which is between the D172 position and called the "rectification controller" and also the selectivity filter), lead to the reduction of the potency and voltage dependence of block spermine. Moreover, the C176 (position between D172 and the M2 helix bundle crossing) resulted in the change of voltage dependency, potency, and kinetics of the spermine block (Kurata et al., 2010).

Transgenic mice with altered levels of polyamine resulting in increased spermidine levels, show a decrease of I_{K1} by almost 38%, without any apparent effect on rectification (Lopatin et al., 2000). Moreover, spermine loss through disruption of spermine synthase gene led to I_{K1} with diminished rectification and no change in current density. This indicates a role of spermine in the rectification of potentials that are positive to E_K, while spermidine dominating at negative potential around E_K. Voltage-dependent manner in all Kir2.x isoforms is characterized by three distinguished components: 2 shallow and 1 steep (Panama & Lopatin, 2006). These components are anticipated to respond in spermidine blockage in 2 distinguished pore sites (Xie et al., 2002). The steep component is the resultant of binding to the negative residues in the cytoplasmic vestibule of the channel and the shallow components are accounted by binding to the rectification controller deeply inside the pore (Kurata et al., 2010; Stanfield et al., 1994). The blockage in the shallow sites is stronger in Kir2.2 than Kir2.3 when considering the rectification and steep components (Panama & Lopatin, 2006).

Chloroquine is used as a primary treatment for malaria and therapy for inflammatory disorders. However, despite its clinical benefits, it has a narrow safety margin, meaning that the difference between the practical and potentially harmful doses is relatively tiny. Chloroquine has a general blocking effect on cardiac potassium (K^+) channels and can block sodium (Na^+) channels at concentrations higher than 20 mM (Orta-Salazar et al., 2002).

Evidence has shown that it led to the prolongation of QT (Heart's ventricular contraction and relaxation time (repolarization phase)) and QRS (Ventricular activation and recovery on ECG (depolarization phase)) in humans (Rodríguez-Menchaca et al., 2008). At higher doses, chloroquine can cause ventricular ectopy and ventricular arrhythmias (Riou et al., 1988). These clinical consequences are due to the prolongation of the cardiac AP duration, automaticity enhancement, and reduction in maximum diastolic potential at the cellular level (Rodríguez-Menchaca et al., 2008). These cellular changes occur due to the I_{K1}, I_{Kr} (Sánchez-Chapula et al., 2001), and also I_{Na} blockage (Orta-Salazar et al., 2002). Similar to the other pore blockers previously mentioned here, chloroquine also blocks the Kir2.x channels in a voltage- and K-dependent manner; this blockage happens from the cytoplasmic surface of Kir2.x channels (Rodríguez-Menchaca et al., 2008). Even when polyamines blocked the Kir2.x channels, chloroquine still can reach its binding site, it has been suggested that there is another binding site for chloroquine that is distinct from the polyamines site. However, chloroquine has a lethal potential, but in certain cardiac diseases patients with

persistent atrial fibrillation who had been prescribed chloroquine (14 days) showed a decrease in the atrial fibrillation which might be a result of blockage of I_{K1}, K_{ACh} and I_{Na} current to prolong the AP of the heart atrium (Orta-Salazar et al., 2002; Takemoto et al., 2018).

1.1.4. Kir2.x channels and regulation by membrane lipids

Kir2.x channels are highly regulated by various membrane lipids, including phosphoinositide, secondary anionic phospholipids, cholesterol, long-chain CoA, and arachidonic acid (Fürst, Mondou, et al., 2014; Levitan, 2009; Shumilina et al., 2006; Wang et al., 2008). In the last decades, signaling lipids came to be considered a main regulator of Kir channels in a variety of tissues including but not limited to neural and heart tissue whose function depends on cell excitability. Studies have shown that neither as a signalling molecule nor as a ligand, but these hydrophobic molecules directly regulate the function of many ion channels in the membrane (Hilgemann, 2007; Huang, 2007). The mechanism of action of the lipids and how they can change the conformation of the protein is poorly understood. The lack of binding between ion channels and lipids makes it more challenging to investigate more about the regulatory role of lipids.

i. Phosphatidylinositol 4,5-bisphosphate (PIP_2)

In 1998, for the first time, D'Avanzo in Doyle's laboratory has shown that lipids can directly activate Kir channels. One of the well-studied signaling lipids is 4,5-bisphosphate (PIP_2), a minor phospholipid in eukaryot membranes (<1%), is necessary and enough to activate K^+ channels (D'Avanzo, Cheng, Doyle, et al., 2010; D'Avanzo, Cheng, Wang, et al., 2010; Huang et al., 1998). Kir2.1 channels are highly dependent on the binding of PIP_2 (Fan & Makielski, 1997). The interaction between Kir2.x channels and PIP_2 occurs through electrostatic attractions between positively charged amino acids in the cytoplasmic domain and negatively charged phosphate groups in the head group of PIP_2 (Suh & Hille, 2008).

Binding of PIP_2 initiates a conformational shift in the tetrameric channel, leading to the opening of the channel and the flow of ions. The crystal structure of Kir2.x has identified critical, preserved amino acid residues across the separate monomers that play a vital role in facilitating PIP_2 binding

(Hansen et al., 2011). It is unsurprising that mutations in this conserved residue lead to *KCNJ2*-related diseases, which will be further studied later on (Ballester et al., 2007; Lopes et al., 2002). The following section discusses a set of drugs that can modify the regulation of Kir2.x by changing its binding capacity with PIP_2.

ii. PIP_2 Interference factor

Thus, PIP_2 is necessary for the function of the Kir2.1 channels. There is a variety of pharmacological compounds that show their effects through interaction and interference with PIP_2-Kir channels. Quinacrine, which was first discovered as an antimalarial drug, has been shown to inhibit Kir2.x channels in neurons of guinea pig (Evans & Surprenant, 1993). One of the important features of Quinacrine is that this pharmacological agent is highly lipophilic, thus interacting with membrane lipids directly. More evidence to prove this suggestion shows that the affinity of PIP_2 has been changed after addition of Quinacrine. However, the mechanism of blocking is not clear, but it is proposed that it can disrupt PIP_2-Kir channel interaction. Moreover, the application of both PIP_2 and Quinacrine demonstrated a decrease in the inhibition of Kir2.x channels (Evans & Surprenant, 1993).

Carvedilol is a popular β blocker that has been used for the treatment of high blood pressure, angina and heart failure (Dunn et al., 1997; Ruffolo & Feuerstein, 1997). In addition to its high potential treatment, it also has been considered as a multichannel blocker such as K_{ATP}, I_{Ks}, and I_{Kr} with different potency (Cheng et al., 1999; Yokoyama et al., 2007). In vitro studies have shown that carvedilol can block the K_{ATP} and K_{ACh} channels without any effect on I_{K1} directly.

It could be explained by the structure of the carvedilol which is highly lipophilic and has an alpha-hydroxyl secondary amine as a functional group. This leads to the insertion of carvedilol into the membrane which disrupts with PIP_2-channel complex interaction and resulting to the inhibition of Kir2.x channels (Ferrer et al., 2011). Kir2.3 has a higher affinity for the PIP_2 compared to Kir2.1 as we can see from their IC_{50} around 0.50 μM for Kir2.3 while >50 μM for Kir2.1 (100-fold difference). Inhibition was calculated based on voltage and concentration; by increasing the affinity of Kir2.3 for PIP_2, the degree of inhibition also decreased with carvedilol (Ferrer et al., 2011).

Interestingly, gambogic acid is another pharmacological agent (anti-cancer), which interferes with both PIP_2 and the blocking of the pore-loop of ion channels (Scherer et al., 2017). It showed a low inhibition effect on the Kir2.x channels when at low micromolar concentrations.

Dronedarone with the trade name of Multaq, is a new class (III) of antiarrhythmic agent that is really used to treat atrial fibrillation (AF) (V Naccarelli & R Kowey, 2014). In vivo studies have implicated that this antiarrhythmic drug also acts as a multichannel inhibitor that blocks the current of a variety of channels including $I_{Ca\text{-}L}$, I_{Ks}, and I_{Kr} (Gautier et al., 2003).

I_{K1} was inhibited but not completely by dronedarone in guinea pig ventricular myocytes in a concentration-dependent manner from 10 to 30 μM. Expression of Kir2.1 in the Xenopus oocytes revealed a blocking effect from dronedarone while this effect was not seen in other subunits such as Kir2.2 and Kir2.3; The blockage was slow and also reversible, and it was not voltage-dependent (Xynogalos et al., 2014).

One of the mutations in Kir2.1 (E224), located in the cytoplasmic pore region, resulted in the loss of the blocking effect of dronedarone. This suggests that the E224 site is responsible for the drug binding and mediating its effects. Data suggesting that I_{K1} is sensitive through modulation of pharmacological agents that blocks the ion channels Review in (Reilly & Eckhardt, 2021).

iii. Cholesterol

Cholesterol is a sterol synthesized in all animals. It is produced in hepatic cell of vertebrate. It plays a very important role in several functions of the body. Constituting 10 to 45% of total lipids, cholesterol is a major component of the lipid membrane. The different levels of membrane cholesterol have been shown to be involved in regulation of membrane proteins such as receptors and ion channels (Rosenhouse-Dantsker, 2019). Most of the Kir channels have been shown to be cholesterol sensitive. The most common effect of cholesterol on Kir channels is the reduction of their activity, which has been suggested to occur due to a decrease in the open probability of the channels. However different effects of cholesterol have been reported on the different type of ion channels including, BK channels (Bukiya et al., 2011), the nicotinic acetylcholine receptor (Addona et al., 2003), and TRPV1(Transient Receptor Potential Cation Channel Subfamily V Member 1) channels (Picazo-Juarez et al., 2011). The increased level of cholesterol has been shown to suppress

the function of the Kir2 channels (Fürst, Nichols, et al., 2014; Romanenko et al., 2004; Romanenko et al., 2002; Rosenhouse-Dantsker et al., 2010). Cholesterol interacts with the binding site of Kir channels directly within the cell membrane, specifically through sterol-protein interaction (D'Avanzo et al., 2011). There are some residues that create structural belts which surround the cytoplasmic domain, close to the TM domain, and are responsible for the modulatory effect of the sterol on Kir channels (Fürst, Nichols, et al., 2014; Rosenhouse-Dantsker et al., 2010; Rosenhouse-Dantsker et al., 2011; Rosenhouse-Dantsker & Levitan, 2012).

It has been suggested that the mechanism of cholesterol-induced suppression might be due to its interaction with Kir channels and PIP_2. However, further studies showed that the cholesterol sensitivity of Kir2.1 and Kir2.3 was not altered by the reducing of the PIP_2 concentration (Epshtein et al., 2009). The suppressive effect of cholesterol was not influenced by anionic phospholipids as well (D'Avanzo et al., 2011).

The first mechanism of cholesterol on membrane came while comparing its two stereoisomers, 3β-hydroxy-5-cholestene (native cholesterol) and 3α-hydroxy-5-cholestene (epicholesterol). However, these enantiomers are different in terms of rotational angle of hydroxyl group in position 3, both have demonstrated a comparable impact on the membrane, affecting lipid packing (Xu & London, 2000). However, further studies determined that, two of these isoforms have a different effect on the Kir channels. As we described above, Kir2.x channels current will be suppressed by cholesterol, the other isoform, epicholesterol, in contrast increased the current of the Kir2.x channels. Recently, it has been identified that, there are two important regions in Kir2.1 which are mainly responsible for the sensitivity of the Kir2.1 to the cholesterol (Fürst, Nichols, et al., 2014). Unexpectedly these regions are not in the CD (Cytoplasmic Domains), but they are located in the cytosolic C-terminus domain. Precisely, the sensitivity regions of the channels is in CD loops a certain region of the C-terminus of the cytosolic domain of the Kir 2.1 channel. Mutations in this loop (L222I) was shown to abolish the sensitivity of Kir2.1 to cholesterol. Two other mutations, N216D and K219Q in the CD loop of Kir2.1 could partially change the cholesterol sensitivity. So they offered that the L222I residue in CD-loop plays an important role in “docking” of the C-terminus of Kir2.1 to the inner leaflet of the membrane thus facilitating its interaction with cholesterol (Levitan, 2009). Other mutations in Kir2.1 (S95H and I171L) have also been reported to significantly decrease the effects of cholesterol depletion (Fürst, Nichols, et al., 2014). Docking analysis has been shown that there is one location that cholesterol bound frequently to the Kirs 2 channel between TM1 and TM2 (Fürst,

Nichols, et al., 2014) and it has been suggested that there are some residues which are crucial for channel gating of the Kir2.x channels and probably cholesterol sensitivity belt does not involve in the formation of the binding site (Fürst, Nichols, et al., 2014; Rosenhouse-Dantsker et al., 2011).

iv. Other membrane lipids

Other phospholipids in the membrane can also affect the sensitivity of the Kir channels (2.1 and 2.2) when it is activated by PIP_2 (Cheng et al., 2011). Experimental studies demonstrated that the sensitivity of the Kir2.1 and 2.2 to PIP_2 will increase 100 times when they are in the presence of 25% secondary anionic phospholipids (PA, PG, PS, PI, DGS-NTA) compared to PIP_2 alone; the impact of modulation of PIP_2-Kir2.1 and -Kir2.2 was dependent on the concentration of these anionic phospholipids. This evidence implies that however anionic lipids cannot affect the Kir channel's function when PIP_2 is absent, but it seems they have a synergistic effect when they are in the presence of PIP_2 in the membrane. Interestingly, increasing the concentration of the secondary anionic phospholipids from 15 to 25% POPG in the presence of 1% PIP_2 resulted in an increase in the open probability of the Kir channels as well as unitary conductance (Cheng et al., 2011). According to the computational data, it seems there is a binding site at the end of the slide helix for secondary anionic phospholipids (D'Avanzo et al., 2013; Lee et al., 2013; Schmidt et al., 2013), which is far from the binding pocket of PIP_2 in Kir2.2 and Kir3.2 (Hansen et al., 2011; Whorton & MacKinnon, 2011). Anionic phospholipids are necessary for channel gating since they probably increase the stabilization of the interaction of the slide helix and cytoplasmic domain (Robertson et al., 2008).

1.1.5. Clinical diseases associated with in *KCNJ2* (Kir2.1 gene)

It has been confirmed for more than two decades that mutations of *KCNJ2* has been linked with many cardiac diseases such as Anderson-Tawil syndrome (ATS) or Long QT syndrome type 7 (LQT7), (Plaster et al., 2001), short QT syndrome type 3 (SQT3), (Priori et al., 2005), familial atrial fibrillation (FAF) (Xia et al., 2005), and catecholaminergic polymorphic ventricular tachycardia (CPVT) (Tester et al., 2006; Vega et al., 2009). SQT3 and FAF are both very rare phenotypic diseases associated with a gain-of-function for Kir2.1. Patients with SQT3 show both ventricular and atrial fibrillation symptoms. In addition, the resting ECG has determined extremely short

repolarization, with QT <300 ms (Priori et al., 2005). As the prevalence of these diseases is very low, their clinical courses are barely understood. It has been revealed experimentally that *KCNJ2* gene gain of function mutation significantly shortens the action potential duration and therefore the ventricular and atrial refractory period.

ATS is considered a rare form of cardiac disease (affects one person in a million) and affects many parts of the body. This genetic syndrome is characterized by periodic paralysis (muscle weakness), ventricular arrhythmia (changes in heart rhythm), and developmental abnormalities (dysmorphic features) (Plaster et al., 2001). Therefore, high expression of Kir2.1 in all excitable cells leads to a great overlapping of Kir2.x mutations with skeletal muscle abnormalities and neurological symptoms in ATS patients (Tristani-Firouzi & Etheridge, 2010).

The result of the electrocardiogram of patients with ATS shows QT prolongation, bigeminy, ventricular ectopy, or bidirectional ventricular tachycardia or polymorphic, and sudden cardiac death (SCD) (Tristani-Firouzi & Etheridge, 2010). Moreover, it seems that more sustained ventricular arrhythmia linked with ATS includes bidirectional ventricular tachycardia (BiVT) and polymorphic ventricular tachycardia (Tristani-Firouzi & Etheridge, 2010). The report of BiVT in patients with ATS is interesting since this is a sign of arrhythmia for CPVT (Figure 5).

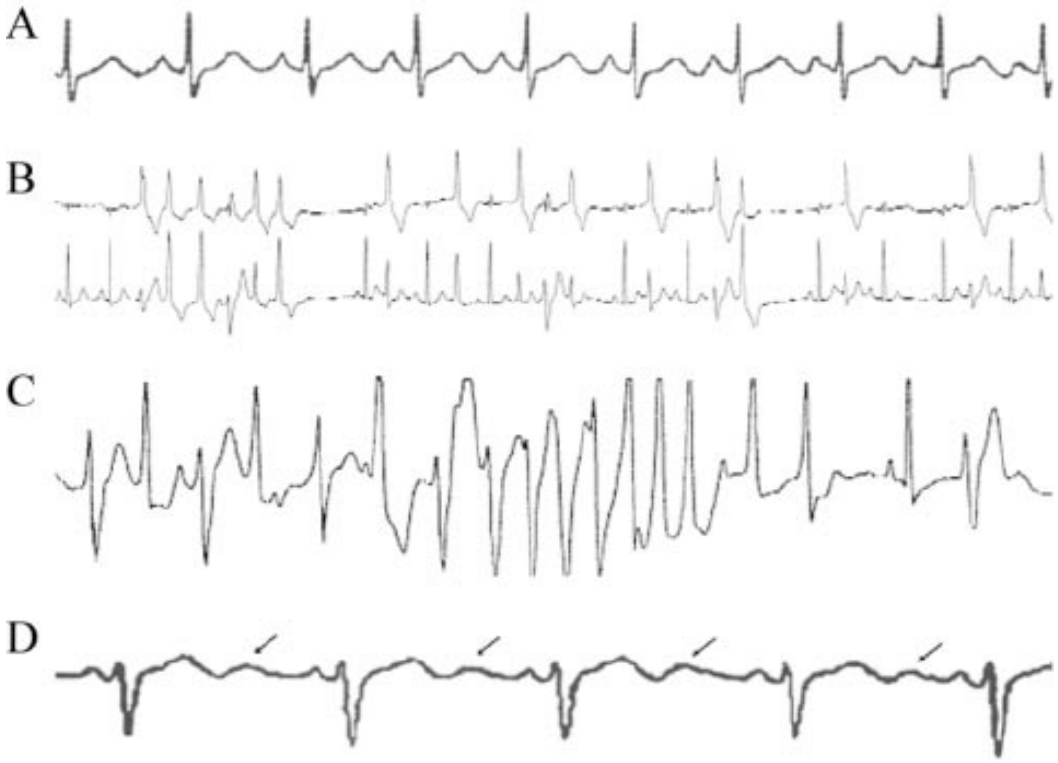


Figure 5. Representational graphs of ECGs from Anderson-Tawil Syndrome patients. A) Prolonged QT intervals. B) Unsuitable polymorphic ventricular tachycardia. C) Bidirectional ventricular tachycardia (BiVT) D) Continuous U wave pointed by arrows. From (Tristani-Firouzi et al., 2002).

1.2. Kir4.1 inwardly rectifying potassium channels subfamily

Kir channel subunit 4.1 is specifically expressed in astrocytes. Kir4.1 subunits are also expressed in the kidney (distal tubular epithelia) and retina or Müller cells.

The constructs Kir4.1 and Kir4.1/5.1 channels are responsible for mediating the spatial K^+ buffering astrocyte's action potential. The *KCNJ10* gene encodes the Kir4.1 subunit which is located on chromosome 1 (Chr1) in humans. This gene codes for 379 amino acids.

The distinctive molecular structure of Kir4.1 subunits is attributed to the ion-selective signature sequence (GYG) and transmembrane regions. By interacting with Kir5.1, they form both homo- and hetero-tetramers. These channels exhibit inward rectification, selectively enabling inward potassium currents Similar to other Kir channels, Kir4.1 channels conduct substantial inward and negligible outward K^+ currents. Ba^{2+} ions can trigger Kir4.1 channels as well (Ohno et al., 2018b) (Figure 6).

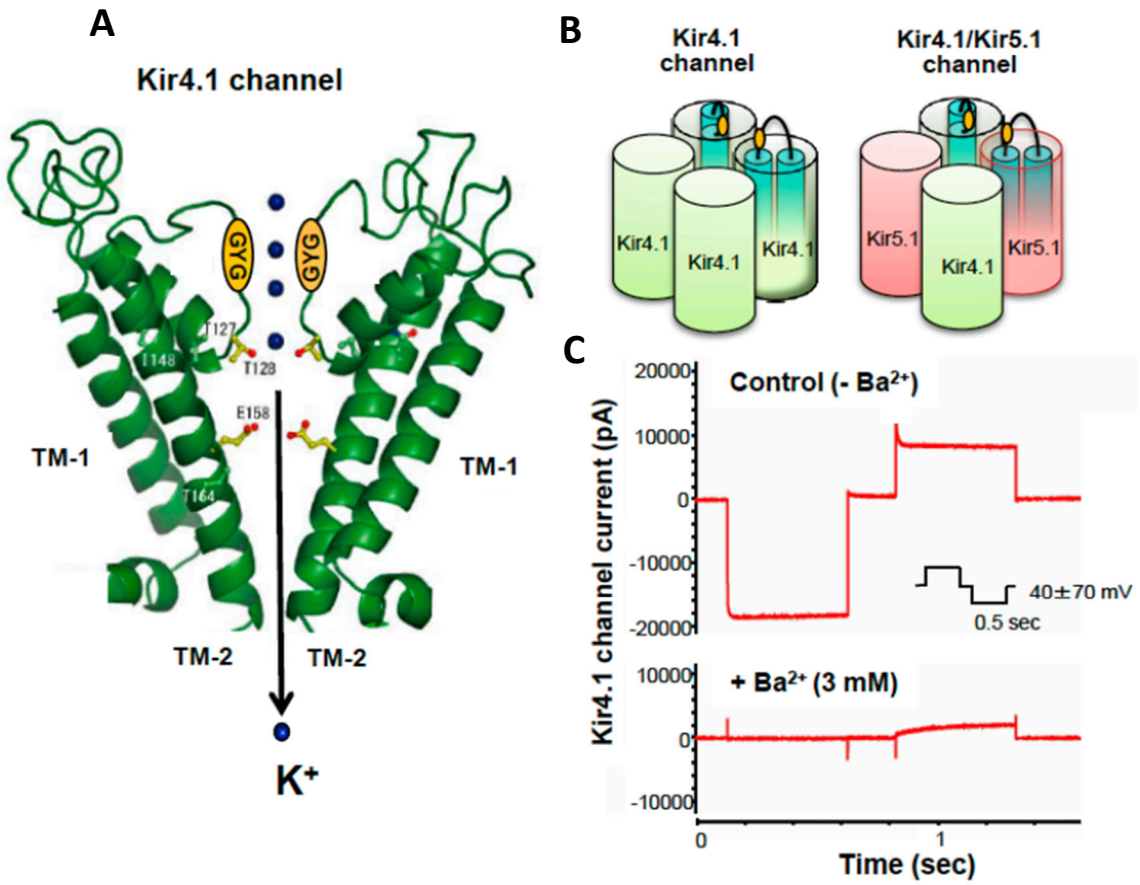


Figure 6. The structure of Kir4.1 channels and their properties. A) Having two TM domains with one extracellular loop which contains a signature (GYG) sequence which participates in ion-selectivity (K^+). B) Two forms of Kir4.1 channels are Kir4.1, the homo-tetramer of Kir4.1, and Kir4.1/5.1, the hetero-tetramer of Kir4.1 and Kir5.1. C) Kir4.1 channels also conduct large inward and little outward K^+ currents (alike other Kir channels). Kir4.1 is also sensitive to Ba^{2+}. From (Ohno et al., 2018a).

It is also known that intracellular substitutes such as polyamines (e.g., spermine) with relatively high concentrations and cations like Mg^{2+} have active roles in the rectifying properties of Kir4.1 and Kir4.1/Kir5.1 channels through deactivation of the channel gating at depolarized membrane potentials (Butt & Kalsi, 2006; Hibino et al., 2010).

Mediating the spatial K^+ buffering by astrocytes is important to control the extracellular concentration of K^+ at synapses and neuronal excitability (Figure 7) (Hibino et al., 2010; Kofuji & Newman, 2004; Steinhäuser et al., 2012). Neural cells release great amounts of K^+ during the action potential repolarization phase and, if this trend is not modulated, extracellular K^+ concentration shoots up to 10 mM or even more, which generates eccentric neuronal discharges and finally causes

depression. The action of spatial K^+ buffering by astrocytes leads to the removal of excess extracellular K^+ concentration and stocks it in areas with lower extracellular K^+ concentrations such as micro vessels (Figure 7). Spatial buffer of K^+ is associated with glutamate uptake by glutamate transporters such as the excitatory amino acid transporter EAAT1 and EAAT2. Part of the aforementioned mechanism is aquaporin-4 (AQP4) involved in water transport into the astrocytes (Hibino et al., 2010; Kofuji & Newman, 2004; Steinhäuser et al., 2012).

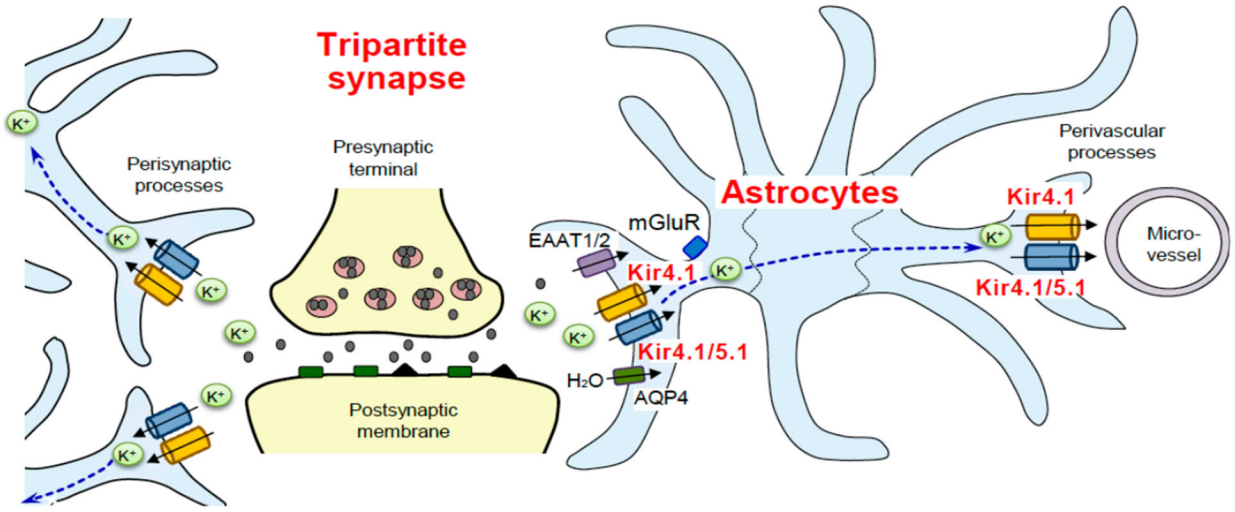


Figure 7. The mediating role of spatial K^+ ion buffering of Kir4.1 channels in astrocytes. The mediating role of spatial K^+ ion buffering of Kir4.1 channels in astrocytes. From (Ohno, 2018).

As part of the previously mentioned spatial K^+ buffering, Kir4.1 and Kir4.1/5.1 channels can remove excessive extracellular K^+ locally raised at synapses; this action depends on the difference between native E_K and astrocytes membrane potential (Hibino et al., 2010; Ishii et al., 1997; Kofuji & Newman, 2004; Poopalasundaram et al., 2000; Steinhäuser et al., 2012). If the function of Kir4.1 channels is interrupted under certain conditions like disease or by drug interactions, it will enhance the neuron's excitability via elevating extracellular K^+ concentrations and the level of extracellular glutamate (Figure 7) (Djukic et al., 2007; Ohno et al., 2015).

1.2.1. Pharmacology of Kir4.1

There are many agents that can affect Kir4.1 channels. Screening analysis of CNS drugs has shown that Kir4.1 channels which were expressed in the HEK293 cell line were reversibly blocked after treatment with anti-depressant drugs. Findings showed that tricyclic antidepressants (TCAs) drugs such as imipramine, desipramine, amitriptyline, and nortriptyline, inhibited Kir4.1 channel through a voltage-dependent way (Su et al., 2007). Moreover, selective serotonin reuptake inhibitors (SSRIs), fluoxetine, sertraline, and fluvoxamine, also blocked the activity of Kir4.1 channels, but not in a voltage-dependent manner (Ohno et al., 2007). The inhibitory role of fluoxetine was observed only for Kir4.1 while it did not have any blocking effect on other Kir channels like Kir1.1 and Kir2.1. The inhibitory concentration effect on Kir4.1, for drugs like fluoxetine, was reported close to the concentration used in patients as antidepressants. In addition, alanine site-direct mutagenesis studies on Kir4.1 in drug-binding studies have shown that SSRI compounds interact within the central cavity of Kir4.1 channels specifically (Furutani et al., 2009). Residues E158 and T128, which are located in the pore region of the Kir4.1 channel, were identified to coordinate the drugs. Moreover, docking simulation analysis revealed that Kir4.1 channels have a pocket, where E158 interacts with the amine moiety of the antidepressant agents with an ionic bond, and the other residue, T128 binds to their benzene ring, hydrogen bond acceptor, with a hydrogen bond (Figure 8).

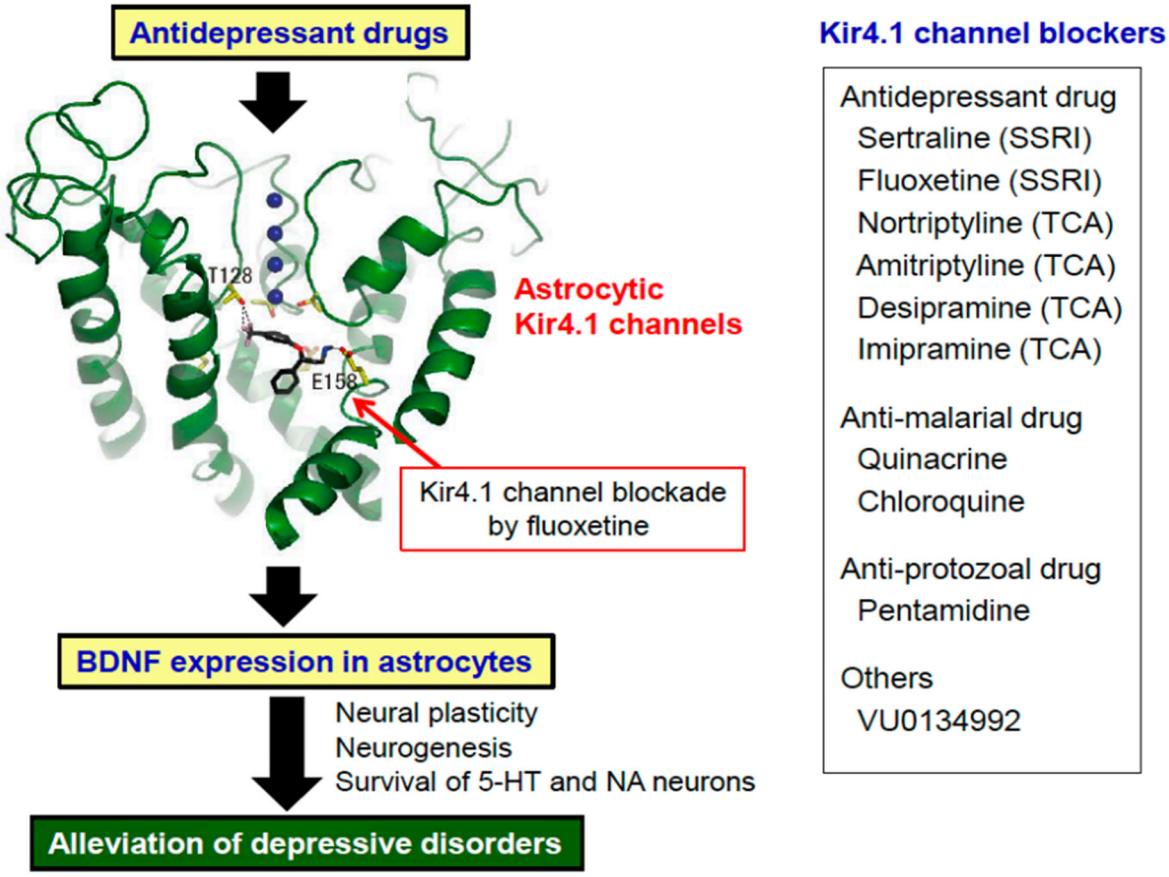


Figure 8. The action mechanism of fluoxetine as an antidepressant drug is through astrocyte brain-derived neurotrophic factor (BDNF) and Kir4.1 channel. From (Ohno et al., 2018a).

Regardless of the blocking effect of antidepressant drugs on Kir4.1, there are other pharmacological agents that can interact with Kir4.1 channels. These groups include chloroquine and quinacrine which are anti-malarial drugs (Marmolejo-Murillo, Aréchiga-Figueroa, Cui, et al., 2017; Marmolejo-Murillo, Aréchiga-Figueroa, Moreno-Galindo, et al., 2017), pentamidine which is a anti-protozoal drug (Aréchiga-Figueroa et al., 2017), and VU0134992, which is a new compound discovered recently (Kharade et al., 2018). Surprisingly, the drugs with the highest inhibitory effect on Kir4.1 like chloroquine, quinacrine, and pentamidine, bind to the T128 and E158, the same location as antidepressant drugs. However, the VU0134992 compound mainly interacted with residues E158 and I159. This finding will help better understand the activity of Kir4.1 channels for developing new ligands in the future (Kharade et al., 2018).

1.2.2. Kir4.1 and lipid regulation

i. PIP_2 regulation of Kir4.1

However, all the Kir channels have been shown to be regulated by the PIP_2, but the affinity of the channels is different depending on the Kir studied. PIP_2 has been shown to increase the Kir4.1/5.1 channel open probability (Yang et al., 2000). Interestingly, none of these effects was seen in the homeric Kir4.1 which shows the strongest interactions with PIP_2 in comparison with all other Kir channels (Du et al., 2004). Some studies suggested that PIP_2 in Kir4.1/5.1 is also responsible for an increase in proton sensitivity (Yang et al., 2000). The addition of 10 μM PIP_2 has led to a shift of 0.22 pH units and reducing sensitivity to pH as well.

ii. Cholesterol

Researchers have found that Kir4.1 is also an the inward rectifier channel (Hibino & Kurachi, 2007). Compared to Kir2.x, Kir4.1 has demonstrated to be inhibited by cholesterol depletion in its methyl-b-cyclodextrin (MbCD) form and led to a loss of current. This lack of current might be due to dissociation of PIP_2, known to act as a regulator and seems to be for many of the ion channels including inward rectifier channels (Hilgemann et al., 2001; Logothetis et al., 2007).

1.2.3. Clinical diseases associated with mutations in *KCNJ10*

The findings of genetic screening analyses have shown that the mutants of *KCNJ10* have led to many neural diseases such as epilepsy, depressive disorders, and other CNS disorders like autism. In addition, studies have also shown that inflammation, central trauma, and ischemia are all linked to a decrease in astrocytic Kir4.1-specific currents. Other neurogenerative diseases like Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and Huntington’s disease are associated with an aberrant activity of Kir4.1 (Nwaobi et al., 2016).

1.2.4. The role of Kir4.1 in epilepsy

Genome sequencing in humans also reported that loss-of-function mutations in the *KCNJ10* gene result in epileptic disorders called EAST/ SeSAME syndrome (Bockenhauer et al., 2009; Scholl et al., 2009).

Patients with EAST all typically show identical symptoms such as ataxia, generalized tonic-clonic seizures, sensorineural deafness, and also renal tubulopathy. Mutations of Kir4.1 in the EAST syndrome were often in the different regions of the cytoplasmic domain such as R65P, G77R, T164I, and A167V, R175Q, R297C and R199X (Reichold et al., 2010; Sala-Rabanal et al., 2010; Tang et al., 2010).

These in vivo studies imply that a decrease in the channels expression level of Kir4.1 channels influences spatial K^+ buffering of astrocytes and leads to hyperexcitation in amygdala neurons (Harada et al., 2013; Morimoto et al., 2004). Several clinical research have also been done in humans such as patients with temporal lobe epilepsy (TLE) that demonstrated down-regulation and dysfunction of Kir4.1, which seems to be a causative factor in TLE (Das et al., 2012; Heuser et al., 2012; Steinhäuser et al., 2012; Zurolo et al., 2012).

i. Depressive disorders syndrome

Depressive disorder are complex psychiatric disorders with different symptoms that not only have negative mood and behavioral effects, but also cause cognitive impairments, somatic symptoms, and sleep disturbances. It has been documented that depressive syndrome development is the result of a reduction in monoamines in the brain, in particular, 5-HT and noradrenaline (Hirschfeld, 2000).

Clinical studies have not yet reported the pathophysiological role of Kir4.1 channels in depressive diseases in humans. However, there are some studies that examined the action of antidepressant drugs in astrocytes, particularly in relationship with Kir4.1 channels (Kinboshi et al., 2017; Ohno, 2018) and suggest the hypothesis that Kir4.1 channels modulate depressive diseases (Su et al., 2007). The crucial action mechanism of Kir4.1 channels in depressive diseases could be explained through different scenarios: 1) the neural excitability regulation through spatial K^+ buffering and 2) BDNF

expression regulation in astrocytes. Studies have shown that the blockage or downregulation of Kir4.1 channels reduces said buffering, and will also increase neuronal excitability through enhancing the extracellular concentration of K^+ and glutamate levels. They in turn modify BDNF expression and ultimately could all lead to the improvement of depressive diseases.

ii. Other related Kir4.1 CNS disorders

Besides mentioned disorders, several studies also have demonstrated that the expression level of the Kir4.1 channels of satellite glial cells surrounding sensory neurons was decreased in a model of chronic pain (Vit et al., 2008). This reduction also has been seen in a herpetic neuralgia model. Moreover, the knockdown of Kir4.1 in the trigeminal ganglion induced facial pain-like behaviors (Silva et al., 2017).

Astrocytic Kir4.1 channels also have shown that play an important role in the initial phase and the development of Huntington's disease (Khakh et al., 2017). Interestingly, the astrocytic Kir4.1 expression level of channels shown downregulated in Huntington's disease models, which results in increased extracellular K^+ levels in the striatum in vivo (Tong et al., 2014).

1.3. Kir7.1 inwardly rectifying potassium channels subfamily

One of the latest channels to be included in the Kir channel family is the Kir7.1 subunits. Kir7.1 was separately identified in three different labs for the first time in 1998, its presence is higher in the secretory epithelial cells of the choroid plexus (Döring et al., 1998), small intestine (Fulford et al., 1998), and central neural cells (Krapivinsky et al., 1998).

Kir7.1's amino acid sequence is the most divergent among the classical subunits of the Kir channel family, sharing only 50% homology with its closely related Kir4.1 and Kir4.2 ion channels (Döring et al., 1998; Krapivinsky et al., 1998). Another significant feature of Kir7.1 is the phosphorylation sites located in the cytoplasmic regions and are suggested to contribute actively to protein trafficking (Schwalbe et al., 1995). Kir7.1 channels exhibit unique current behaviour in terms of voltage-dependence. When comparing their I-V curves to other Kir channels, the current increases in the outward direction with negative potentials. This means that as the membrane potential becomes

more negative, the outward current through Kir7.1 channels becomes larger, which is unlike the typical inward rectification observed in other Kir channels. Heterologous expression studies have shown that these channels have a very small single-channel conductance (50fS) as well (Hibino et al., 2010).

The retinal pigment epithelium (RPE), an essential layer of the retina (Figure 9.A). This layer is between the choroids and the neuroretina and contains potassium channel Kir7.1. It is essential for maintaining the ionic equilibrium required for appropriate photoreceptor (PR) function and the processing of visual signals, as it controls potassium ion flux Shifting of the membrane potential towards negative voltages (left shift) is mainly because of the reduction in the concentration of the extracellular K^+ from 5 mM to 2 mM which leads to an increase in pure potassium current (Figure 9.B). Moreover, Kir7.1 inward rectification is weak when the extracellular concentration of K^+ is low but strongly rectifies in high K^+ (Figure 9.B). They have shown low sensitivity to the common Kir channel blockers like Ba^{2+} and Cs^{2+}. This is due to the presence of M125 in the pore region of the Kir7.1 channel. Upon substituting M125 with an arginine, the conductance of the channel and sensitivity to Ba^{2+} increases around 20 and 10-folds respectively (Hibino et al., 2010).

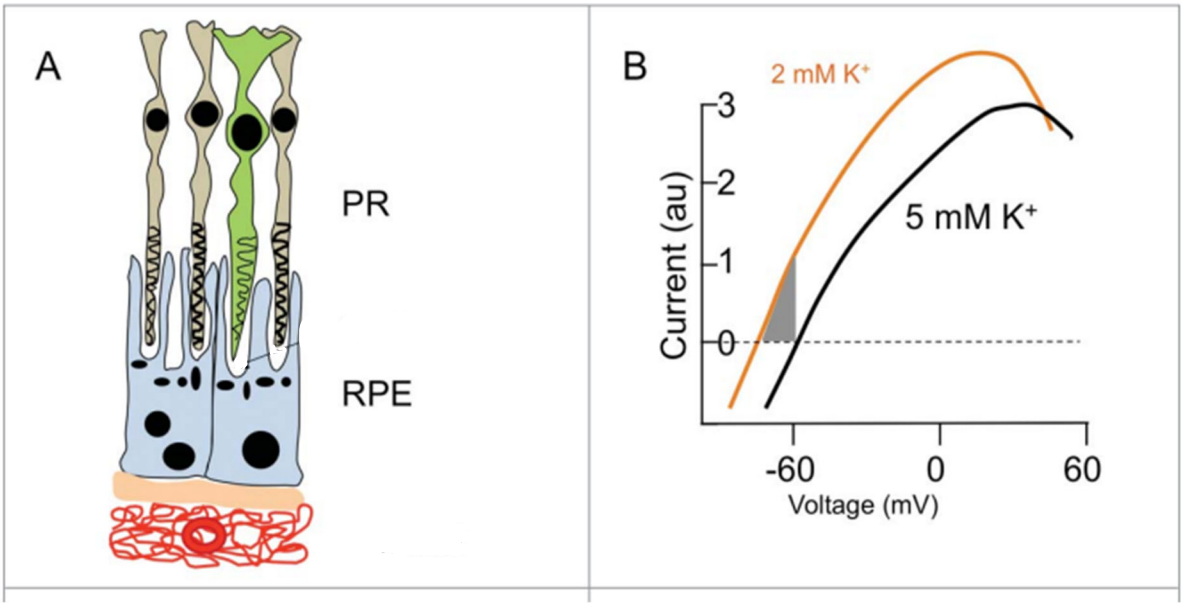


Figure 9. A) Diagram of a normal retinal system. (B) The hyperpolarizing shift of Kir7.1 current in membrane potential by switching extracellular concentrations of K^+. Changing the extracellular K^+ concentration causes the current activation which is close to the resting membrane potential of the retinal pigmented epithelium (grey triangle). From (Kumar & Pattnaik, 2014).

1.3.1 Pharmacology of Kir7.1

1.3.2 Regulators of Kir7.1

Similar to all members of the Kir family, Kir7.1 is also regulated by PIP_2 (Pattnaik & Hughes, 2009). In contrast with all Kir channels family, Kir 7.1 and Kir1.1 have shown different sensitivities to cholesterol. Enrichment of cholesterol led to the enhancement of the activity of the Kir7.1 rather than decrease as observed for Kir2.1 (Rosenhouse-Dantsker et al., 2010).

There are a group of membrane components which interact with intrinsic amino acid residues and can modulate the Kir7.1 channel. One of the studies has shown that cAMP-dependent protein kinase A (PKA) and protein kinase C can regulate the Kir7.1 ion channel of the kidney (Zhang et al., 2008). Findings implied that there was an increase in the current of Kir7.1 which was correlated with increasing of intracellular concentration of cAMP. Consequently, the current of Kir7.1 decreased with the mutation of PKA at the sole site S287. Findings also confirmed that Kir7.1 current are inhibited by the mutation site S201 in PKC while the S169 and S14 sites of PKC do not change the Kir7.1 sensitivity to this phosphorylase.

One of the other factors that can affect the function of Kir7.1 is pH, both extracellular and intracellular. However, extracellular pH can alter the conductance of Kir7.1; for instance, between the range of 6.5 to 9, the conductance of Kir7.1 is almost independent of the pH, while lower ranges below 6.0 strongly inhibit the conductance. It seems that minimal acidification of the cytoplasm (pH 7.2-6.8) activates the current of the Kir7.1 channel and further lowering of the pH (6-5.5) leads to the inactivation of the current offering a biphasic response of Kir7.1's conductance to the pH (Yuan et al., 2003).

During alkalization of the cytoplasm, the effects on the Kir7.1 involves a reversible but fast inhibition (Hughes & Swaminathan, 2008; Yuan et al., 2003). Mutations H26R and H26A in Kir7.1 affect both proton-induced inhibition and activation informing that this position is a pH sensor for the ion channel (Thumann, 2001).

1.3.3 Clinical diseases associated with mutations in Kir7.1

One of the regions with the highest expression of Kir7.1 is the retinal pigment epithelium (RPE). Kir7.1 channel dysregulation has been held responsible for the visual impairment which is probably accompanying pathogenic mutations (Kumar & Pattnaik, 2014; Sepúlveda et al., 2015; Vera et al., 2019). Kir7.1 is localized at the membrane of the RPE apically and it contributes to the homeostasis of extracellular K^+ in the subretinal space (La Cour, 1985). There is a link between mutations in *KCNJ13* which encodes Kir7.1 channels in humans with retinal diseases and congenital blindness. Various autosomal-recessive pathogenic variants of *KCNJ13*, in addition to compound heterozygous mutations, are linked with leber congenital amaurosis (LCA16) (Khan et al., 2015; Pattnaik et al., 2015; Sergouniotis et al., 2011) and snowflake vitreoretinal degeneration (SVD), an eye altering the retina and vitreous functions. Here we will focus on SVD syndrome. SVD is one of the progressive and developmental hereditary eye syndromes that impact the retina and vitreous (Gheiler et al., 1982). SVD is considered vitreoretinal degeneration which will be characterized by early onset cataract, vitreous humor congenital liquefaction, and aberration of the interface between the vitreous and retina resulting in an increased risk of retinal detachment and the formation of tiny crystalline deposits in the peripheral retina (Lee et al., 2003). A single point mutation in the *KCNJ13*, R162W is linked with an inherited autosomal-dominant SVD form with symptoms such as vitreous degeneration, Fuchs corneal dystrophy, high risk of retinal detachment, and mild degeneration of the retina (Hejtmancik et al., 2008). It has been shown that aforementioned mutation led to the accumulation of Ca^{2+} in the cells and consequently, cell death due to premature depolarization (Hagemeister & Sheridan, 2008). However, the mechanism involved in SVD is not clear; it could probably be due to the insufficient activity of Kir7.1 channels. Researchers suggested that Kir7.1 mutations happen close to a hot spot that seem to contribute to the activation of the Kir7.1 channel by PIP_2 (Hagemeister & Sheridan, 2008).

LCA is another syndrome that is a loss-of-function of *KCNJ13* that in turn leads to blindness. Unfortunately, the mutation of Kir7.1 which cause LCA is not characterized very well (Pattnaik et al., 2015). Researchers have demonstrated that the LCA16 mutations can influence the cytoplasmic domain or the selectivity sequence (G-Y-G). This mutation impairs the protein trafficking to the membrane and consequently, lack of Kir7.1 current leads to cell death (Sergouniotis et al., 2011).

1.4. New class of lipids and ion channel regulation

1.4.1. Endocannabinoids:

Cannabinoids are a class of molecules, whether they are naturally occurring in plants or artificially created through synthesis or semi-synthesis, are considered ligands that bind to specific cannabinoid receptors (CBR1 and CBR2) (Harry, 2022). Cannabinoids are categorized into three classes, phytocannabinoids, synthetic cannabinoids, and endocannabinoids (endogenous cannabinoids). Plant-derived cannabinoids are synthesized in the plants such as *Cannabis sativa.* One of the principal phytocannabinoid with psychoactive effects is Δ-^{9}-tetrahydrocannabinol (THC). Synthetic cannabinoids are not naturally occurring; this man-made product can be used for drugs development for a therapeutic effect. Endogenous cannabinoids which are naturally produced in the mammalian cell are lipid signaling molecules; they can mimic the activity of the THC. Anandamide and 2-arachidonoylglycerol, are two important classes of endocannabinoids synthesized from phospholipids of the membrane in the heart and other cardiovascular tissues (Atakan, 2012).

The Endocannabinoid system (ESC) plays a crucial role in the development of the central neuron system in our body. It also has an impressive contribution in the modulation of mature neurons for their function and networking activity. ESC also plays an active role in reducing heart activity, especially causing a relaxation of coronary arteries. Endocannabinoids are also activated in the stress response system in circulatory shock and myocardial infarction where they will be upregulated under acute stress circumstances. They are very well-known due to their protective properties as they were shown to decrease arrhythmias and reduce tissue damage in myocardial infarction (Pacher et al., 2006). Endocannabinoids can modulate vascular function through reduction of oxidative stress, and inflammation which are the main underlying mechanisms in development and progression of atherosclerosis (F Tuma & Steffens, 2012); these impacts might be helpful in the reduction of atherosclerosis progression. It has been shown that the stimulation of the CBR2 could inhibit lesion progression. Inactivation of one of the endocannabinoids (FAAH) in mice has led to the reduction of cardiac dysfunction compared to the control group. However, the mechanisms of action of the endocannabinoids are not fully understood but evidence has shown that they can affect gene expression in inflammation (Pacher et al., 2006).

ESC consists of proteins (enzymes in charge of the transportation, synthesis, and degradation of endocannabinoids), cannabinoid receptors (CBRs), and endogenous cannabinoids, or so-called endocannabinoids (Figure 10). Interestingly, almost all the components of the ESC are multifunctional. Thus, this open system can influence various systems in our body while it can be influenced by many signaling pathways. This property is very important, and it needs to take into consideration when ECS is targeted by drugs (Lu & Mackie, 2016).

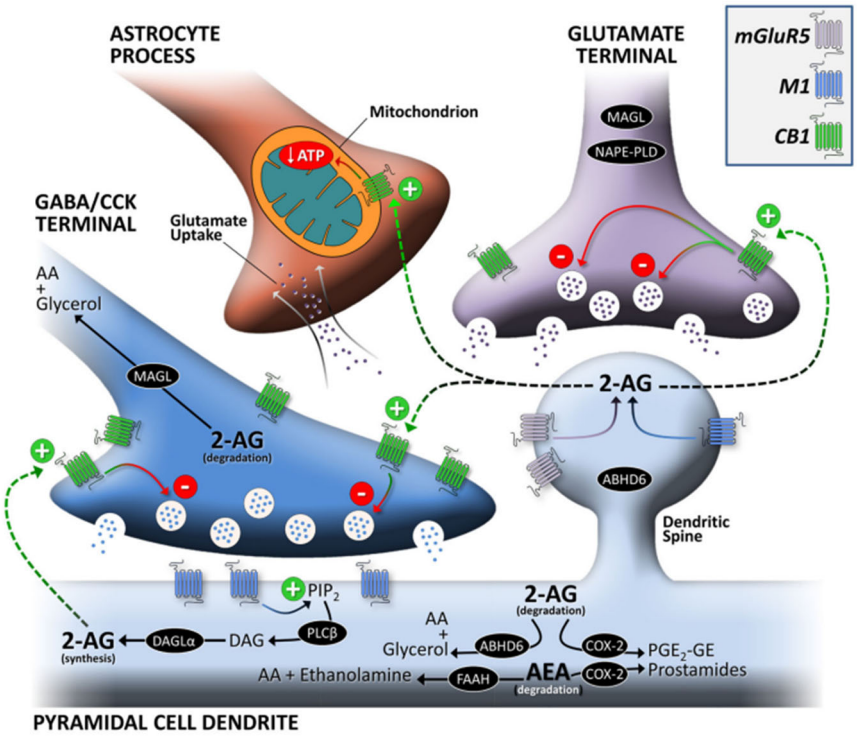


Figure 10. The endocannabinoid system in neurons. The crucial role of the cannabinoids, endocannabinoids, cannabinoid receptors and enzymes involved in the synthesis and degradation, built the neural endocannabinoid system. The secretion of neurotransmitters in CNS has been done by most of the CB1 receptors. They are generally found in preterminal axon segments and axon terminals while expressed sparsely in the active zone of synapses. From (Lu & Mackie, 2016).

i. Endocannabinoids and their specific receptors (CBR1 and CBR2)

However, it has been already thought that due to the lipophilic nature of endocannabinoids, they can bind to the membrane non-specifically and lead to various signaling pathways (Figure 11).

Endocannabinoids have a specific binding site known as CBRs, which is similar to the GPCR structure. These endocannabinoid receptors possess seven transmembrane domains, in addition to an intracellular C-terminal tail and extracellular N-terminal tail. It has been shown that these receptors are pretty much activated by three classes of ligands including 1) cannabinoids and related synthetic compounds, 2) eicosanoids (AEA and 2-AG), and the last antagonist 3) aminoalkyl indoles. In addition, there are a couple of other compounds which have been designed either as agonists, inverse agonists, antagonists, or modulators of these receptors (just for CBR1).

CBR1 is encoded by the CNR1 gene which is mostly dependent on cannabis (Hartman et al., 2009). This receptor with three known isoforms is predominantly expressed in the brain (human). It is also expressed in other tissues such as skeletal muscles, the liver, and pancreatic islets (González-Mariscal et al., 2016). In most parts of the brain, CBR1 is expressed in presynaptic terminals of neurons of both gamma-aminobutyric acid (GABA)-ergic and glutamatergic neurons (Iannotti et al., 2016). While researchers have reported that homomeric CBR1 expresses in presynaptic neurons as well as form heterodimers with other GPCRs including dopamine D2, orexin type-1 receptors or adenosine A2 (Ferré et al., 2010). CBR1 is also expressed on the surface of the non-neuronal cells of the brain (astrocyte) where its deactivation leads to the release of the neurotransmitters (Ward et al., 2011). The activation of the CBR1 results in the release of intracellular Ca^{2+}, this high intracellular Ca^{2+} also promote the release of the glutamate and consequently activation of presynaptic metabotropic glutamate receptors (Navarrete & Araque, 2010). Any endogenous or exogenous stimulus on CBR1 can inhibit the adenylate cyclase activity and subsequently lead to a decrease in the level of the cyclic adenosine monophosphate (cAMP) or induce the activity of the mitogen-activated protein kinase (MAPK). Some studies have demonstrated that CBR1 in some specific cell types can regulate adenylyl cyclase (AC) (Turu & Hunyady, 2010). The AC/ cAMP signals can play important role in many physiological processes such as differentiation, proliferation, and cell survival. In addition, cAMP is involved in regulating many classes of ion channels and transporters like Na^{+} and Ca^{2+} ion channels (Sánchez et al., 2003). The modifications of cAMP occur through various mechanisms, including direct activation of CNG (Cyclic Nucleotide Gated) ion channels (Biel & Michalakis, 2009), and modulation of voltage gated ion channels (Ca^{2+}) by increasing the open probability (Catterall, 2011). This receptor can also modulate the activity of Kir3.x ion channels (Robbe et al., 2001).

These reports show the importance of CBR1 in cell fate and its function on neural electrical activity as an important player in the neurotransmitter-releasing cascades. The crucial role of this receptor is maintaining cellular homeostasis (Iannotti et al., 2016). Moreover, CBR1 receptors are suggested to trigger the effects of the endocannabinoids in the myocardium of humans (Bonz et al., 2003), rats (Bátkai et al., 2004), and mice (Pacher et al., 2005).

Another GPCR of endocannabinoids is CBR2 which is encoded by the CNR2 gene and is functionally related to CBR1. In contrast with CBR1, low levels of it are expressed in the brain, mainly restricted to the microglia and astrocytes (Demuth & Molleman, 2006). Further evidence has also shown that CBR2 is predominantly expressed in immune cells such as macrophages, monocytes, and B- and T-cells (Staiano et al., 2016). It can control the migration of the immune cells, which shows the regulatory inflammatory and nociceptive response (Malan Jr et al., 2003). The overexpression of CBR2 can also cause inhibition of the Ca^{2+} channels (Demuth & Molleman, 2006).

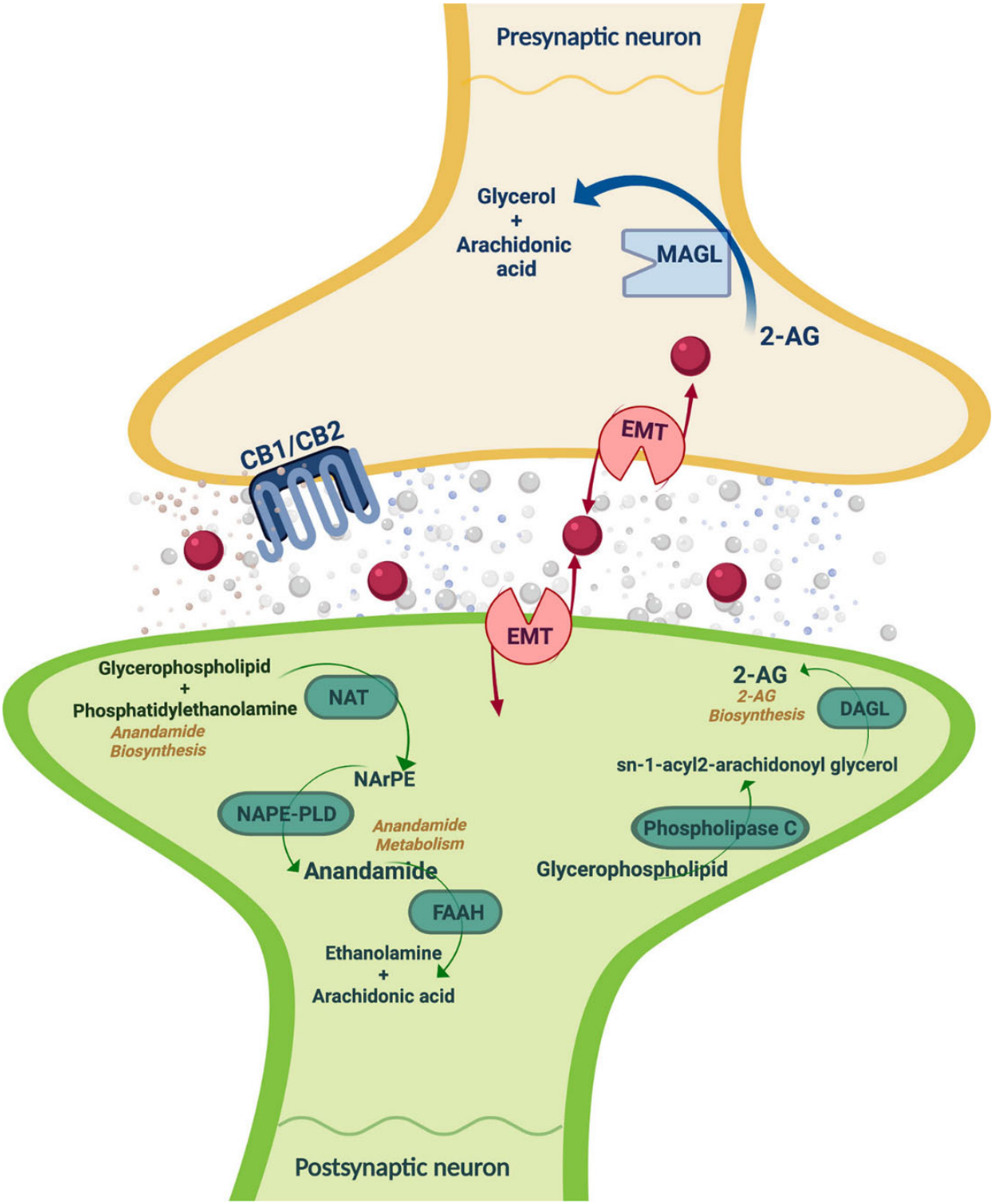


Figure 11. Overview of the main components of the endocannabinoid system and the metabolizing routes of eCBs. From (Navarrete et al., 2020).

ii. **Endocannabinoids** interaction **with ion channels independently of CBR1 and CBR2**

As we mentioned above, the interaction of the cannabinoids and cannabinoids receptor has been widely studied. While the huge body of recent studies showing that endocannabinoids can also interact with membrane proteins directly independent of their downstream signaling effect with CBR1 and CBR2 (Iannotti et al., 2014; Poling et al., 1996; Rimmerman et al., 2013; Ryan et al., 2009).

1.4.2. Ion channels

i. Ca^{2+} channels interactions

It has already been reported that even before the discovery of the AEA (Devane et al., 1992), a specific isolated lipid from the bovine brain can affect the function of low voltage-gated calcium channels (L-type) in rat cardiac membranes and in GH3 pituitary cells which cause the block Ca^{2+} currents (Janis et al., 1988). Further studies suggested that these lipophilic endogenous compounds which are necessary as modulators of the L-type VGCC in cortical membranes (Johnson et al., 1993). T-tubules are one of the sources of the said channel, which plays a very important role in contractions (Oz & Frank, 1991). Studies have shown that AEA can disrupt the binding of some Ca^{2+} channels agonists such as dihydropyridine (DHP) ([3H]PN200-110), phenylalkylamine ([3H] D888), and 1,5-benzothiazapine ([3H]diltiazem) with IC_{50} values of 4 μM, 8 μM, and 29 μM, respectively (Shimasue et al., 1996). In other studies, AEA and its analog methanandamide (mAEA) in the T-tubule membrane showed that they can inhibit the depolarization-induced Ca^{2+} fluxes (Oz et al., 2000). Further studies also demonstrated that 2-AG with its IC_{50} value of 1–10 μM inhibited Ca^{2+} fluxes and disrupted the binding of the DHP on Ca^{2+} channel (Oz et al., 2004). In another study, it was reported that AEA can have an inhibitory effect on T-type VGCC which was expressed in many cell lines including HEK-293, neuroblastoma NG108-15, COS, CHO, and also in Xenopus oocytes (Chemin et al., 2001). The currents of high voltage-activated Ca^{2+} channels inhibited with a concentration of 100 nM of AEA in dorsal root ganglion neurons of rats, while the L-type Ca^{2+} channels were not sensitive to SR141716A (Evans et al., 2004).

ii. Transient receptor potential (TRPV) channels

Within the mammalian TRP superfamily, 28 cation-permeable TRP channels are highly conserved membrane proteins. Following how closely the sequences of these channels matched up, they divided into six subfamilies.

The classical TRP (TRPC) subfamily of TRP channels contains the first mammalian TRP channel to be identified. Due to the founding member's heat sensitivity, the TRPV subfamily has received much research. Unique ion channels in the TRPM subfamily are bifunctional since they have functional enzymatic domains. One chemo-nociceptor channel related to the TRPA subfamily has been suggested as a potential analgesic drug target. The fourth type of mucolipidosis (ML-IV) is predominantly caused by TRPML subfamily channels, primarily in intracellular compartments. The TRPP subfamily, which includes a variety of proteins, is also connected to autosomal dominant polycystic kidney disease (Samanta et al., 2018).

The TRPV1 has shown that is regulated by a variety of molecules including vanilloids and capsaicin (naturally occurring) (Caterina & Julius, 1999). There are similarities between the structure of capsaicin and AEA; based on these similarities many studies have reported that AEA can also regulate the activity of the TRPV1 with EC_{50} in the range of 0.7–10 μM (Smart et al., 2000; Zygmunt et al., 1999). AEA did not have any effects on the antagonists of CBR1 and CBR2, while the effects of AEA can be blocked by capsazepine (a TRPV1 antagonist) in TRPV1-expressing Xenopus oocytes or in mammalian cell lines that are transfected with TRPV1 without endogenous CBR1 and CBR2 (Zygmunt et al., 1999). Further studies have also shown that the regulatory effects on TRPV1 is not limited to AEA, for other lipid molecules such as lipoxygenase (LOX), a metabolite of AA and members of fatty acid amides have also shown agonistic modulation (Benham et al., 2002; Hardie, 2003). The TRPV4 channel, another member of the TRPV channels with 45% sequence identity with TRPV1 is activated by several subsets of endogenous compounds in particular AA (Nilius et al., 2004). AEA and 2-AG within the range of 1–10 μM caused the increase in intracellular levels of Ca^{2+} and activation of the whole cell current. It is considered that this current change is due to the production of AA as a metabolite of AEA and 2-AG (Watanabe et al., 2003). Conversely, AEA and 2-AG up to a concentration of 10 μM did not have any effects on TRPA1 channels, another member of the TRP family (Jordt et al., 2004); which suggests that different subtypes of that family could have different sensitivity to endocannabinoids.

It is worth mentioning that the Nobel Prize in Physiology or Medicine for 2021 was granted to David Julius and Ardem Patapoutian in recognition of their groundbreaking discoveries related to receptors for temperature and touch. Their work led to identifying TRPV1 and PIEZO1/2, essential receptors involved in sensing temperature and tactile stimuli (Reeh & Fischer, 2022).

iii. Na^+ Channels

The common effect of endocannabinoids on the voltage-gated Na^+ channels has been the inhibition of its current by way of blockage (Duan et al., 2008; Okada et al., 2005). Endocannabinoid 2-PG was reported to decrease the Na^+ current of the parathyroid cells in frogs that do not express any cannabinoid receptors. These findings imply that endocannabinoids can bind sites that are not exclusive to known cannabinoid receptors leading to whole cell current alteration. Applying 50 uM of 2-PG did not change the basal properties of the parathyroid frog cells but the peak of the Na^+ current decreased significantly (36%) at -24 mV (Okada et al., 2005). An aminoalkylindole derivative compound that acts like THC and CBD was also applied on the Nav channels and resulted in a left-shift of the voltage (Okada et al., 2005). The study found that various cannabinoids and endocannabinoids had a similar effect of decreasing the current when applied to the extracellular environment. However, the effect of CBD was not tested in this particular study (Duan et al., 2008). Further studies have shown that CBD does not have any inhibitory effect on Nav channels. Generally, except CBD, these findings suggest that cannabinoids and endocannabinoids affect the biophysical properties of the Nav channels and also its current density.

iv. K^+ channels

To support the idea of direct interaction of the cannabinoids and endocannabinoids compound and ion channels, other membrane proteins have also been studied. For example, the Kv1.2 channels which was transfected in B82 fibroblasts were fully inhibited by applying 750 nM of anandamide. That study eliminated CBR1's functional effect using a common CBR1 inhibitor (SR141716A) (Poling et al., 1996).

The effect of the endocannabinoids and cannabinoids was tested on ligand-gated K^+ channels. Application of the anandamide has been shown to account for reduced K_{ATP} currents during the cromakalim experiments by 50%. The latter induces outward current in follicle-enclosed oocytes when transfected with K_{ATP} in the presence of 8.1 μM anandamide. The channel was blocked by applying 100 μM (Oz et al., 2007).

1.5. Hypothesis and Aims:

This dataset suggests that endocannabinoids can directly regulate the function of numerous ion channels independently of CBRs. This work will show how some of the two different classes of endocannabinoids' fatty acid ethanolamides (FAEs) and two 2-Monoacylglycerols (2-MGs) will regulate inward rectifier K^+ channels.

Since inward rectifier K^+ channels are highly regulated by membrane lipids, we hypothesized that endocannabinoids are a novel class of Kir regulatory lipids. The specific aims of this study are as follows:

-Aim 1: To assess if Kir2.1 channels are regulated by endocannabinoids, independently of CBRs?

-Aim 2: Do endocannabinoids have an effect on mutants responsible for ATS/ LQT7.

-Aim 3: To assess if the regulation of Kir2.1 by endocannabinoids is conserved among other Kirs.

2. Methods and Materials

2.1. Endocannabinoids panels and reagents

The two classes of endocannabinoids (Cayman Chemicals Company; USA), fatty acid ethanolamides (FAEs) and 2-monoacylglycerols (2-MGs) were diluted in 99.8% ethanol at a concentration of 10 mg/mL from stock to a working concentration of 10 mM and stored at -20°C. The horse serum, penicillin-streptomycin, and kanamycin stock solutions (ThermoFisher Scientific, Gibco Cell Culture, USA) were used pure and undiluted.

2.2. Expression of recombinant Kir channels in Xenopus oocytes

cDNA from human wild-type Kir2.1 and its mutants G144S and V302M, Kir4.1 and Kir7.1 were successfully subcloned into the Xenopus oocyte expression vector pGEM-HE or pGEM-SH, as previously described (1,2). Briefly, to obtain polyadenylated mRNA for injection, MluI digestion (New England Biolabs, MA, USA) was performed to linearize all the pGEM-Kir plasmids. Standard in vitro transcription synthesis using ~1.0 µg of linearized cDNA was achieved with the mMESSAGE mMACHINE™ T7 Transcription kit (ThermoFisher, LifeTechnologies, USA). Extracted and purified RNA samples were validated by absorbance measurements at 260 nm.

Mutations of hKir2.1 (cloned in pGEM-HE) were generated by oligonucleotide-mediated site-directed mutagenesis with the Q5 2X Master Mix kit (M0491S, New England Biolabs). The mutagenesis primers were: G144S forward: CCCAGACAACCATAAGCTATGGTTTCAGATG, G144S reverse: CATCTGAAACCATAGCTTATGGTTGTCTGGG, V302M forward: TACTGG-AAGGCATGATGGAAGCCACTGCCAT, V302M reverse: ATGGCAGTGGCTTCCATCA-TGCCTTCCAGTA.

2.3. Oocyte expression

All electrophysiological experiments were carried out on unfertilized oocytes (stages V and VI). Preparation and handling of Xenopus oocytes were performed as described. Defolliculation was performed with collagenase type 1A (C9891 Sigma, Oakville, Canada) in a Ca^{2+}-free solution (1

mg/ml). Then oocytes were injected with 4.6-9.2 of mRNA hKir (1µg/µL) by a Drummond Nanoject II injector (Drummond Scientific, Broomall, PA). The Kir2.1 mutations were also typically co-expressed with wild-type Kir2.1 in a 1:1 ratio which led to a 20-50% reduction of wild-type current.

Before injecting RNA, oocytes are stored in an incubator at 18 °C in Barth supplemented with antibiotic medium (90 mM NaCl, 3 mM KCl, 0.41 mM $CaCl_2.2H_2O$, 5 mM HEPES, 0.82 $MgSO_4$.7 H2O, 100 U/mL of penicillin-streptomycin, 10 mg/mL of kanamycin). Post-injection oocytes were placed in the previous media supplemented with 2-5% horse serum. One to two days later, channel-expressing oocytes was assessed by two-electrode voltage clamp.

2.4. Electrophysiological recordings

Briefly, electrophysiological recordings of oocytes' currents were recorded at 22-25°C with 0.5-2 MΩ microelectrode pipettes (30-30-1, FHC Inc, ME, USA). Pipettes were filled with a 1 M KCl solution and oocytes bathed in a MES-buffered high [K^+] solution (96 mM KCl, 2 mM NaCl, 1.0 mM $CaCl_2$,1 mM $MgCl_2$, and 5 mM Na-HEPES, pH 7.4). The microscopic currents of inward rectifier K^+ channels were recorded by the Oocyte Voltage Clamp amplifier (OC-725C, Warner Instruments, CT, USA) and the currents were digitized using the Digidata 1322A data acquisition apparatus (Molecular Devices, CA, USA). Data acquisition was obtained using Clampex 10.5 software at a sampling rate of 5 kHz, filtered at 1 kHz.

Electrophysiology recordings were performed before and after the incremental addition of different endocannabinoids from both FAE and 2-MG classes to the bath solution. Cells were voltage-clamped at different holding potentials in the range from +150 mV to -150 mV until the current traces were stable (did not changed by addition of the endocannabinoids). Oocytes expressing mutant Kir2.1 were recorded in the same MES-buffered high [K^+] solution. Briefly, oocytes were held at voltages ranging from +150 to -150 mV in incremental steps of +10 mV (Figure 12).

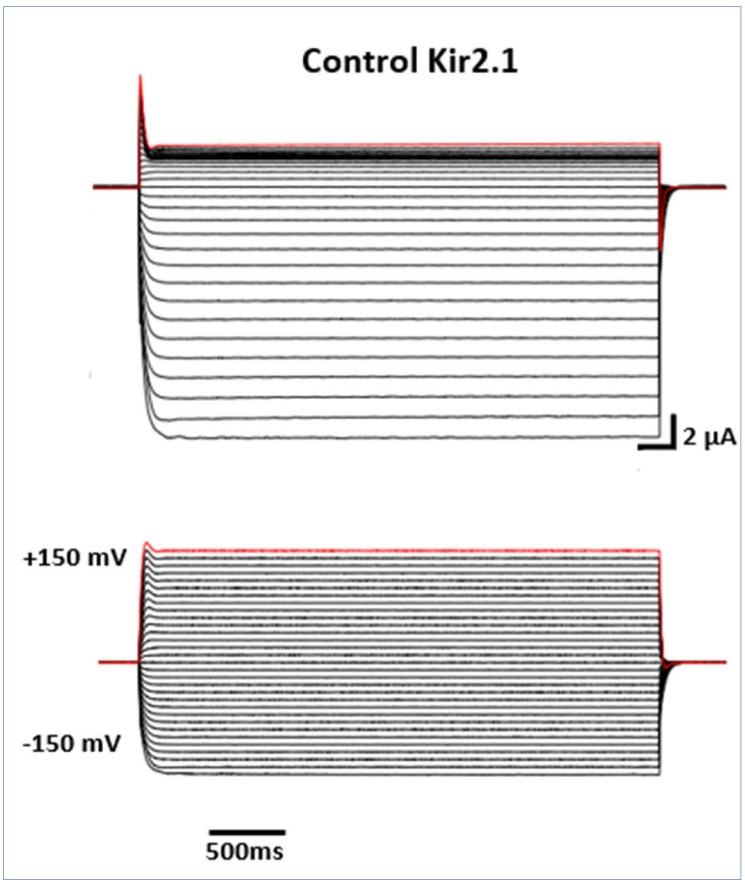


Figure 12. The top figure represents inward current records, and the bottom recordings the voltage steps applied by TEVC.

2.5. Data analysis and statistics

Data were analyzed using the Clampfit software and Origin v8.0 (Northampton, MA, USA) for post-analysis and data plotting.

Current-voltage (I-V) relationships were measured using the built-in pClamp software outputting voltage and current traces. I-V curves are fitted to a Boltzmann equation (Equation 1):

Equation 1: Boltzmann equation

$$I = \frac{(V_m - V_{rev})g_{max}}{1 - e^{\frac{V_m - V_{\frac{1}{2}}}{k}}}$$

Where I denotes the current amplitude, V_m the membrane voltage, V_{rev} the reversal potential, $V_{1/2}$ the voltage at half-maximal current, and G_{max} is the maximal slope of the curve.

Currents recorded in each cell were then normalized to the control current value (0 µM endocannabinoid) recorded at -150 mV enabling pair-wise evaluation of the changes in currents induced by each condition to be averaged across cells. The resulting I-V curve and its standard error of the mean (SEM) is presented in the figures.

To investigate the concentration-dependent effects of specific subsets of endocannabinoids on Kir channel currents, a pair-wise comparison methodology was utilized. The relative increase in the Kir function induced by each endocannabinoid at a given concentration (X µM) was determined by evaluating 1 – [G_{max}(X µM)/G_{max}(0 µM)] ensuring the appropriate propagation of errors. This relative increase in G_{max} was plotted against the concentration for each endocannabinoid to establish a dose-response curve. EC_{50} and E_{max} values were obtained by fitting the Hill equation (Equation 2);

$$\Delta G_{max} = (E_{max} * [L]^n) / (EC_{50}^n + [L]^n)$$

Where E_{max} is the maximum response, and L is the ligand concentration, in our case, endocannabinoids (µM), n, the Hill coefficient, and EC_{50}, the effective concentration providing half a response. Fitting our data to a Hill equation (Origin v8), we determined

*After calculation of the EC_{50} and the E_{max} value, a 5% increase in relative G_{max} was established as a threshold to identify endocannabinoids that have an effect on Kir2.1 function based on the slight increase in current observed at high concentrations of the vehicle (ethanol) used as a solvent. Therefore, a two-sample T-test was used to test the hypothesis that E_{max} of a particular cannabinoid was greater than that of ethanol.

3. Results

3.1 Kir2.1 channels, regulated by endocannabinoids, independently of CBRs.

This study uses *Xenopus laevis* oocytes as model since it does not express cannabinoid receptors (Hejazi et al., 2006). By way of TEVC, we evaluated if Kir2.1 currents are modulated by endocannabinoids, independently of cannabinoid receptors' influence.

1.1.1 Regulatory effect of fatty acid ethanolamides (POEA and OEA) on Kir2.1 currents

We observed a remarkable increase in the total Kir2.1 channel current following the incremental addition of POEA and OEA. Maximal Kir2.1 currents at -150 mV (Figure 13 &14) were observed through the addition of 20 μM POEA. Upon the addition of 10 μM POEA, we observe a small increase in the Kir2.1 currents compared to the control. Greater concentrations (30-50 μM) also increased the current (Figure 13.D). Similarly, different concentrations of OEA produced an increase in Kir2.1 channel current, which showed the almost same effect similar to the POEA effect. The addition of 40 μM and 50 μM OEA at -150 mV produced the maximal increase in inward rectifying current, observable at -150 mV (Figure 14.D). The addition of 30 μM NEA obtained the maximum Kir2.1current. There was no significant enhancement in the current of Kir2.1 currents upon the addition of 40 and 50 μM of NEA, compared to 30 μM NEA (Figure 15.D).

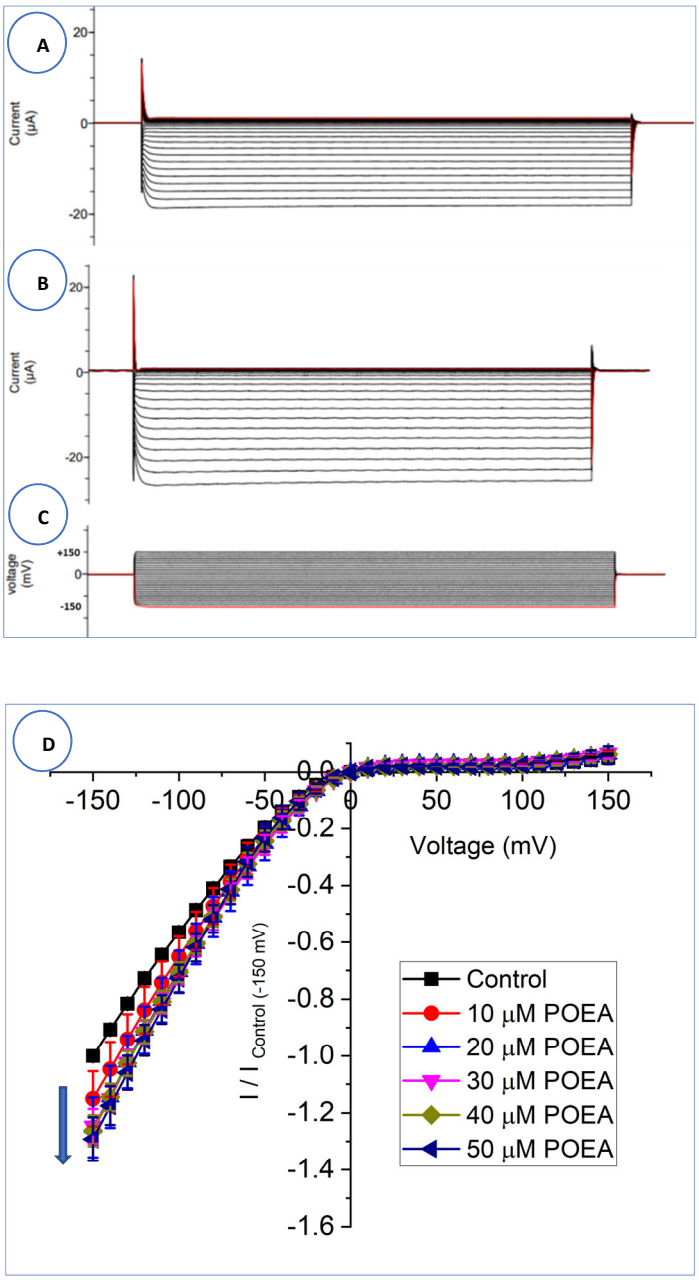


Figure 13. Effects of fatty acid ethanolamides: POEA on Kir2.1 channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 channels without endocannabinoid treatment. B) Recording currents of Kir2.1 channels following treatment with various concentrations of POEA. C) Application of the voltage protocol (-150 to +150 mV). D) Current-voltage (I-V) relationship of Kir2.1 channels following the incremental addition of POEA. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 µM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

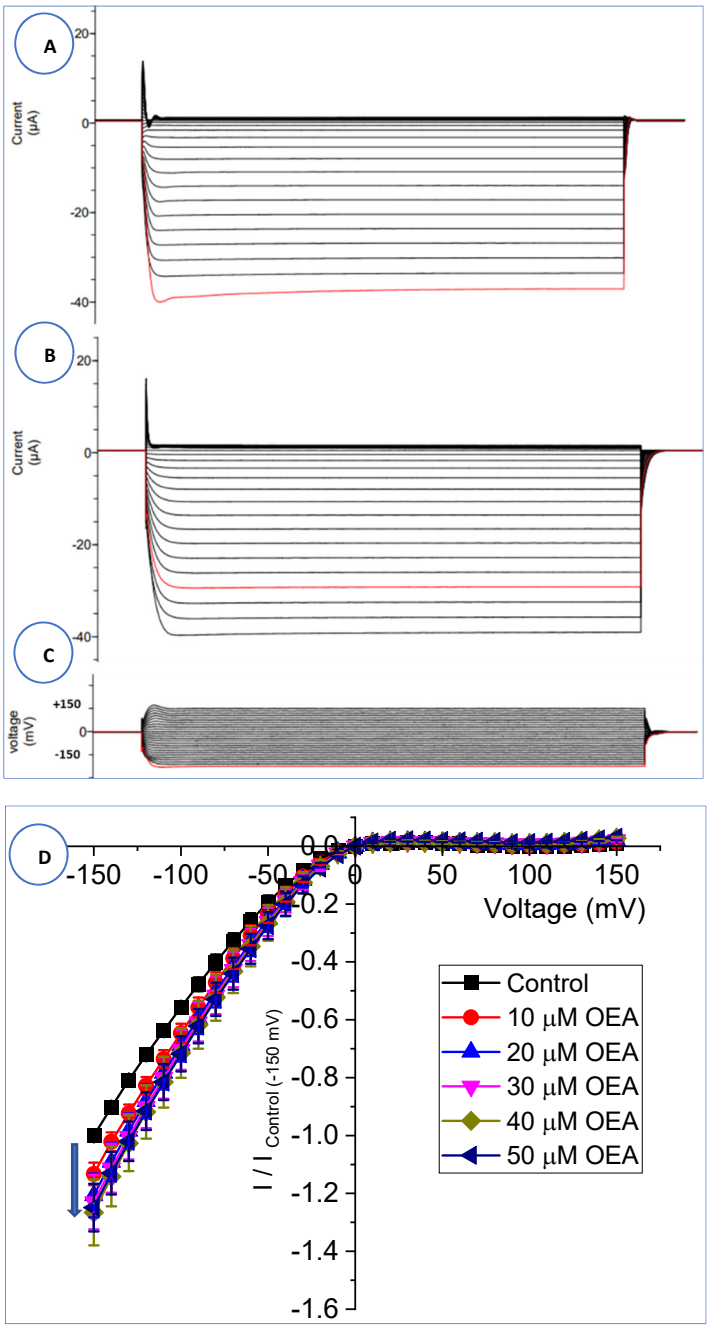


Figure 14. Effects of fatty acid ethanolamides: OEA on Kir2.1 channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 channels without endocannabinoid treatment. B) Recording currents of Kir2.1 channels following treatment with various concentrations of OEA. C) Application of the voltage protocol (-150 to +150 mV). D) I-V relationship of Kir2.1 channels following the incremental addition of OEA. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 µM endocannabinoid). This enabled the averaging of pair-wise data from different cells The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

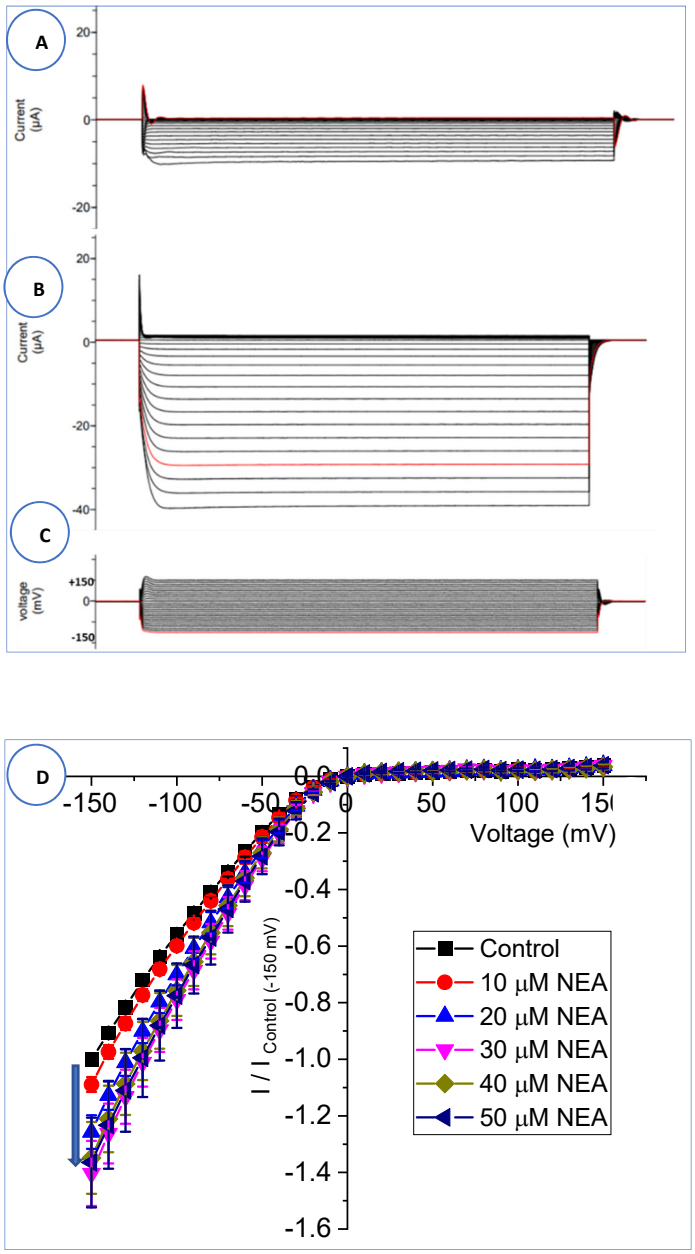


Figure 15. Effects of fatty acid ethanolamides: NEA on Kir2.1 channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 channels without endocannabinoid treatment. B) Recording currents of Kir2.1 channels following treatment with various concentrations of NEA. C) Application of the voltage protocol (-150 to +150). D) I-V relationship of Kir2.1 channels following the incremental addition of NEA. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM of K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

1.1.2 Regulatory effect of 2-MG endocannabinoids (1-AG) on Kir2.1 currents

A remarkable increase in the total Kir2.1 channel current following the incremental addition of 1-AG was observed (Figure 16). The Maximum Kir2.1 current was obtained by the addition of 30 µM 1-AG. The addition of 40 and 50 µM of 1-AG did not result in any notable increase in Kir2.1 currents, when compared to the addition of 30 µM 1-AG (Figure 16.D).

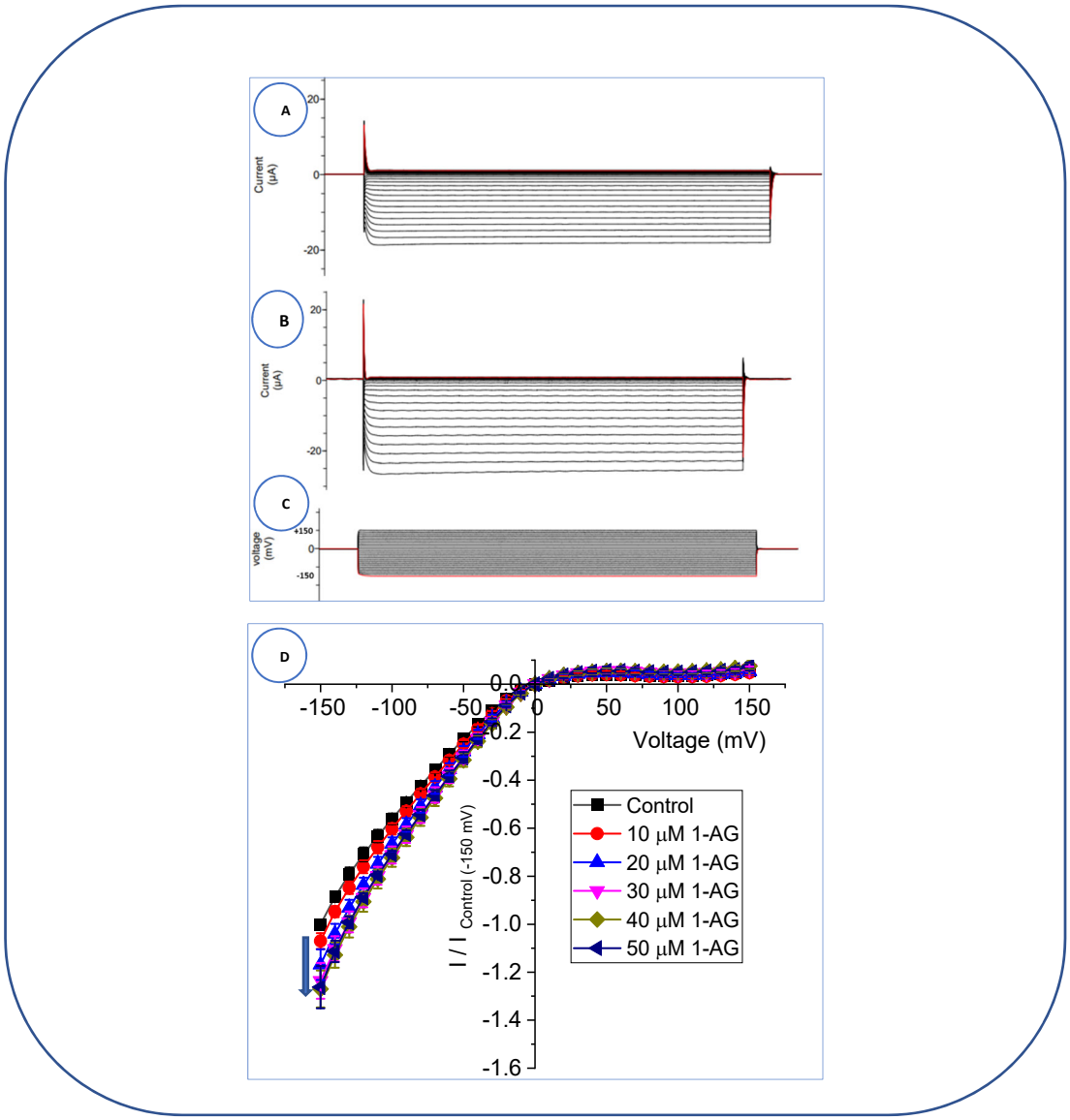


Figure 16. Effects of fatty acid ethanolamides: 1-AG on Kir2.1 channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 channels without endocannabinoid treatment. B) Recording currents of Kir2.1 channels following treatment with various concentrations of 1-AG. C) Application of the voltage protocol (-150 to +150). D) I-V relationship of Kir2.1 channels following the incremental addition of 1-AG. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 µM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM of K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

1.1.3 Calculation of EC_{50} and Maximal effect (E_{max})

Concentration dependence curves were calculated for both classes of endocannabinoids, namely POEA, OEA, 1-AG and NEA by plotting the relative increase in slope conductance (G_{max}) compared to the control (Figure 17). Data was then fitted to a dose-response function to determine EC_{50} and E_{max} of these compounds. Half-maximal effective concentration for POEA and OEA, was almost 9.30.7 µM. In addition, both endocannabinoids showed a comparable maximal effect value of 21and 23±1.3%, respectively (Table 1) ($P<0.05$).

Both 1-AG and NEA exhibited EC_{50} values of 20.3 µM and 23.3 µM, respectively. Notably, NEA demonstrated a significant increase in the maximal response, with a value close to 50% (48.2%±4.5), while 1-AG exhibited relative increase in G_{max} about 20% (20.3±0.6). The results indicated a significant ($P<0.05$) enhancement on Kir2.1 channel function by both endocannabinoids of the 2-MG class (Table 1).

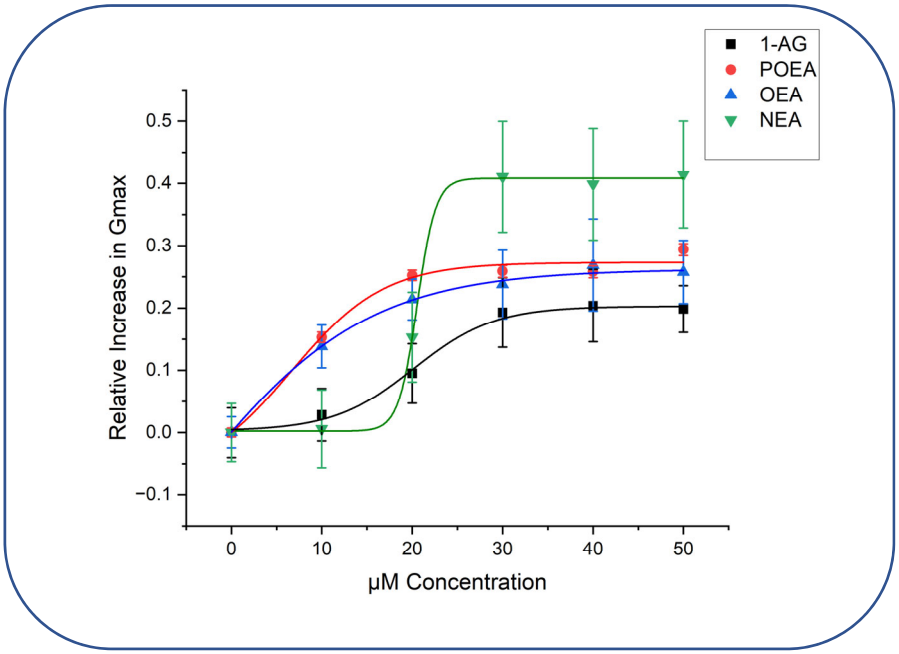


Figure 17. Concentration dependence curves were calculated for endocannabinoids POEA, OEA, 1-AG and NEA to obtain the relative increase in G_{max} compared to the control. Data was then fitted to a dose-response function to collect the EC_{50} and E_{max} ($6\leq n<9$).

Endocannabinoids	EC_{50} (µM)	E_{max} (%)
Ethanol (Vehicle)	-	3.5 ± 0.7
POEA	9.3	21.2*
OEA	9.2±0.0	23.5*
1-AG	20.0±0.9	20.30.6*
NEA	23.3	48.2*

Table 1. E_{max} and EC_{50} obtained for 1-Ag, POEA, OEA and NEA. A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir2.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent. * ($P \leq 0.05$, ($6 \leq n < 9$)).

1.2 Endocannabinoid regulation of I_{K1} as a treatment for LQT7

To understand the effects of endocannabinoids of ATS/ LQT7 mutants of Kir2.1 channel on its function, we examined two critical mutations linked to ATS/ LQT7 syndrome, G144S and V302M. This study provides an essential foundation for the evaluation of potential treatments for ATS/LQT7 syndrome using endocannabinoids and whether these can restore normal Kir2.1 function in cells expressing LQT7 mutants.

1.2.1 Regulatory effect of ArEA on Kir2.1 ATS/ LQT7 mutations

Additions of ArEA to the bath solution, led to a significant increase in the current of Kir2.1 G144S mutation (Figure 18). By comparing the I-V curves to the control, it was found that a concentration of 40 µM of ArEA at -150 mV generated the highest amplitude of inward current (Figure 18.D).

In addition, the endocannabinoid ArEA also had an increased effect on the current of Kir2.1 V302M mutant channel (Figure 19). In general, an increase in the current was seen by the incremental addition of the ArEA (Figure 19.D).

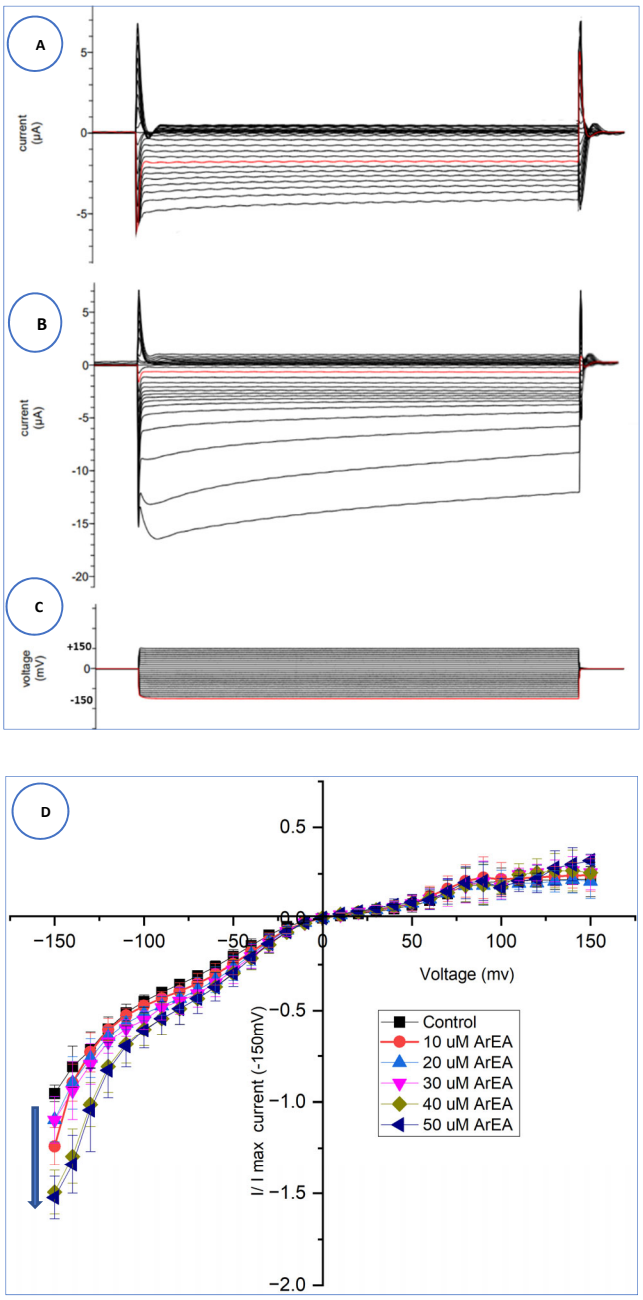


Figure 18. The effect of endocannabinoid ArEA observed on the current of Kir2.1 mutant (G144S) expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 mutation (G144S) without endocannabinoid treatment. B) Recording currents of Kir2.1 channels mutation (G144S) following treatment with various concentrations of ArEA. C) Application of the voltage protocol (-150 to +150). D) I-V relationship of Kir2.1 channels mutation (G144S) following the incremental addition of ArEA. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM of K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

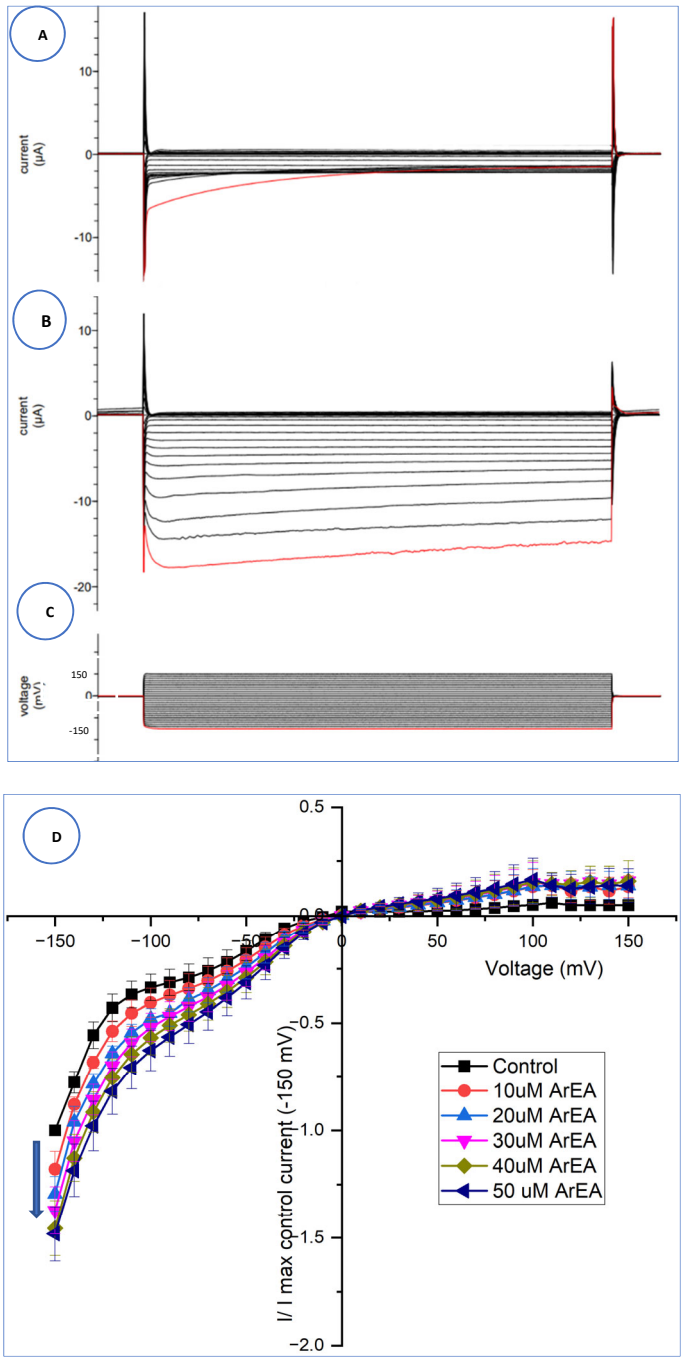


Figure 19. The effect of endocannabinoid ArEA observed on the current of Kir2.1 mutant (V302M) expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir2.1 mutation (V302M) without endocannabinoid treatment. B) Recording currents of Kir2.1 channels mutation (V302M) following treatment with various concentrations of ArEA. C) Application of the voltage protocol (-150 to +150). D) I-V relationship of Kir2.1 channels mutation (V302M) following the incremental addition of ArEA. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM of K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

3.2.2. Regulatory effect of 2-PG on Kir2.1-V302M currents

There was an increase in the current Kir2.1 mutation (V302M) following the incremental addition of endocannabinoids 2-PG (Figure 20). However, the first concentrations from 20 to 30 µM of 2-PG led to a small increase in the inward current of the mutation, but the current increased significantly after the addition of 40 and 50 µM of 2-PG (Figure 20.D).

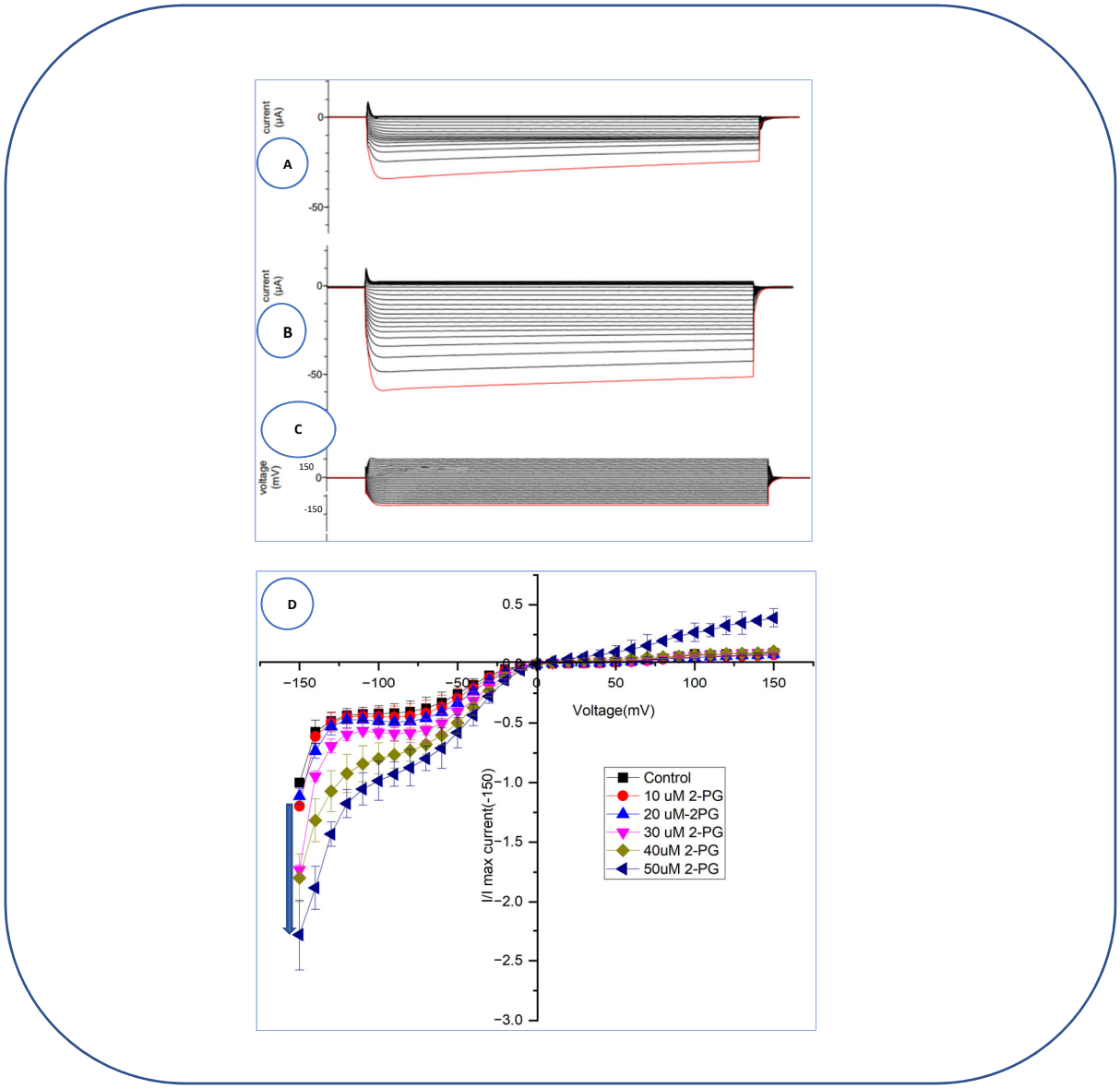


Figure 20. The effect of endocannabinoid 2-PG observed on the current of Kir2.1 mutant (V302M). A) Control condition involving the recording of Kir2.1 mutation (V302M) without endocannabinoid treatment. B) Recording currents of Kir2.1 channels mutation (V302M) following treatment with various concentrations of 2-PG. C) Application of the voltage protocol. D) Current-voltage (I-V) relationship of Kir2.1 channels mutation (V302M) following the incremental addition of 2-PG. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 µM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM of K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

3.2.1.1 Calculation of endocannabinoid dose dependence on ATS/ LQT7 mutant Kir2.1 channels

The potency and maximal effect of endocannabinoid ArEA were calculated for the mutation of Kir2.1 G144S An EC_{50} value of 1.4± 2.1 µM was calculated by plotting the dose-dependent curve for ArEA (Figure 21). A significant maximal effect was calculated which was 59% ± 0.44 (P≤0.05) (Table 2). We also calculated the potency and maximal effect of endocannabinoid ArEA and 2-PG for the Kir2.1 V302M mutation (Figure 19). An EC_{50} value of 1.29± 1.44 and 3.78±6.64 µM were obtained by plotting the dose-dependent curve of ArEA and 2-PG, respectively. The concentration required for maximum effect for 2-PG was 116% (±0.68), while this value for ArEA was 20% (±0.17) (Table 2).

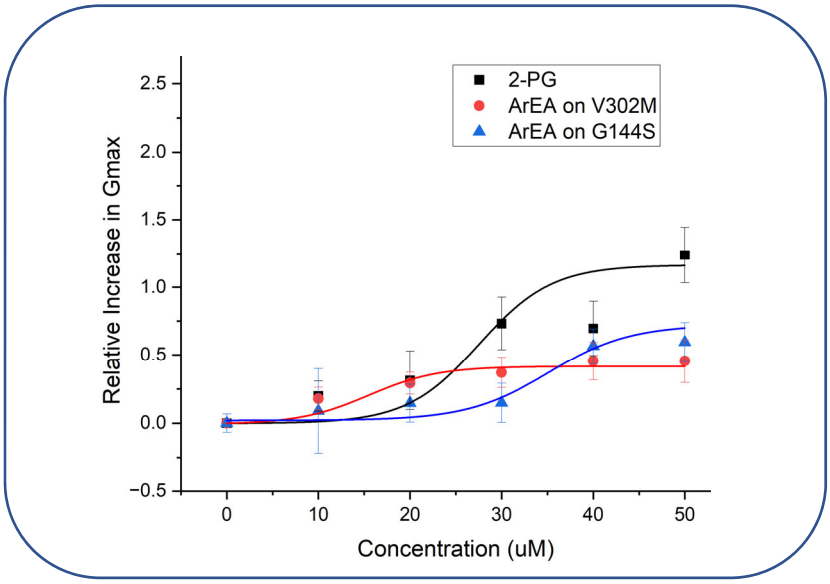


Figure 21. Concentration dependence curves of Kir2.1 mutations (V302M and G144S) calculated for endocannabinoids: ArEA and 2-PG to obtain the relative increase in G_{max} compared to the control. Data was then fitted to a dose-response function to collect the EC_{50} and E_{max} (5≤n≤7).

Endocannabinoids	**EC_{50} (µM)**	**E_{max} (%)**
Ethanol (Vehicle)	-	3.5 ± 0.7
ArEA (V302M mutation)	1.29± 1.44	49±0.17*
2-PG	3.78 6.64	123±0.68*
ArEA (G144S mutation)	1.41± 2.19	59± 0.44*

Table 2. Emax and EC50 obtained for ArEA and 2-PG tested for mutation of the Kir2.1 V302M. A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir2.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent (data was not shown). A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir2.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent.*(P≤0.05, 5≤n≤7).

1.3 . Is Kir2.1 regulation by other endocannabinoids conserved among Kirs?

Lipid effects on Kir channels are complex and vary among family members. Moreover, the amino acid sequences of the transmembrane helices in Kir channels differ significantly, particularly in regions that are likely to form potential binding pockets. Considering these differences, we investigated the impact of endocannabinoids on other Kir channels first among the family, specifically Kir7.1 and Kir4.1, which are classified as a weak and intermediate inward rectifier, respectively. These channels often exhibit distinct responses to membrane lipids when compared to Kir2.1 currents.

1.3.1 Regulatory effect of Kir4.1 by different concentrations of endocannabinoid ArEA

Upon addition of two different concentration ranges (5-20 μM and 10-50 μM) of ArEA to Kir4.1 channels, a significant increase in the inward current was observed (Figure 22 & 23). These results were obtained at various time intervals, yet a consistent increase was observed for each concentration of ArEA (Figure 22.D & 23.D).

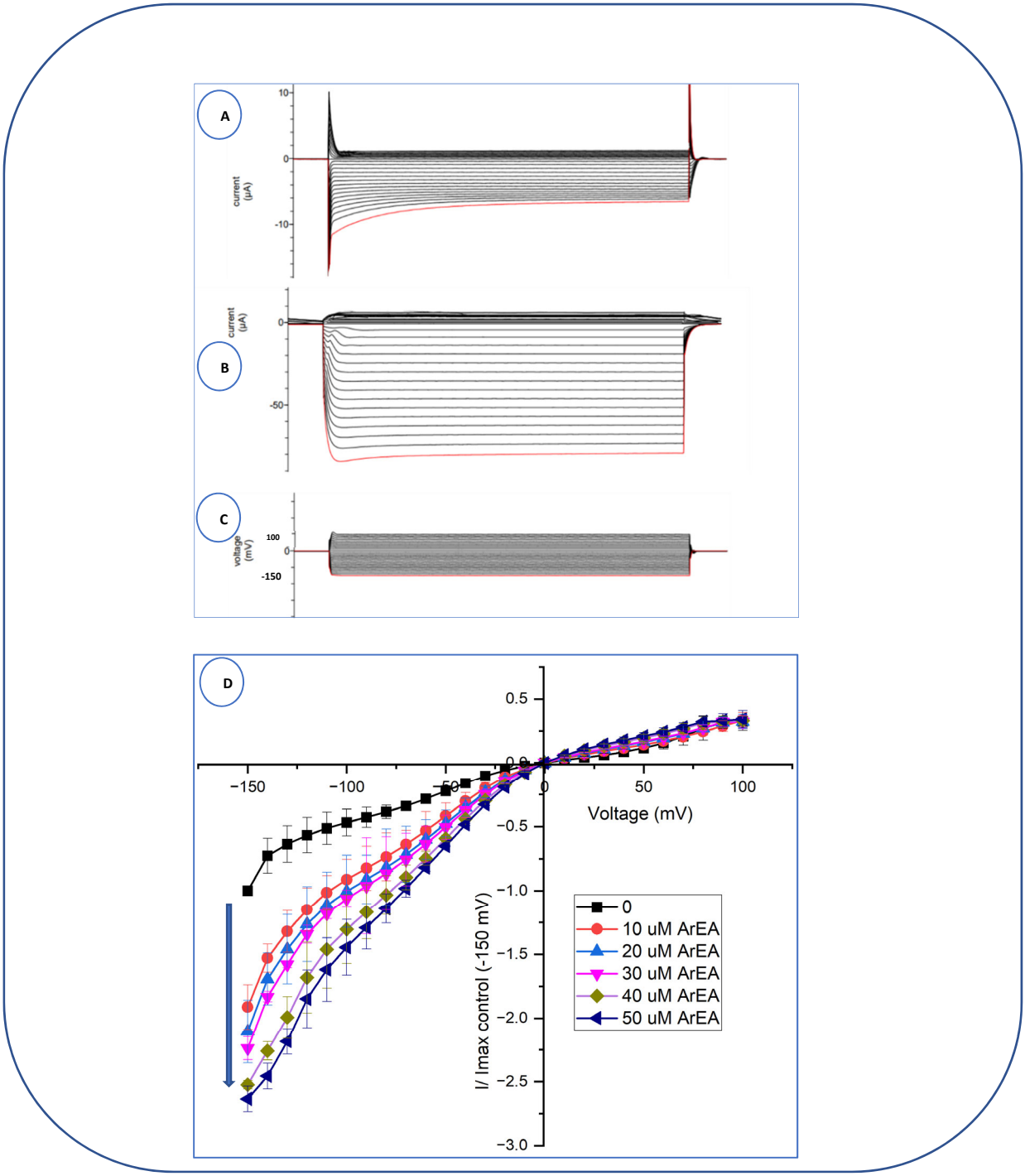


Figure 22. The effect of endocannabinoid ArEA, 10-50 μM on Kir4.1 current expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir4.1 channels without endocannabinoid treatment. B) Recording currents of Kir4.1 channels following treatment with various concentrations of ArEA. C) Application of the voltage protocol (-150 to 100mV). D) I-V relationship of Kir4.1 channels following the incremental addition of ArEA endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

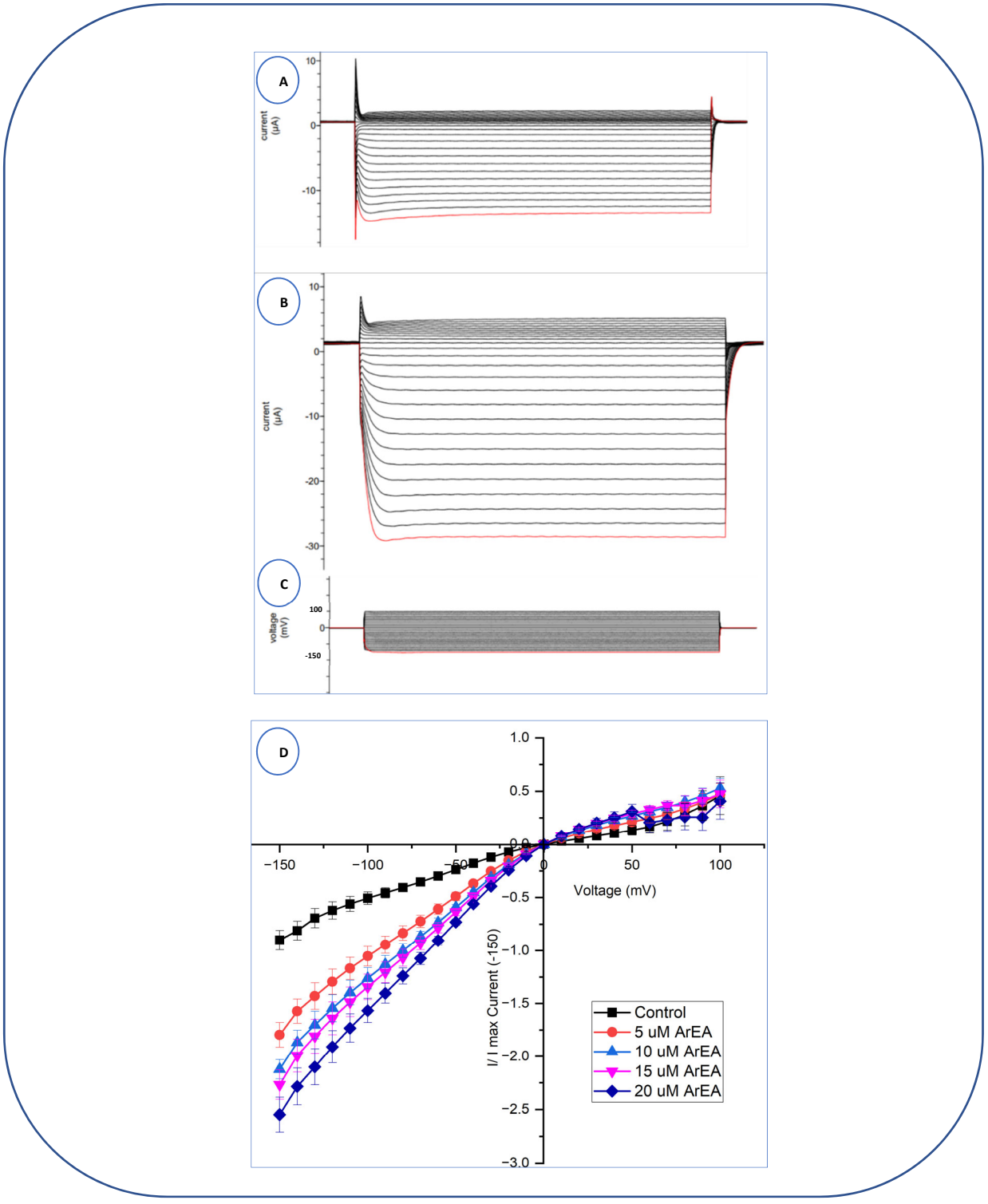


Figure 23. The effect of endocannabinoid ArEA, 5-20 μM on Kir4.1 current expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir4.1 channels without endocannabinoid treatment. B) Recording currents of Kir4.1 channels following treatment with various concentrations of ArEA. C) Application of the voltage protocol (-150 to +100mV)). D) I-V relationship of Kir4.1 channels following the incremental addition of ArEA endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

1.3.2 Regulatory effect of Kir4.1 by different concentrations of endocannabinoid 2-PG

Incremental addition of the 2-PG on Kir4.1 channels showed a great increase in the current of Kir4.1 (Figure 24). After the addition of the first concentration of 2-PG on Kir4.1, a substantial increase in the current was observed (Figure 24.D).

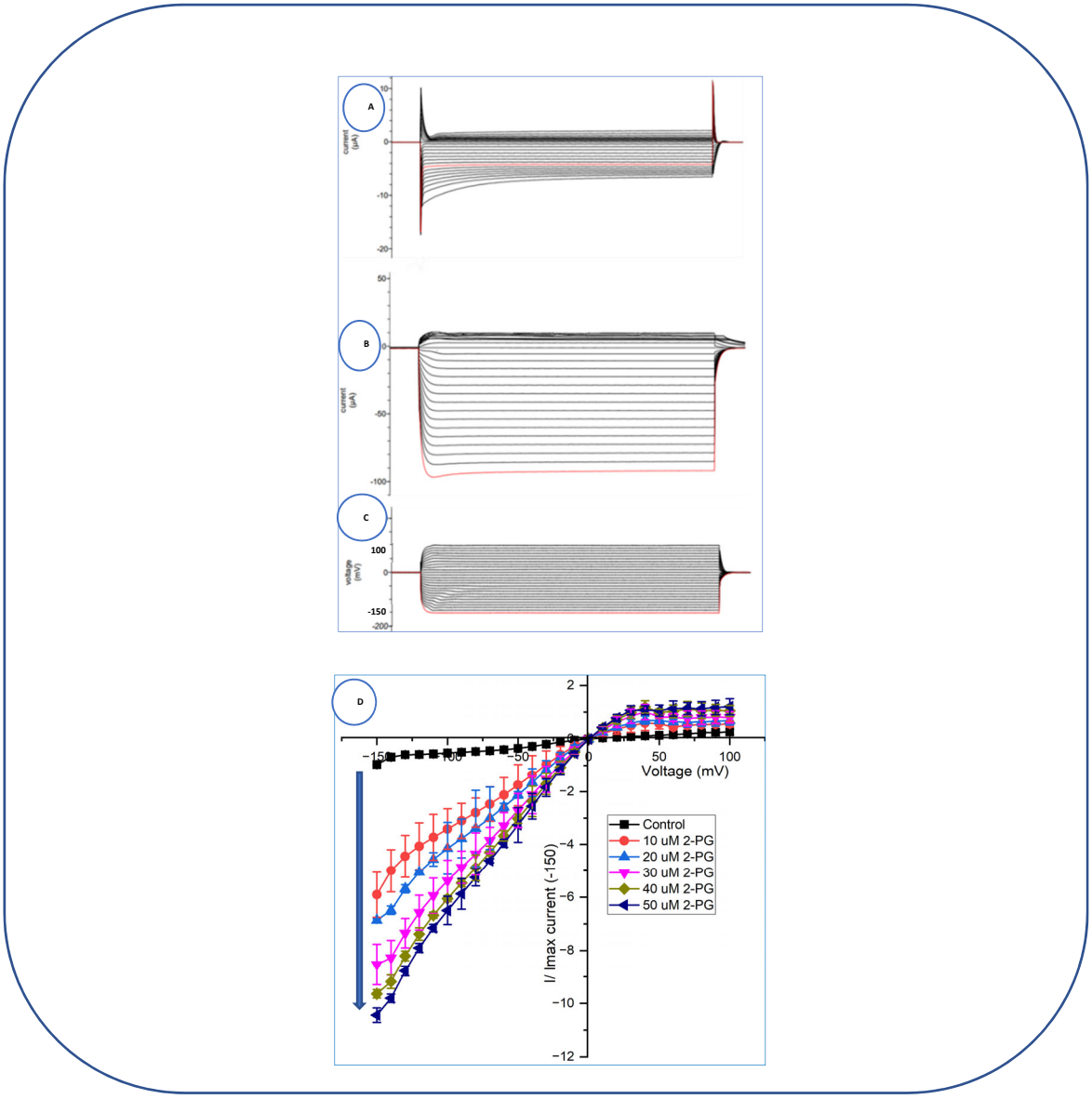


Figure 24. The effect of endocannabinoid: 2-PG on Kir4.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir4.1 channels without endocannabinoid treatment. B) Recording currents of Kir4.1 channels following treatment with various concentrations of 2-PG. C) Application of the voltage protocol (-150 to 100mV)). D) Current-voltage (I-V) relationship of Kir4.1 channels following the incremental addition of 2-PG endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 µM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($5 \leq n \leq 7$).

1.3.3 Calculation of EC_{50} and E_{max}

A dose-response curve was plotted by calculating the relative increase in G_{max} of ArEA and 2-PG on the Kir4.1 channel (Figure 25). Additionally, the half-potency (EC_{50}) and maximal effect of the endocannabinoid ArEA (at regular and lower concentrations) and 2-PG were calculated and are presented in Table 3. The maximal effect of 2-PG was extraordinarily high, reaching 810% with an EC_{50} value of around 3 µM (±1.1). On the other hand, ArEA exhibited a highly significant maximal potency of 163% (±1.01) with an EC_{50} value of 9.96 µM (±0.96)."

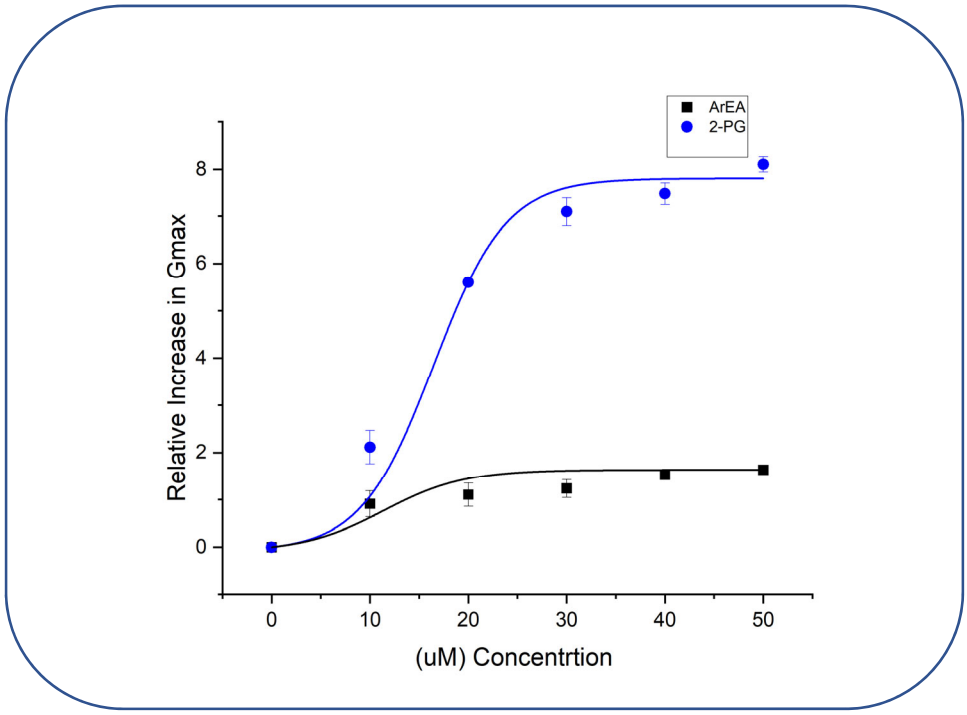


Figure 25. Concentration dependence curves were calculated for endocannabinoid ArEA with 2 concentrations to obtain the relative increase in G_{max} compared to the control. Data was then fitted to a dose-response function to collect the EC_{50} and E_{max} (5≤n≤7).

Endocannabinoids	**EC_{50} (µM)**	**E_{max} (%)**
ArEA	9.96±0.96	163±1.01*
2-PG	3.0	810± 0.01*

Table 3. Emax and EC50 for ArEA and 2-PG tested for Kir4.1. A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir4.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent. A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir2.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent. (*(P≤0.5, (5≤n≤7)).

1.3.4 Regulatory effect of Kir7.1 by endocannabinoids 2-PG and OEA

The effect of 2-PG on the current of Kir7.1 channels was investigated, and it was found that it led to a change in the current. Unlike the results observed with other Kir channels such as Kir2.1 and Kir4.1, where exposure to endocannabinoids resulted in changes in the inward rectification current, Kir7.1 exhibited different responses. In some cases, the current remained unaffected, while in others, it even decreased upon incremental addition of the endocannabinoids. For instance, the addition of different concentrations of 2-PG did not affect the inward current of the Kir channel (Figure 26. D). On the other hand, the incremental addition of OEA led to a decrease in the Kir7.1 channel current (Figure 27. D).

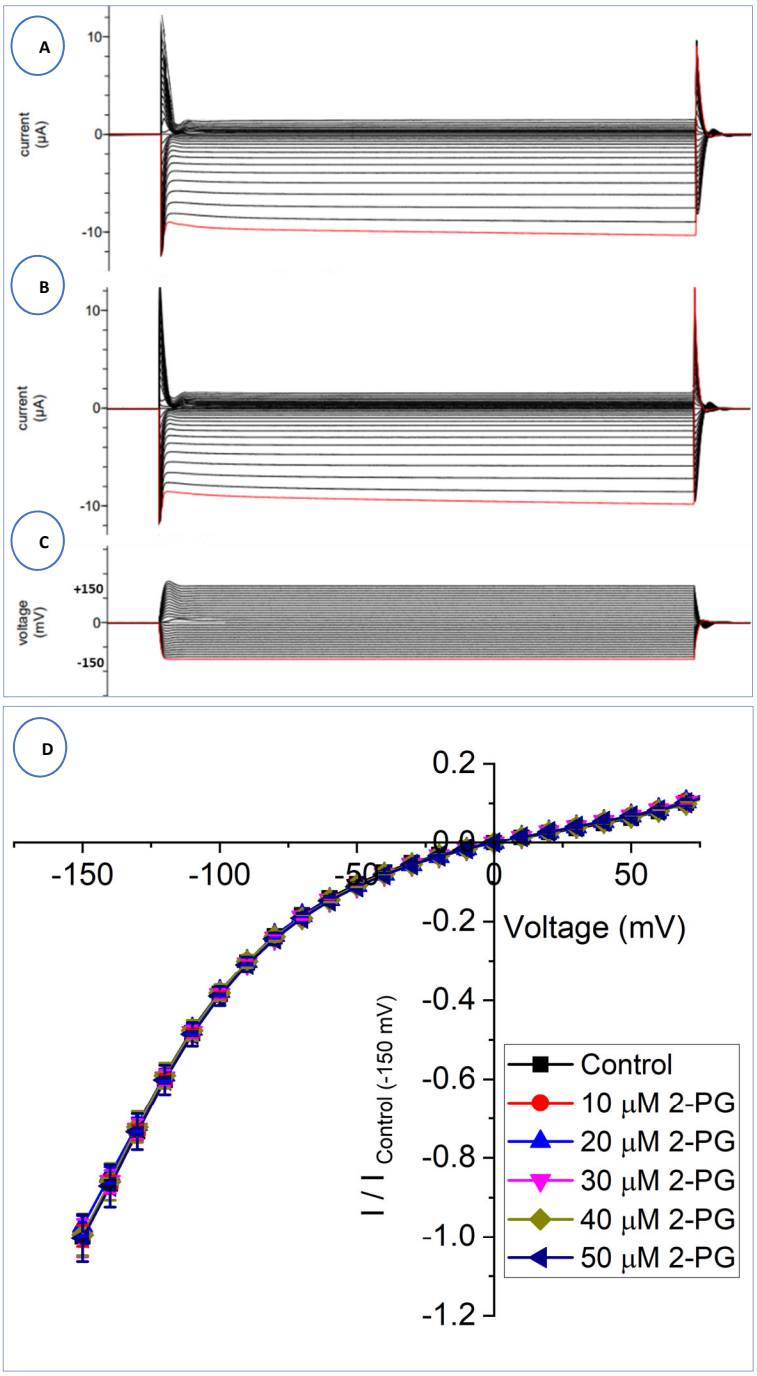
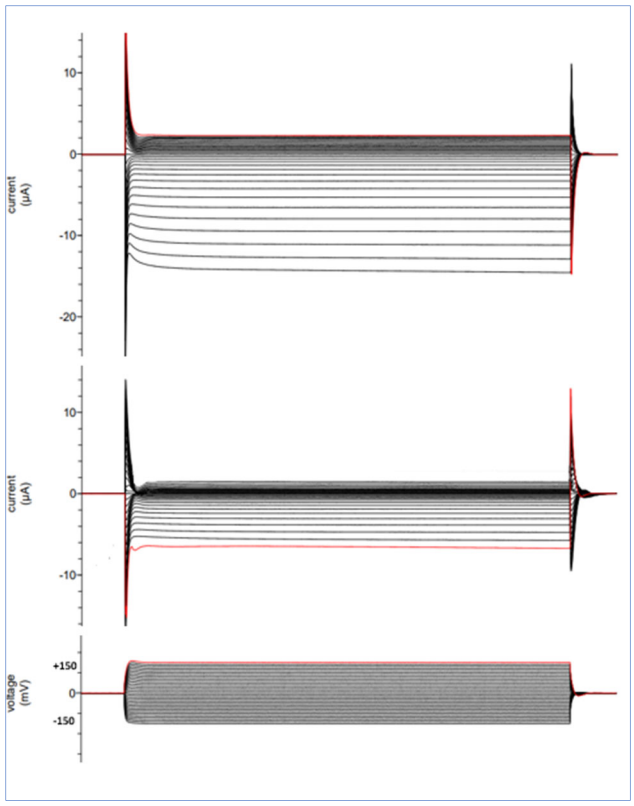


Figure 26. The effect of endocannabinoid: 2-PG on Kir7.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir7.1 channels without endocannabinoid treatment. B) Recording currents of Kir7.1 channels following treatment with various concentrations of 2-PG. C) Application of the voltage protocol. D) I-V relationship of Kir7.1 channels following the incremental addition of 2-PG endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).



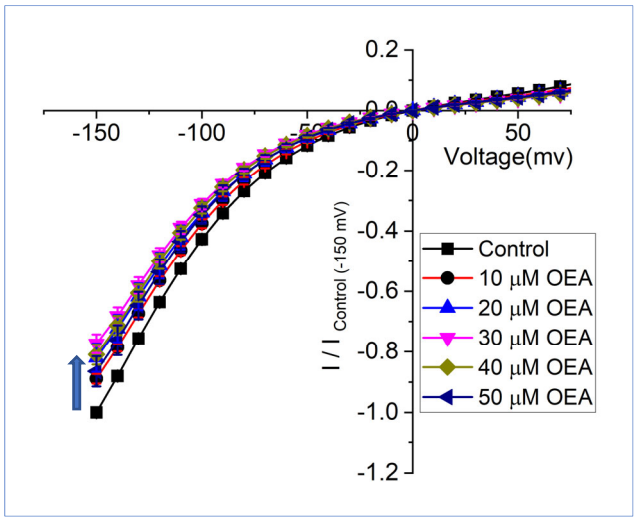


Figure 27. The effect of endocannabinoids: OEA on Kir7.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir7.1 channels without endocannabinoid treatment. B) Recording currents of Kir7.1 channels following treatment with various concentrations of OEA. C) Application of the voltage protocol. D) Current-voltage (I-V) relationship of Kir7.1 channels following the incremental addition of 2-PG endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

1.3.5 Regulatory effect of Kir7.1 by endocannabinoids NEA and LEA

When NEA was added to the bath solution, a significant decrease in the current of Kir7.1 was observed (Figure 28). The final concentration of NEA (50 μM) resulted in a substantial decrease in current compared to the control (Figure 28.D).

On the other hand, the regulatory effect of LEA on Kir7.1 did not cause any considerable change in the current (Figure 29). The incremental addition of LEA did not affect the inward rectification current of Kir7.1 channels (Figure 29. D).

Similarly, a minor change in the current of Kir7.1 was observed when the endocannabinoid ArEA was introduced (Figure 30).

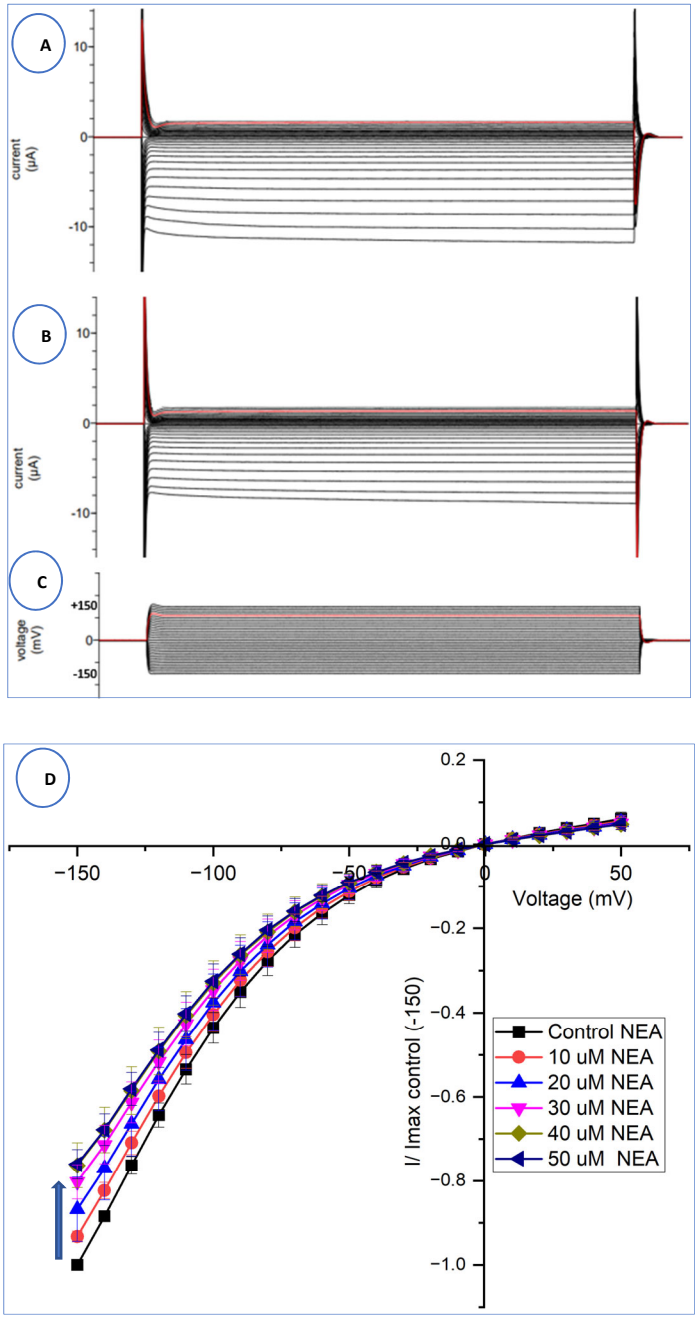


Figure 28. The effect of endocannabinoid: NEA on Kir7.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir7.1 channels without endocannabinoid treatment. B) Recording currents of Kir7.1 channels following treatment with various concentrations of NEA. C) Application of the voltage protocol (-150 to +150mV). D) I-V relationship of Kir7.1 channels following the incremental addition of NEA endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

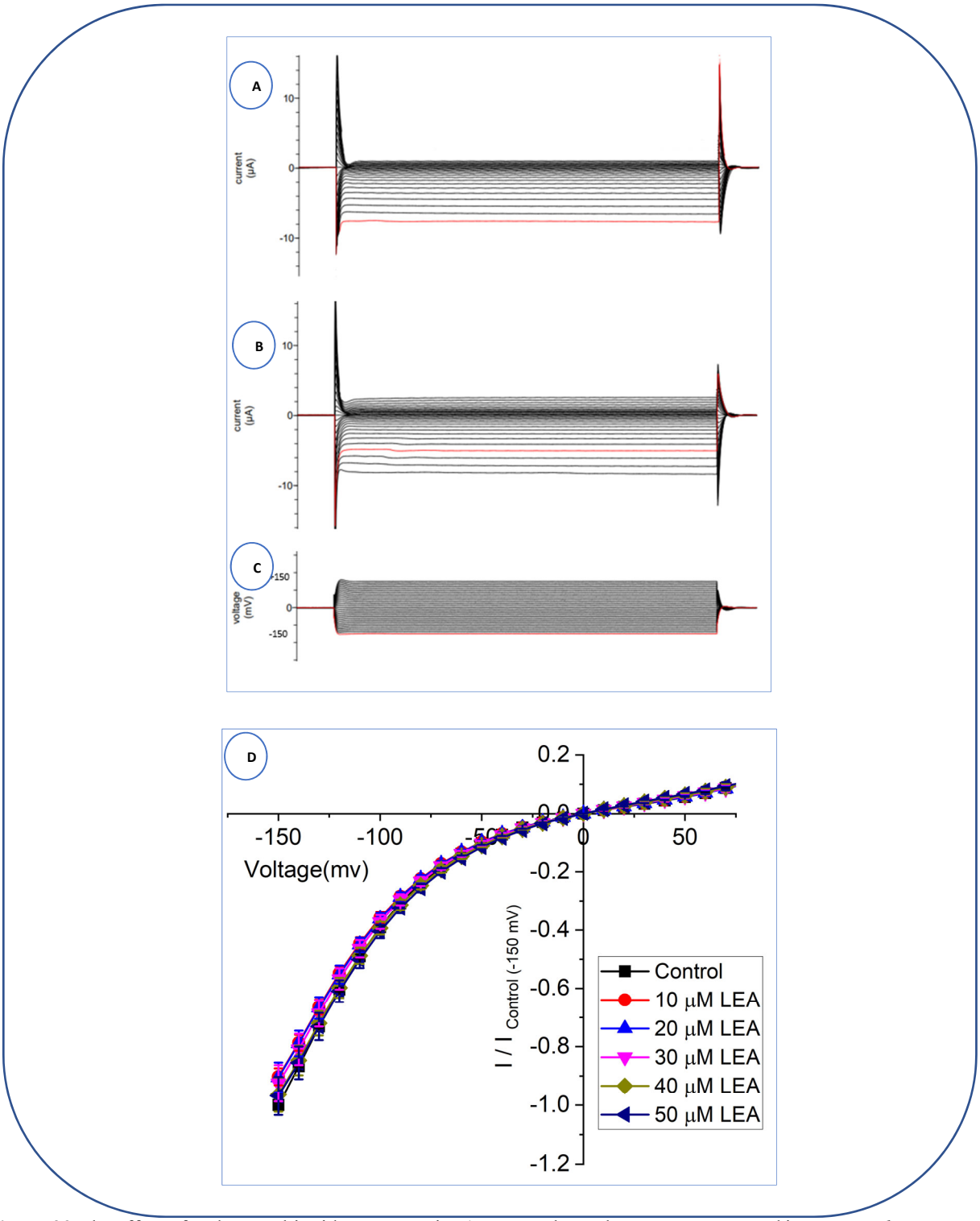


Figure 29. The effect of endocannabinoid: LEA on Kir7.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir7.1 channels without endocannabinoid treatment. B) Recording currents of Kir7.1 channels following treatment with various concentrations of LEA. C) Application of the voltage protocol (-150 to +150mV). D) I-V relationship of Kir7.1 channels following the incremental addition of LEA endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

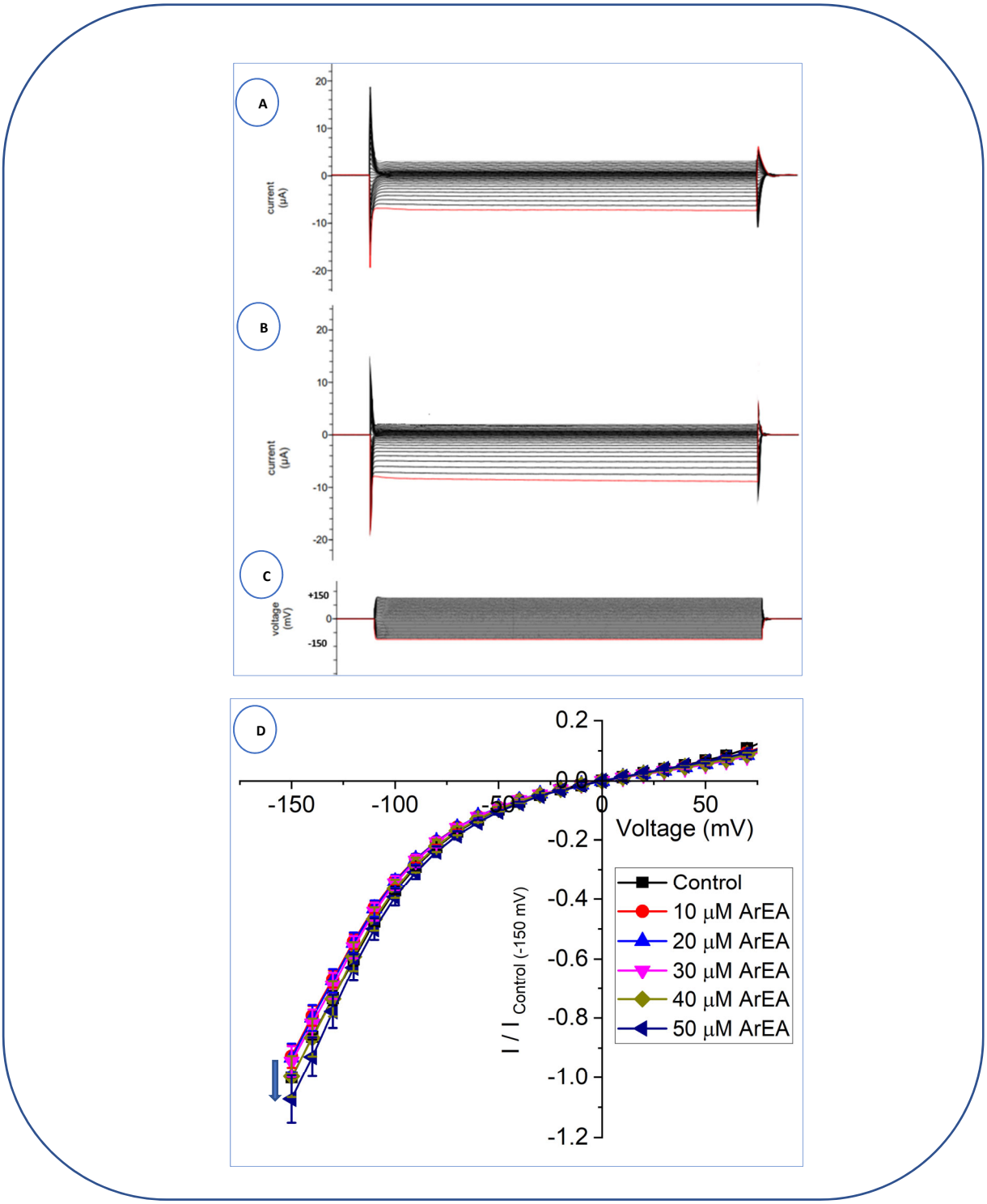


Figure 30. The effect of endocannabinoid: ArEA on Kir7.1 current channels currents expressed in *Xenopus laevis* oocytes. A) Control condition involving the recording of Kir7.1 channels without endocannabinoid treatment. B) Recording currents of Kir7.1 channels following treatment with various concentrations of ArEA. C) Application of the voltage protocol (-150 to +150mV). D) I-V relationship of Kir7.1 channels following the incremental addition of ArEA endocannabinoids. For each cell, currents were normalized to the current elicited at -150 mV for control conditions (0 μM endocannabinoid). This enabled the averaging of pair-wise data from different cells. The extracellular solution used during the recordings contained 89 mM K^+. Each cell recording took 6-8 hours per day ($6 \leq n < 9$).

1.3.6 Calculation of EC_{50} and E_{max} for 2-PG and ArEA endocannabinoid

Concentration dependence curves were calculated for 2-PG, OEA, ArEA, LEA, and NEA endocannabinoids by plotting the relative increase in slope conductance (G_{max}) compared to the control (Figure 31). Data was then fit to a dose-response function to determine EC_{50} of 2-PG and OEA, which were small amounts, 1.33±1.15 and 1.87±1.09 µM, respectively. In addition, both mentioned endocannabinoids showed different values for maximal effect, which was 6.9% (±0.06) for 2-PG and a negative value for OEA (-13±0.049) ($P \leq 0.5$) (Table 4).

By plotting the dose-dependent curve, both ArEA and NEA need the amount of 5.8 and 6.1 µM, respectively, to produce a 7.0% (±0.078) and -23% (±0.035) maximal effect. LEA also has shown a minimal maximal effect (Table 4).

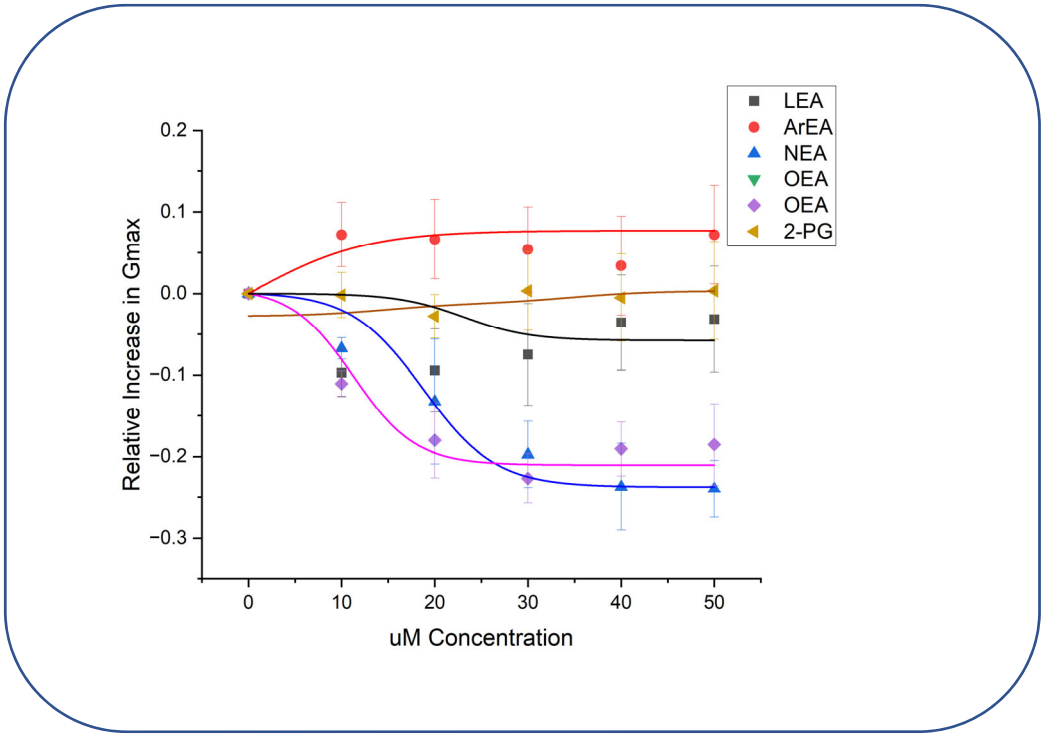


Figure 31. Concentration dependence curves of Kir7.1 calculated for 2-PG NEA to obtain the relative increase G_{max} compared to the control. Data was then fitted to a dose-response function to collect the EC_{50} and E_{max} ($6 \leq n \leq 9$).

Endocannabinoids	EC_{50} (µM)	E_{max} (%)
2-PG	1.33±1.15	6.9±0.06*
LEA	1.22±1.34	3.1±0.06*
NEA	6.1±1.50	-23±0.035*
ArEA	5.8±0.0	7.0±0.078*
OEA	1.87±1.09	-130.049*

Table 4. Emax and EC50 for 2-PG, LEA, NEA, ArEA, and OEA endocannabinoid for the Kir7.1. E_{max} and EC_{50} for 2-PG, LEA, NEA, ArEA, and OEA endocannabinoid for the Kir7.1. A 5% increase in current was established as a threshold to identify endocannabinoids that have an effect on Kir7.1 function based on the slight increase in current observed at high concentrations of the vehicle (Ethanol) used as a solvent *($P \leq 0.5$, ($6 \leq n \leq 9$)).

4. Discussion:

In the heart, I_{K1} stabilizes the resting membrane potential and is responsible for the shaping of the initial depolarization phase and final repolarization phase of AP (Tourneur, 1986). I_{K1} plays a key role in cell excitability of myocytes, especially in the ventricle (Ibarra et al., 1991). Heterogeneous I_{K1} is the result of Kir2 isoforms' predominant current of which Kir2.1 shows a very strong inward current and a tiny outward one in the repolarization phase of the AP. The Kir2.x family mediated this current in both the atrium and ventricule, compared to the other inward rectifiers that contribute to cardiac excitability.

I_{K1} has been shown to undergo changes by endogenous lipophilic compounds through the modulation of Kir2.x isoform function. Various membrane lipids, including phosphoinositides, secondary anionic phospholipids, cholesterol, long-chain CoA, and arachidonic acid was shown to affect I_{K1} current through regulation of the Kir channels directly (Cheng et al., 2011; D'Avanzo, Cheng, Doyle, et al., 2010; Fürst, Mondou, et al., 2014; Levitan, 2009; Shumilina et al., 2006; Wang et al., 2008). Alternation of Kir2.1 function is a critical determinant of proper heart function (Veerapandiyan & Statland). Thus, any gain- and loss-of-function mutations of Kir2.1 would affect I_{K1} amplitude. I_{K1} is tightly downregulated in heart failure of human patients and genetically suppressed in Andersen-Tawil syndrome (LQT7).

Endocannabinoids are lipidic signalling molecules with strong therapeutic potential in many diseases through the activation of CB1 and CB2. Apart from the activation of cannabinoid receptors, they simultaneously have been shown to affect the function of ion channels independent of the interaction with their receptors. Previous studies have proved that membrane lipids can modulate the function of K^+ channels (Forte et al., 1981; van Dalen & de Kruijff, 2004).

To understand the regulatory effect of the endocannabinoids on Kir2.1 using biochemical, mutagenesis, and electrophysical techniques, we showed the following findings: 1) Kir2.1 is activated by the endocannabinoids directly without affecting the cannabinoids receptor, 2) Some endocannabinoids can increase the current of the Kir2.1 in the oocyte model which expressed the mutations linked with LQT7 syndrome, 3) we observed that the endocannabinoids' regulatory effect is not conserved only in Kir2.1 channels, it also can modulate the function of Kir4.1 and Kir7.1 with

different sensitivities. In this section, we will discuss and compare our findings with other studies that are either similar or different from our own.

It is also worth mentioning that in our study, we investigated the regulatory impact of endocannabinoids on Kir channels with different concentrations. This exploration involved a range of endocannabinoid concentrations 10 to 50 μM to understand their effects comprehensively. The application of dose-response experiments allowed us to identify concentration ranges where endocannabinoids significantly affect Kir channel behaviour. Using concentrations higher than physiological levels was intentional, as it enables the detection of effects that might remain latent at physiological concentrations. As dynamic signalling molecules, endocannabinoids are present in human circulation; they are derived from various organs and tissues (brain, adipose tissue, muscle, and circulating cells (Hillard, 2018). Their concentrations rely on numerous physiological and pathophysiological factors within the body. For instance, serum concentrations of AEA typically range from 1 to 5 nM, while 2-AG concentrations in serum span 10 to 500 nM (Hillard, 2018). Higher ligand concentrations are used in experimental studies in the literature. For instance, research involving exogenous PIP_2 application on Kir2.1 channels in Xenopus oocytes has demonstrated activation of the Kir2.1 at micromolar concentrations (10 μM) (Xie et al., 2005). Effective ligand (PIP_2) activation often requires concentrations beyond the physiological range (1-3%). By embracing higher concentrations, the study navigated the complexities of ligand-channel interactions and their implications for cellular responses. Thus, higher concentrations can facilitate the effective delivery and interaction of exogenous endocannabinoids with Kir channels (Xie et al., 2005).

4.1 Kir2.1 channels regulated by endocannabinoids, independently of CBRs

A large body of evidence supported that Kir2.x isoforms (Kir2.1, Kir2.2, Kir3.1) have well-defined roles in biological (heart rhythm and muscle contraction) and pathological functions. Using the TEVC technique, we have shown that the ionic current of Kir2.1 channels is activated by endocannabinoids, fatty acid ethanolamides (FAEs), and 2-monoacylglycerols (2-MGs), on *Xenopus laevis* oocytes, after the addition of different concentrations (10-50 μM).

Many ion channels of which Ca^{2+} channels (Devane et al., 1992), Na^{+} channels (Nicholson et al., 2003), TRPV channels (Montell et al., 2002), and K^{+} channels (Poling et al., 1996) have been shown to be directly modulated by cannabinoids (reviewed (Oz, 2006)). The nature of the mechanism of

action is suggested to be through lipophilic interactions. Specifically, a study in 1990 showed that THC can decrease the inward current of Na^+ in neuroblastoma cells (Turkanis et al., 1991). Later on another research group reported that Kir current was activated by WIN 55,212-2, a synthetic version of anandamide, in A-2 cells (Mackie et al., 1995). Our findings are very consistent with this early study, even though at the time, they believed that this regulation might be due to the presence of CBRs in neural cells. In this present study, we show this regulatory effect of natural endocannabinoids on *Xenopus laevis* oocyte cells which do not express CBRs. Furthermore, we identified the Kir2.1 current in oocytes when using a low concentration of Ba^{2+} (2 μM), resulting in the inhibition of the K^+ current. According to our results, Kir2.1 current was regulated only with a handful of endocannabinoids, and they differ in their sensitivities. For instance, OEA and POEA from FAEs increased the current of the Kir2.1 channels at -150 mV with EC_{50} at about 9 μM, and the maximal effect was 21.2% and 23.5% increase respectively. Half maximal effect for 1-AG, was 20.0 μM with a maximal effect around 20.3%, while with almost the same EC_{50} (23.3 μM), NEA showed a significant E_{max} with a value of 48.2%. Here in our study, we are reporting NEA as the endocannabinoid with the greatest affinity for Kir2.1, the high affinity of 1-AG has previously been reported by other groups for GPCR (Godlewski et al., 2009), but it was not reported for NEA. Activation of Kir2.1 current facilitates quick repolarization and excitability in cardiac cells.

In addition, endocannabinoids are metabolic product of the polyunsaturated fatty acids (PUFAs), and the role of PUFAs is critical for maintaining cell membrane structure and function. In the human body, two essential PUFAs, linoleic acid and alpha-linolenic acid, are involved in the synthesis of omega-6 and omega-3 fatty acids, respectively. This process involves adding of a double bond between two carbon atoms (saturation) and addition of two carbon atoms (elongation) (Ander et al., 2003). Omega-6 fatty acids mainly contribute the synthesis of two main endocannabinoids, namely AEA and 2-AG. Dietary omega-3 fatty acids are necessary to form prostaglandins and leukotrienes, as well as some classes of omega-3 derived endocannabinoids including docosahexaenoylethanolamide (DHEA), eicosapentanoyl ethanolamide (EPEA) (Watson et al., 2019). Moreover, endocannabinoid of AEA or arachidonoylethanolamine is the ethanolamide conjugate of arachidonic acid (ARA; 20:4n-6) which shows its close structure to PUFAs (Komarnytsky et al., 2021). A subset of PUFAs (omega-3 and omega) can also interact and modulate the function of most ion channels (Kahn-Kirby et al., 2004). PUFAs affect the function of many ion channels with specific mechanisms. In order to alter the function of ion channels, it is necessary for them to have a carboxyl group that is charged and two double bonds in cis geometry (Elinder &

Liin, 2017). In 1990, it has been reported by a research group that PUFA has a direct mechanism of action on the function of the K^+ channels such as activation of Kv channels (Rouzaire-Dubois et al., 1991). PUFAs showing a direct regulatory effect have been observed to induce the current of the K^+ channels (Ferroni et al., 2003; Horimoto et al., 1997{Ferroni, 2003 #470). A most recent study has also investigated the interactions of KCNQ1 channels with PUFAs, demonstrating the direct interaction of the KCNQ1-PUFA through two different binding sites, including the voltage sensor and the pore domain. This research suggested that binding pockets of the KCNQ1 channel secure or hold the negative charge of PUFAs by interacting with specific amino acids of the channel (Yazdi et al., 2021). Therefore, in our study, this information supports our idea that endocannabinoids as a signalling derivative of PUFAs can regulate the function of Kir channels and possibly interact directly behind the pore domain as PUFAs have been shown to do for other K^+ channels.

4.2 Endocannabinoid regulation of I_{K1} as a treatment for LQT7

Since activation of Kir2.1 leads to faster repolarization in the heart, we examined if endocannabinoids can be used to treat LQT7/ATS which is associated with the deficit of the *KCNJ2* gene (Tawil et al., 1994). Using electrophysiology techniques combined with biochemical and mutagenesis techniques, we showed that the mutants G144S and V302M when co-expressed with wild-type channels in a ratio 1:1 actively decreased the ionic current of the endogenous K^+ measured at -150 mV. ATS is an autosomal-dominant disorder associated with loss-of-function *KCNJ2* mutations (Plaster et al., 2001). Our observations indicated that mutations of the Kir2.1 channel did not exhibit functional activity unless they were co-expressed with an equal concentration of Kir2.1 WT mRNA in a 1:1 ratio. This finding implies that the presence of Kir2.1 WT mRNA is essential for restoring functional properties to the mutated Kir2.1 channel. These findings are in line with initial studies on Kir2.1-G144S mutant (Plaster et al., 2001) and another study on mutation V302M (Ma et al., 2007) that confirms the introduction of the equal amount of Kir2.1 WT mRNA alongside the mutant counterpart seemed to be a prerequisite for the proper functioning of the mutated Kir2.1 channel.

Moreover, another study has provided valuable information about the functional consequences of the G144S mutation. This study demonstrated that when the G144S mutation was transfected into inner ear hair cells alongside Kir2.1 wild type, a significant effect was observed on I_{K1} current in utricle hair cells in mice. This impact stemmed from the loss of function of the Kir2.1 channel, which

was attributed to the G144S mutation's dominant-negative effect. Specifically, the G144S mutation resulted in a defective selectivity filter within the Kir2.1 channel. This defect led to the abolishment of Kir2.1 currents and, consequently, the I_{K1} current. The study clarified the role of the G144S mutation in disrupting the normal function of Kir2.1 channels while highlighting how the interaction between mutated and wild-type components influences channel activity (Levin & Holt, 2012). When we observed the Kir2.1-G144S mutant density was smaller than the Kir2.1 wild type (20-40%). According to early studies, mutation G144S is an important mutant linked to ATS that does not generate measurable currents and leads to current density smaller than wild type (Ballester et al., 2006). G144S disrupts the selectivity filter GYG domain function, a signature sequence of K^+ channels and position 144 is considered a highly conserved K^+ channel residue thus, not surprisingly leading to a non-functional channel (Plaster et al., 2001). Other relevant mutations in the M1 pore region (G144A, G156D) have been characterized in ATS patients (Zhang et al., 2005). These findings are also consistent with the dominant negative effects of these mutations, which our studies align with.

Mutation V302M is in the C-terminus region and it has been reported that residue 302 is in the G-loop (Ma et al., 2007) and considered as a flexible side chain (barrier) for K^+ permeation at the cytoplasmic pore through the G-loop (Pegan et al., 2006). V302M has been shown to also disrupt current of Kir2.1 channels compared to the wild-type (Ma et al., 2007) which is consistence with our electrophysiological recording for this mutation as well. Because based our recordings the current of the mutant Kir2.1 was lower compared to the wild-type, mainly due to the dominant negative effects mutation.

Our findings showed that the incremental addition of ArEA to the co-assembly of Kir2.1-wt/G144S channels increased the maximal effect by approximately 59%. The half-maximal effect was only 1.4 μM. Exposing the V302M mutation to varying concentrations of ArEA resulted in an increase in the inward rectifier of K^+ current, with a maximal effect value of 20% for ArEA (evaluated at -150 mV). However, with an EC_{50} of 3.78 μM, 2-PG showed a sizeable maximal effect of approximately 116% and significantly enhanced the current of the Kir2.1-V302M mutant at hyperpolarized voltage.

Regarding physiological relevance, we can highlight that the voltage range of -150 to 150 mV used in our study encompasses the physiological voltages experienced by cells. This range includes cells with resting potentials like cardiac myocytes, which are approximately -90 mV (Grant, 2009). By employing higher physiological concentrations of extracellular K^+, we aimed to shift the equilibrium

potential for potassium (EK=0) to more positive potentials. This adjustment enabled us to investigate the effects of specific endocannabinoids on Kir2.1 channels at more negative potentials. Consequently, under physiological conditions, Kir channels exhibit a significant K^+ conductance at potentials negative to EK while generating reduced current flow at potentials positive to EK (outward current) (Hibino et al., 2010). This dynamic suggests that changes in Kir channel behaviour due to endocannabinoid effects may carry physiological importance. So, we believe that certain endocannabinoids' observed significant maximal effect on Kir channels could indicate a notable alteration in channel behaviour. This degree of change could potentially affect cellular excitability and resting membrane potential, particularly in cells expressing a substantial Kir current. Such cells are anticipated to possess a resting membrane potential (Eres) close to EK, which in turn can impact the overall electrical characteristics of excitable cells, including their firing patterns and responsiveness to various stimuli (Hibino et al., 2010).

Based on our findings, we propose that these heterozygous mutations G144S and V302M might have the potential for modulation by certain endocannabinoids. However, it is essential to note that our study provides initial evidence suggesting the feasibility of such rescue. However, comprehensive investigation, including detailed mechanistic studies, is required to understand the potential interaction between these mutations (G144S and V302M) and certain endocannabinoids we examined in our study.

We lack evidence of a specific mechanism that can explain the action of endocannabinoids on Kir2.1 mutations. Exploring the underlying mechanisms behind these interactions remains an intriguing subject for future research. However, according to several studies on mutations in specific regions of *KCNJ2*; it has been suggested that diminishing of Kir2.1 affinity for PIP_2 binding site is a key mechanism involved in many ATS mutations (Bendahhou et al., 2003; Donaldson et al., 2004; Lopes et al., 2002). Any changes in the amino acid sequence in the vicinity of valine-302 residue affects the channels activity and PIP_2 sensitivity which is strongly sensitive to changes in size and hydrophobicity of the side chain in the C-terminus position (Ma et al., 2007). Other associated ATS mutants such as N216H, and P186L showed an altered function of the Kir2.1 channel within the region binding the signalling phospholipid PIP_2 (Tinker et al., 1996; Zhang et al., 1999). These early studies strongly present evidence that the cytoplasmic structure of Kir2.1 possesses clusters of amino acid sequences which form regulatory domains that interact with cellular metabolites to control the opening and closing of the channel (Pattnaik et al., 2012).

Another possible explanation is that the Kir2.1-G144S mutant has already been shown to disrupt the function of the selectivity filter, which leads to the inhibition of I_{K1}. This disruption is caused by alterations or modifications in the selectivity filter, resulting in abnormal function of the Kir2.1 channel (Levin & Holt, 2012). Based on both our findings and previous studies, it is likely that the observed active endocannabinoids may modulate the sensitivity of Kir2.1 channel mutations G144S and V302M. Interactions with the PIP_2 binding site, which promote channel opening, could represent a potential mechanism. Another possibility is that active endocannabinoids possess direct and specific binding sites on Kir2.1 channels independent of other binding sites, such as PIP2. While our research has provided preliminary data on the regulatory effects of certain endocannabinoids, we acknowledge the lack of direct experimental proof to definitively support this mechanism of action. Future investigations are necessary to confirm and elucidate the underlying mechanisms governing the effects of endocannabinoids on Kir channel behavior, particularly in the context of these specific mutations, G144S and V302M.

Moreover, it has already been reported that agonists PUFAs or DHA bind a specific site on ion channels, as seen in pLGICs on the channel's outer helices (Basak et al., 2017).

In addition, PUFAs, as endogenous and exogenous amphipathic molecules, have been suggested to have an antiarrhythmic effect (Endo & Arita, 2016), especially on the LQT syndrome. PUFAs have been shown to modulate I_{Ks} currents in cardiac cells and have a therapeutic effect on the LQT syndrome through the interaction of ion channels such as Kv7.1, along with *KCNE1* as an auxiliary subunit to mediate the K^+ currents in the heart (Deal et al., 1996; Noble & Tsien, 1969). In cardiac cells, PUFAs (DHA and EPA) can bind to Na_V and Ca_V channels and lead to inhibition of the activity of these channels through binding to their voltage-sensing S4 segments, which are almost homologous (Kang et al., 1995).

They have suggested that analogues of PUFAs, which exhibit selectivity for Kv7.1/KCNE1 channels, might have the capacity to decrease the duration of ventricular action potentials in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (Bohannon et al., 2020). These findings align with our results and support the potential effects of endocannabinoids in cardiac syndromes such as ATS/ LQT7 syndrome.

4.3 Is the regulation of Kir2.1 by endocannabinoids conserved among Kirs?

While Kir4.1 and Kir7.1 are not primarily found in the heart tissue, studying their response to endocannabinoids helped us better understand the regulatory mechanisms of Kir channels. This broader perspective enhances our understanding of these endogenous lipids' action on other Kir channels beyond cardiac contexts. Here we have found that endocannabinoids also can regulate the inward current of other Kir channels (Kir4.1 and Kir7.1) with different sensitivities. Several studies have shown that membrane lipids affect different family members of the Kir channels in terms of channel gating with different sensitivities to the agonist (Hilgemann & Ball, 1996; Huang et al., 1998; Logothetis et al., 2015). Our interpretation of the results of these and earlier studies is that the regulation effect of the endocannabinoids on Kir2.1 channel function is not conserved among other members of the Kir family (Kir4.1 and Kir7.1). For instance, phosphoinositides (PIPs) are critical for the activation of Kir channels and previous studies have shown that different Kir channel subfamilies could be categorized based on the stereospecificity and affinity to PIPs. Some Kir channels are reported to have the highest sensitivity to PIP_2 such as Kir2.1, Kir2.4, and Kir4.1, while others, the likes of Kir6, Kir7 and Kir3, are less specific or have lower affinities (Lopes et al., 2002).

In addition to PIP_2 regulation, there is evidence to suggest that PUFAs, such as arachidonic acid, which are also constituents of cell membrane lipids, can modulate K^+ conductance (Hamilton & Kamp, 1999). The effects of arachidonic acid are conserved only for some of the Kir channels. For instance, arachidonic acid only affects the Kir3 family (Rogalski & Chavkin, 2001) and its metabolites, 11,12-epoxyeicosatrienoic acid (11,12-EET), has been shown to activate the Kir6.1 channel (Ye, 2005). Among the Kir2.x family, the only member affected by arachidonic acid is Kir2.3 (Liu et al., 2001). In line with our study, this lipid component has also been shown to directly regulate Kir2.3 (Liu et al., 2002) with an EC_{50} value of 0.59 μM, but only in the presence of PIP_2. Indeed, arachidonic acid alone is not enough to induce current in Kir2.3 channels. It requires the synergistic effect of PIP_2, as well as either an isoleucine or leucine in the Kir2.3 in the interaction site of Kir2.3-PIP2 (Wang et al., 2008). Previous studies have shown that, arachidonic acid has no significant regulatory effect on Kir2.1 (wild type or mutants), Kir2.2 or Kir2.4 channels which are known as strong rectifiers (Liu et al., 2001; Liu et al., 2002). Based on earlier studies, it seems that the reason arachidonic acid cannot affect the current of Kir2.1 (Liu et al., 2001) is due to the strong sensitivity of Kir2.1 to PIP_2. This is because arachidonic acid shares structural features with PIP_2. One of the other explanations is that there are certain binding sites for arachidonic acid in the

transmembrane domain that affects the regulation of Kir2.3. However, the effect of arachidonic acid on I_{K1} has not been tested yet (Wang et al., 2008).

Furthermore, the impact of cholesterol on Kir channels is unique. While cholesterol reduces the current density of Kir2.1 channels, Kir4.1 exhibits decreased activity as a result of cholesterol depletion (Levitan, 2009).

While our study needs additional work, based on these and earlier studies, it seems that endocannabinoids are enough for the direct regulation of Kir channels, with differing sensitivities among members (further data and experiments are needed to confirm these findings.

Kir4.1 channels in astrocytes mediate the K^+ buffering action, a process by which astrocytes regulate the extracellular potassium level (Ohno, 2018). However, several studies have reported increased Kir4.1 current due to human loss-of-function mutations (Nwaobi et al., 2016; Ohno, 2018; Schirmer et al., 2018). Surprisingly, a few recent studies have claimed a significant decrease in Kir4.1 protein expression in a Huntington's disease mouse model compared to the control group (Tong et al., 2014). Furthermore, a more recent study conducted in 2023 by the same research group has shown that the dysregulation of Kir4.1 in glial cells leads to a reduction in Kir4.1 current. To investigate this further, they treated ischemic mice with luteolin, a natural flavonoid, and observed an increase in Kir4.1 current in glial cells of type 2 (NG2 glia or oligodendrocyte progenitor cells). These new findings have resulted in the promotion of axon remyelination, reduction in infarction area, and increased lifespan of the ischemic mice (Hong et al., 2023). The findings of this recent study (Hong et al., 2023) suggest that endocannabinoids may also play a role in Kir4.1 mutations associated with Huntington's disease. Our study has demonstrated that endocannabinoids significantly increase the ionic current of Kir4.1 channels, which might support their involvement in disease-associated mutations.

In contrast to increases in the current of Kir4.1, the addition of a subset of endocannabinoids decreased or barely changed the current of Kir7.1 compared to the active endocannabinoids which regulated the Kir2.1 strongly. It can be explained based on the findings of previous studies in which researchers assessed the strength of Kir channel interactions with PIP2, focusing on the most robust interacting channel, Kir4.1. According to this analysis, Kir channel subfamilies have been ranked in the following order (from strongest to weakest PIP_2 interaction): Kir4 > Kir1 ~ Kir2 > Kir6 > Kir7 > Kir3 (Du et al., 2004; Lopes et al., 2002). These studies go along with our results and our

interpretation withhold the regulatory effect hypothesis of endocannabinoids meaning that it could be close to the effect that has already been shown for PIP_2 (Pattnaik & Hughes, 2009).

Following other research (Lammel et al., 2012; Sartorius et al., 2010), PIP_2 predominantly interacts with Kir channels featuring leucine at this position, including Kir2.1, Kir2.4, Kir4.1, Kir4.2, and Kir1.1. Conversely, channels like Kir2.2, Kir2.3, Kir3 family, and Kir6 family, which possess isoleucine at this position, generally exhibit weaker or moderate affinity for PIP_2.

The presence of either leucine or isoleucine at this position is crucial in affecting Kir channel interactions with PIP_2. However, Kir7.1 channels could be an exception. Despite having leucine at this position, they demonstrate weak affinity interactions with PIP_2 (Du et al., 2004), these findings are in line with our data.

In contrast to our observations, cholesterol also has shown a distinct effect on the Kir7.1 channel as it led to an increase of the current of Kir7.1, and it emphasizes the complex effect of lipids on Kir channels. Among the group of endocannabinoids, we tested (2-PG, OEA, NEA, LEA, and ArEA), only ArEA and 2-PG showed significant modulatory effects, with highest maximal effects observed at only 7%. ArEA had an EC_{50} value of 5.8 μM, while NEA requiring only 1.3 μM to produce half of the maximal effect, indicating a significant effect of 2-PG on Kirs. The maximal effect of LEA was very small, around 3.1%. On the other hand, NEA and OEA showed negative maximal effects.

Moreover, numerous studies corroborate our findings, indicating that Kir7.1 lacks pronounced rectification characteristics. Evidence suggests that Kir7.1 exhibits minimal sensitivity to variations in external K^+ concentration, conducting a minute K^+ current (Döring et al., 1998); These conclusions align with our own results.

Another factor that can impact the weak rectification of Kir7.1, leading to unusual and unique K+ permeation, is the pore region of Kir7.1. Adjacent to the conserved G-Y-G motif in all Kirs, there are two other residues in Kir7.1 that make them more similar to Kv channels than other Kirs. In the pore region of Kir7.1 at position 125 (or +2 after GYG), there is a methionine, while other Kir channels have an arginine at this position. Additionally, there is a proline at position +4 after G-Y-G, which is absent in other Kirs but present in all Kv channels. It has been suggested that this position plays a critical role in K^+ permeability (Döring et al., 1998). These key factors also can lead to the weak rectification of Kir7.1 channels compared to other Kirs.

Distinct specificities of Kir channels are mostly due to the different binding pockets which are at the end of the slide helix, and it contains basic amino acids for each subunit of Kir channels. These findings suggest that different patterns of phospholipid binding lead to different phospholipid specificities, resulting in specific interactions that are crucial for the activation or inhibition of Kir channels by these phospholipids (Fürst, Mondou, et al., 2014). A crystallization study of PIP_2 has proven that TMD and CTD are fully involved in the membrane slide helix interaction (Hansen et al., 2011). It has been reported that the complexity of anionic phospholipids, such as PIP2, is dependent on Kir channels. This implies that different levels of PIP2 are required to activate the Kir channels. These lipids uniformly affect all Kirs channels in the same manner, but they also significantly change the function in a different way even within a subfamily (Logothetis et al., 2015). This current research presents evidence of the complex effects of a subset of endocannabinoids, which have demonstrated different effects on different members of Kir channels. This emphasizes the need for further investigation to understand the mechanisms and binding site interactions between endocannabinoids and Kir channels.

4.4 Limitations:

While our presented research sheds light on the regulatory effects of endocannabinoids on Kir channels, specifically Kir2.1 and its mutations, Kir4.1, and Kir7.1 channels, it is necessary to acknowledge the most obvious limitations that arise to consideration—firstly, using Two-Electrode Voltage-Clamp (TEVC) technique as an electrophysiological method for functional assessments. One of our limitations in the present study is that using TEVC does not enable us to translate these findings into the pharmacological environment, such as in vivo.

In addition, we did not have an adequate sample size for some of the tests, which could affect variation in our results.

Furthermore, higher extracellular K^+ concentrations in our experiments made the tests more sensitive. While this allowed us to see the potential effects of endocannabinoids in a more negative possible range, it might not entirely reflect the conditions these Kir channels experience in the body. Despite this, our study emphasizes the need for more research in different physiological situations and provides valuable insights into how endocannabinoids influence Kir channels. We additionally

acknowledge the possibility for further investigation, including studying different Kir channel subtypes, including more mutations connected to Kir4.1 and Kir7.1. Future research may expand on these limitations to fill in the gaps and improve our comprehension of the intricate regulatory functions of endocannabinoids on numerous Kir channels.

4.5 Future Direction:

4.5.1 Short term

i. Other family of Kir channels

Kir channels are potential drug targets for treating various medical conditions, such as hypertension, cardiac arrhythmias, and neurological diseases (Weaver & Denton, 2021). In addition, there is an association between mutations in Kir channel genes and certain genetic disorders, including Bartter's syndrome , Andersen-Tawil syndrome, EAST/SeSAME syndrome, Cantu syndrome and congenital hyperinsulinism (Denton & Delpire, 2023). On the other hand, endocannabinoids as endogenous lipid components contribute to several physiological processes, including pain modulation, appetite regulation, mood, and inflammation (Atakan, 2012). Studying how these endocannabinoids regulate the function of Kir channel family could give us a better understanding of their complex role in targeting various pathogenic signals. However, Kir 2.1 and also Kir4.1 are very well-known in terms of function due to their different physiological role in the cardiac and central nervous systems, respectively (Hager et al., 2022; Ohno et al., 2021). We still recommend an investigation of other subunits of these families, such as 2. 2, 2. 3, 2. 4, 2. 6 and Kir4. 2. Regardless of their effect, which may or may not show the same sensitivity due to their complex interaction with endocannabinoids, this investigation could provide a deep understanding between endocannabinoids and different members of Kirs. We also recommend examining the interaction of other subsets of endocannabinoids on different Kir family members. It is necessary to emphasize the essential mutations related to these Kir channels. For instance, the Kir6. 2 (KCNJ11) mutation is one of the critical mutations linked to congenital hyperinsulinism. This rare genetic disorder causes low blood sugar levels in infants and children (Loechner et al., 2011). Additionally, according to our findings we have seen a significant effect of some of the endocannabinoids on Kir4. 1, we also advise looking

at this effect on Kir4. 1 mutations which are associated with various neurological disorders, including seizures, would be valuable.

In conclusion, further examination of the broader subsets of the endocannabinoids on different isoforms of the Kir channels and important related mutations gives us a deeper comprehensive function of their interaction. It can help us set other pharmacological goals to help patients in near future.

4.5.2 Long term

i. Patch-clamp electrophysiology

There are several essential advantages in studying ion channels; we can examine the Kir channel's function using the patch clamp technique instead of the TEVC. The patch clamp allows us to record Kir channels at boosted resolution, one of the most notable advantages. The above technique easily simplifies the examination of single-cell behaviour. Furthermore, it enables us to use several configurations, including whole-cell and cell-attached. Ion channel activity can be easily monitored while the integrity of the cell membrane uses cell-attached patching. Also, the whole-cell configuration could give us data regarding detailed intracellular signalling.

Last but not least, it has been seen as a valuable approach for drug screening and discovery, helping us measure accurate current. A patch clamp enables us to investigate how various endocannabinoids affect Kir channel activation. As a result, research employing patch clam enables us to assess the potential of certain endocannabinoids proposed by this present study. Thus, we recommend precise electrophysiological recordings better to understand the impact of screened endocannabinoids on Kir currents. Here is a summary of the methodology: It involves the transfection of Kir2. 1 cDNA-containing plasmid into an external expression system like CHO-K1 or HEK293 cells, which have minimal inherent ion channel expression and do not naturally exhibit endocannabinoid receptors. These cells are commonly used to study various cardiac and neurological diseases and can be utilized to investigate I_{K1}'s current components through a patch-clamp electrophysiological technique. The effects of screened endocannabinoids on endogenous potassium currents in HEK293 cells can be examined using the whole-cell version of the voltage-clamp technique. The bath solution will have a composition of NaCl 137 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, D-glucose 10 mM,

HEPES 10 mM, and pH (NaOH) 7.40, while the pipette solution will contain KCl 130 mM, $MgCl_2$ 1 mM, MgATP 5 mM, HEPES 10 mM, EGTA 5 mM, and pH (KOH) 7.20. Kir2.1 currents will be measured by applying voltage ramps between -120 mV and +60 mV, with a holding potential of -60 mV. Cells will continuously be perfused with a mixture of extracellular solution and screened endocannabinoids or incubated with the selected endocannabinoid before recording.

ii. Structural study

The mechanism of action of endocannabinoids with the channels within the membrane remains unclear. Although several structural studies have identified the binding interaction site of Kir2.1 and PIP2, further research is needed to fully understand the mechanisms of activation of the Kir channels (Cukras et al., 2002; Pegan et al., 2005; Rohács et al., 2003). These studies have shown that there are some positively charged residues, including R67, R82, H53, K182, K185, K187, K188, R189, R218, K219, K228, and R312 in the C- and N-terminals of Kir channels that play a very important role in the activation of Kir-PIP_2 (Huang et al., 1998; Rohács et al., 1999). Most of the Kir channels are sensitive to polyamines, spermine, and spermidine, with micromolar concentrations underlying the molecular mechanism of Kir channels. Specific residues (e.g. Kir2.1 E224, F254, E299, D255 and, D259, D172) from the transmembrane pore domain are targeted by polyamines (De Boer et al., 2010), with an exact mechanisms recognized for Mg^{2+} but with weaker potency. The exact interaction mechanism between Kir2.1 channels and active endocannabinoids is still unknown. To gain a better understanding of how these endocannabinoids activate Kir channels, it is suggested that a combination of computational and structural approaches, along with electrophysiological and biochemical methods, is necessary. These approaches will help assess the potential binding sites of endocannabinoids to Kir2.1 channels and identify the location and structure of these binding sites. It is likely that specific residues within Kir2.1 interact with these endogenous lipids, leading to the regulatory effects observed. Further studies involving structural analysis, biochemical experiments, and mutagenesis studies are needed to investigate the binding site residues and unravel the specific mechanisms through which endocannabinoids interact with Kir2.1 channels. For instance, there are several techniques that can be used to analyze the protein-protein interactions between Kir channels and endocannabinoids, such as Western blot, binding assays like ELISA or radioactivity, crosslinking, and crystallization. These methods can offer significant insights into the binding mechanisms and potential functional implications of these interactions. Our findings that Kir2.1 is

directly regulated by endocannabinoids may have broader implications on the function of cardiac, neuronal, and immune cells.

iii. Computational studies

In the future, one possible approach to studying how endocannabinoids directly regulate Kir channels (Kir2.1, Kir4.1) could involve using molecular dynamics simulations. By examining the atomic-level interaction between endocannabinoids and Kirs channels and assessing its impact on channel function, we could potentially identify binding sites for endocannabinoids on Kirs (Kir2.1, Kir4.1) channels and determine how binding affects channel gating and ion selectivity.

Another method for investigating endocannabinoid modulation of Kir channels could involve virtual screening, in which an extensive database of endocannabinoid compounds is screened against a Kirs channel model to identify compounds that can modulate the activity of the channel. Promising compounds could then be further validated through experimental testing to determine their efficacy. Finally, machine learning algorithms could be used to develop predictive models for Kir channel activity and regulation by endocannabinoids based on the channel's structural and sequence information.

4.6 Conclusion

The critical endocannabinoid system's role in our body is undeniable. However, the evidence has shown that the endocannabinoid system's regulatory effect on cardiovascular function is not strong.

However, we are reporting the potential promising effects of selected endogenous lipids on Kir2.1 channels on oocytes; concerning all differences, it can apply in the striated muscles of atrial and ventricular heart cells, where these channels are highly expressed. We believe that this perspective can help us to understand the connection between the endocannabinoid system and heart function more effectively.

Kir 2.1 channels regulate the resting membrane potential and contraction of cardiac and smooth muscle cells by generating the current I_{K1}. Conversely, endocannabinoids have distinctive heart effects, such as heart rate changes, blood pressure, and contractility. The relaxation of coronary and

other arteries is aided by endocannabinoids, which significantly reduce the cardiac workload. However, they do not play a role in regulating cardiovascular function in a consistent manner. These effects are modulated through the interaction of endocannabinoids with several receptors, including CBRs, TRPV1, PPARs and other ion channels expressed in the heart. Our results suggest that the direct interaction of endocannabinoids with Kir2.1 channels could contribute to cardiac function as a promising treatment for cardiac syndromes such as ATS/ LQT7.

In our study, we used a subset of the endocannabinoids from two classes of endocannabinoids, fatty acid ethanolamides (FAEs) and 2-monoacylglycerols (2-MGs), to study the regulatory effects on Kir2.1 and its mutation (G144S and V302M). We used an electrophysiological technique called a two-electrode voltage clamp (TEVC) on Xenopus oocytes. By incrementally adding some of the endocannabinoids, we observed a significant increase in the K^+ current in both wild-type and Kir2.1 mutation channels. Among these endocannabinoids, NEA and 2- induced the largest increase in Kir2.1 function, while 2-PG showed a maximal increase in the current in Kir2.1 mutations (V302M). Our findings confirm that the direct regulation of endocannabinoids is not only conserved in Kir2.1 channels but also in Kir4.1 channels, where some selected endogenous lipids increase the K^+ current significantly more than Kir2.1. However, the inward current of Kir7.1 channels did not change with the micromolar concentration of several endocannabinoids. This finding could have broad implications in the fields of neurology and cardiology, particularly in the development of drugs for treating Andersen-Tawil syndrome (ATS). ATS is caused by loss-of-function mutations in the Kir2.1 channel, and our findings suggest that targeting the Kir2.1 mutations by endocannabinoids could potentially be used to treat this condition.

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