THE RESPONSE OF SYNTHETIC 4-HYDROXYBENZOIC ACID ON Kv1.4 POTASSIUM CHANNEL SUBUNIT EXPRESSED IN *Xenopus laevis* OOCYTES

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UNIVERSITI SAINS MALAYSIA

2016

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By

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Thesis submitted in partial fulfillment of the requirement

for the degree of

Master of Neuroscience

JUNE 2016

TINDAK BALAS SINTETIK 4-HYDROXYBENZOIK ASID KE ATAS SALURAN KALIUM Kv1.4 YANG DI EKSPRESIKAN KE DALAM OOSIT *Xenopus laevis*.

oleh

FATIN HILYANI MOHAMAD

Tesis diserahkan untuk memenuhi sebahagian keperluan bagi

Ijazah Sarjana Neurosains

JUN 2016

ACKNOWLEDGEMENT

For,

All lecturers and supervisors; Dr Jingli Zhang, Prof. Dato' Dr Jafri Malin Abdullah, Assoc. Prof. Dr Muzaimi Mustapha, Prof. Dr Shaharum Shamsuddin (PPSK) and Dr Wan Amir Nizam Wan Ahmad (PPSK).

Special thanks to Prof. Dr Robert Bahring (University of Hamburg, Germany) who kindly donated DNA of Kv1.4 potassium channel for the DAAD Electrophysiology Workshop in Universiti Sains Malaysia (USM) in June 2009 and gave permission to subsequently use it for the Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia for future experiments.

Friends whom I owe much gratitude and respect; Jia Hui Wong, Mazira Ghazali (Neuro Dept), Sui Mei Kee, Nuraza Othman (Neuro Lab) and all Integrated Neuroscience (INP) and Neuroscience Department family,

Mohamad and Wan Naimah, the parent who have no idea what I am doing, but is still proud of me anyway,

Thank you,

Fatin H. Mohamad,
(11th July, 2016)

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LIST OF ABBREVIATIONS AND ACRONYMS

3-Hba	3-hydroxybenzoic acid
3,4-DHba	3,4-dihydroxybenzoic acid
4-AP	4 – aminopyridine
4-Hba	4-hydroxybenzoic acid
AEDs	Anti epileptic drugs
BADs	Benzoic acid Derivatives
Ca^{2+}	Calcium ion
cDNA	Competence DNA
Cl^-	Chloride ion
cRNA	Competence RNA
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GA	Gallic acid
IC50	Inhibitory concentration (50%)
$[K]_o$	Extracellular potassium ion concentration
K^+	Potassium ion
Kv	Voltage-gated potassium channel
mM	mili molar
ms	mili second
mV	mili volt
Na^+	Sodium ion
ND96	Frog's Ringer or buffer solution

OR-2	Buffer solution to wash away oocytes follicles
RNA	Ribonucleic acid
RPM	Rotation per minute
SA	Salicylic acid
TEA	Tetraethylammonium
TEVC	Two-electrode voltage clamp
µA	micro ampere
µM	micro molar
X.laevis	*Xenopus laevis*

TINDAK BALAS SINTETIK 4-HYDROXYBENZOIK ASID KE ATAS SALURAN KALIUM Kv1.4 YANG DI EKSPRESIKAN KE DALAM OOSIT *Xenopus laevis*

ABSTRAK

Kajian yang mendalam sedang dijalankan ke atas produk semulajadi terutamanya pokok-pokok herba yang telah lama dipraktikkan di dalam perubatan tradisional seperti Cina dan Ayurveda untuk merawat penyakit saraf seperti sawan dan sakit kepala. 4-hydroxybenzoik asid adalah fenol tidak flavonoid yang boleh ditemui dari pucuk *Dendrocalamus asper* (buluh), buah-buahan (strawberi dan epal) dan bunga-bungaan. Di dalam kajian ini, tindak balas 4-hydroxybenzoik asid diuji ke atas saluran kalium Kv1.4 yang telah diekspresikan ke dalam oosit *Xenopus laevis* sebagai model sistem. Kv1.4 adalah saluran kalium dari keluarga *Shaker* yang pantas dinyahaktifkan melalui dua mekanisma; jenis N yang pantas dan jenis C yang perlahan. Ianya memainkan peranan penting dalam repolarisasi, hyperpolarisasi dan mengembalikan potensi membran melalui pengawalan pergerakan K^+ menyeberangi luar membran sel. cRNA Kv1.4 yang telah disediakan dalam kerja molecular disuntik ke dalam oosit sihat yang telah diambil melalui pembedahan *X.laevis* di bahagian abdomen bawah. Arus dihasilkan daripada K ions dikesan oleh voltan apitan dua-elektrod-mikro (TEVC), dengan potensi kawalan dari -80mV dan peningkatan 20mV sehingga +80mV. Bacaaan dari rawatan oleh 0.1% DMSO, konsentrasi 4-Hba dan penghalang saluran kalium diambil pada +60mV. Analisis dijalankan menggunakan perisian pClamp diikuti t-test pelajar. Nisbah amplitud

akhir / puncak adalah merupakan indeks aktiviti saluran Kv1.4 dengan $n \geq 6$ (bilangan oosit yang diuiji). Nisbah yang rendah menunjukkan potensi membran yang rendah (repolarisasi) dan penambahan nyahaktif saluran Kv1.4. Pengurangan nisbah dari 5 konsentrasi yang berbeza (1µM, 10µM, 100µM, 1mM dan 2.5mM) dibandingkan dengan 0.1% DMSO sebagai kawalan. Kesemua konsentrasi menunjukkan keputusan signifikasi statistik dengan $p < 0.05$ kecuali untuk 100µM. Peningkatan arus konsentrasi yang dinormalisasikan melalui perbandingan dengan penghalang saluran kalium (TEA dan 4-AP) menunjukkan signifikasi statistik bagi kesemua konsentrasi. Kajian ini juga menunjukkan tempoh masa yang diambil oleh setiap konsentrasi untuk mempengaruhi nyahaktiviti Kv1.4 didapati tidak memainkan apa-apa peranan penting. Kesimpulannya, 4-hydroxybenzoik asid dikenal pasti dapat menambah baik kesan nyahaktiviti Kv1.4 dalam mengurangkan atau merepolarisasikan potensi membran supaya ledakan saraf yang tidak normal dapat dihalang. Ini dapat dilihat melalui perbandingan dengan DMSO dan juga penghalang saluran kalium. IC50 didapati sedikit tinggi dari 10µM dan konsentrasi yang lebih tinggi (100µM, 1mM dan 2.5mM) menujukkan kesan sampingan toksik. Oleh itu, konsentrasi yang terbaik dari kajian ini adalah 10µM dengan curaman Hill (*slope*) 0.1799.

THE RESPONSE OF SYNTHETIC 4-HYDROXYBENZOIC ACID ON Kv1.4 POTASSIUM CHANNEL SUBUNIT EXPRESSED IN *Xenopus laevis* OOCYTES

ABSTRACT

Extensive researches are being made on natural products especially herbs and plants that have long been practiced in traditional medicines such as Chinese and Ayurvedic that have been used to treat neuronal disorders such as convulsive, dizziness and headaches. 4-hydroxybenzoic acid is a non-flavonoid phenol found abundantly in *Dendrocalamus asper* shoots (bamboo), fruits (strawberries and apples) and flowers. In this study, the response of synthetic 4-hydroxybenzoic acid was tested on Kv1.4 potassium channel that was expressed in *Xenopus laevis* oocytes as the model system. Kv1.4 is a rapidly inactivating *Shaker*-related member of the voltage-gated potassium channels with two inactivation mechanisms; the fast N-type and slow C-type. It plays vital roles in repolarization, hyperpolarization and signaling the restoration of resting membrane potential through the regulation of the movement of K^+ across the cellular membrane. cRNA of Kv1.4 prepared during molecular work was injected into viable oocytes that was extracted through surgery at the lower abdomen of *X.laevis*. The current produced from K ions were detected by the two-microelectrode voltage clamp (TEVC) method, holding potential starting from -80mV with 20mV step-up until +80mV. Readings of treatments with 0.1% DMSO, 4-Hba concentrations and K channel blockers were taken at +60mV. The ratio of tail / peak amplitude is the index of the activity of the Kv1.4 channels with n

≥ 6 (number of oocytes tested). Lower ratio signifies lower membrane potential and enhancement of Kv1.4 channel inactivation. The decreases of the ratios of 5 different concentrations (1µM, 10µM, 100µM, 1mM and 2.5mM) were compared with 0.1% DMSO as the control. All concentration showed statistically significant results with $p < 0.05$ except for 100µM. The normalized current of the 4-hba concentrations were compared with potassium channel blockers (TEA and 4-AP) and all groups showed statistically significant results. This study also showed that time taken for each concentration to affect Kv1.4 does not play any significant roles. In conclusion, 4-hydroxybenzoic acid was found to be able to enhance the inactivation of Kv1.4 by lowering the membrane potential so that the abnormal neuronal firing can be inhibited which can be seen through comparison of DMSO and potassium channel blockers. With IC50 slightly higher than 10µM, increasing concentrations (100µM, 1mM and 2.5mM) had shown to exhibit toxicity effects. The best concentration from this study is 10µM with Hill slope of 0.1799.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Following the regulation of membrane potential by the movements of inhibitory ions such as K^+ and Cl^- and excitatory ions such as Na^+ and Ca^{2+}, depolarization or more positive membrane potential could results in neuronal firing whereas repolarization and hyperpolarization or more negative membrane potential contribute to neuronal inhibition (Purves, et al. 2012). Any irregularities or disruption to this mechanism would interfere with the normal neuron action and inactivation which could result in many neuronal disorders.

Based on previous studies and researches, potassium ions have been identified as the most diverse ion channels that can be found in almost every part of the human body encoded by more than 70 genes classified into 12 subfamilies (Kv1 – 12) (D'Adamo, et al. 2013). Nevertheless, limited studies have been done on A-type transient current producing with double inactivation mechanisms (N-type and C-type), Kv1.4 channel (Jefferys, J.G.R. 2010; Chen H. et al. 2013; Oliva, et al. 2005). Kv1.4 channel can be found abundantly in *Schaffer* collateral axons and part of the molecular layer

of the dentate gyrus. It also formed heteromerization with Kv1.1 and Kvβ1.1 subunits in the mossy fiber boutons that synapse with the pyramidal neurons in CA3. Mutations from this heteromeric formation can contribute to the episodic ataxia type 1 (EA1) seizure (D'Adamo, et al. 2013).

Kv1.4 is responsible in regulating the amplitude of back-propagating action potentials of the neuron through its double inactivation mechanisms which could result in non-conductance of K^+ and repolarization (Rasmusson, et al. 1998). Therefore, the enhancement Kv1.4 inactivation will help to overcome the abnormal high frequency of action potential which underlies many neuronal disorders such as convulsive, fits, epilepsy and even stroke (Wulff, et al. 2009). The double inactivations are hypothesized to occur through the permeation and allosteric mechanisms which are influenced by many factors such as pH, oxidation and extracellular [K] (Claydon, et al. 2004; Xu, et al. 2001).

In this study, the efficacy of synthetic 4-Hydroxybenzoic acid (4-Hba) in promoting and enhancing potassium Kv1.4 channel inactivation was tested by expressing the channel via cRNA injection in the model system of *Xenopus laevis* oocytes. 4-Hba is a non-flavonoid phenolic compound (Khadem and Marles, 2010) that can be found in many natural products such as *Dendrocalamus asper* and *Veronica peregrina* L. (Kim, et al. 2014). Extraction of *Dendrocalamus asper* shoots by our collaborator Universiti Malaysia Terengganu (UMT), found abundance of 4-hydroxybenzaldehyde.

However, as the compound is in excess of valence electrons for bonding, it can be easily oxidized to 4-hydroxybenzoic acid (Dobhal, et al. 2010) which is a more stable structure. In addition, based on previous study by Bilal (2015), preliminary screening of the effects of 4-hba, palmitic acid and lauric acid found that 4-hba can enhance the inhibitory current of GABA (A) channels which were expressed in *X.laevis* oocytes unlike palmitic and lauric acids. Nevertheless, the effect of this compound on Kv1.4 channels which also helps to lower the membrane potential has never been tested, making it as the objective of this study.

Every cell functions and regulates itself by the movement of ions, proteins and molecules across the cellular membrane that separates the intracellular from the extracellular environment. These movements of ions with charges across a barrier lead to electrical potential difference or membrane potential that can be detected by electrodes. This is called the electrophysiology study techniques (Bierwirtz and Schwarz, 2014). Two-electrode voltage clamp (TEVC) technique used in this experiment allows the measurement of ions flow across the oocytes membrane by injecting two microelectrodes, one for detecting the voltage and another for current injection. The voltage is clamped at -80mV and any current and membrane potential changes read by the electrodes are compared to calculate the differences which can vary due to additional Kv1.4 expression of cRNA in the oocytes.

The extraction of *Xenopus laevis* oocytes were carried out by surgery. Incision less than 1cm were made on the lower abdomen of the anesthetized selected frog and all the lobes of oocytes were pulled out using forceps. A normal female frog usually has more than 5 lobes of oocytes that produce hundreds of them. The incised muscle and skin were sutured again so that the frog can live for another oocytes extraction on the opposite side of the abdomen if necessary (at least 3 months apart). These oocytes will be individually screened and selected for bigger oocytes with clear separation of yellowish 'vegetal' and dark brown 'animal' poles (Sigel and Minier, 2005). Then, the oocytes were incubated for a night before cRNA injection of the Kv1.4 using the micropipette can be done.

The voltage reading of the injected oocytes under 0.1% DMSO was compared with five different concentrations of 4-Hba (1µM, 10µM, 100µM, 1mM and 2.5mM). At least 6 different viable oocytes were used for each concentration. The solutions were controlled by the perfusion system whilst the oocytes were impaled by the double electrodes. The voltage is hold at -80mV, with step-up of 20mV until +80mV. Recordings were taken at +60mV which is the potential at which potassium channel is activated. The oocytes are bathed in the neutral ND96 (1 min) before 0.1% DMSO (1 min) followed by 4-Hba (5 mins) and finally the potassium channel blockers (12.5 mM TEA and 5mM 4-AP) (5 mins). In total, there were 12 voltage readings taken for every voltage-clamping of each oocytes.

In conclusion, this study investigates the response of 4-Hba effect on Kv1.4 potassium channels inactivations which are responsible in controlling the repolarization and restoring the resting membrane potential of the neurons. These channels are expressed in *Xenopus laevis* oocytes that act as model system so that the electrophysiological changes elicited can be read by the two-electrode voltage clamp technique. Lowering of membrane potential shows enhancement action of 4-Hba on the inactivation mechanisms of Kv1.4, resulting in prolonged repolarization which is the targeted action to overcome abnormal continuous neuronal firing.

1.2 Rationale of Study

In general, this study aims to investigate the response of synthetic 4-Hba which can be found abundantly in natural products. This is to find additional and new potential compounds that are able to enhance repolarization of membrane potential as a targeted mechanism for abnormal continuous neuronal firing (convulsant, fits) treatment through natural resources such as herbs and plants that are less toxic and with lower prolong effects. Although there are many previous studies investigating potential compounds that can enhance repolarization, there have been none on 4-Hba based on our findings and researches. In addition, there are also fewer studies on the inactivation response of Kv1.4 channels against natural compound and its importance in affecting membrane potential and inhibiting the neuronal excitability. If this research provides positive answers, it could offer additional discovery on the

mechanism and importance of Kv1.4 channel along with proving the practicality of natural compounds in treating disorders and deregulations that cannot be answered by drugs.

1.3 EXPERIMENT GROUP

There are 4 channels filled with different solutions for every recording. The first and second channels are constant but the third and fourth channels are manipulated depending on the concentrations of 4-hydroxybenzoic acid groups.

1) First channel = 50ml ND96 solution
2) Second channel = 50µl DMSO + 50ml ND96
3) Third channel = experimental group (50µl of 1µM or 10µM or 100µM or 1mM or 2.5mM 4-hydroxybenzoic acid + 50ml ND96)
4) Fourth channel = negative control group (50µl of 1µM or 10µM or 100µM or 1mM or 2.5mM 4-hydroxybenzoic acid + 50µl 12.5mM TEA + 50µl 5mM 4-AP + 50ml ND96)

1.4 DATA COLLECTION

The current obtained from recordings were saved in the computer and analyzed with p-Clamp10 (Axon Instruments, USA) software and statistically tested with student t-test with SigmaPlot12 (Systat Inc, USA) software and Prism6 (GraphPad Software, USA).

CHAPTER 2

LITERATURE REVIEW

2.1 The Regulation of Membrane Potential

Movement of ions across cellular membrane creates potential difference or electrical gradient due to difference of ionic positive and negative charges between extracellular and intracellular membrane. This current-like potential difference is called membrane potential and it can be detected using microelectrodes reading. There are basically four important and influential ions that can regulate the membrane potential. These are the Na^+, K^+, Cl^- and Ca^{2+}. However, the firing, inhibition and resting of the neurons are majorly affected by the influx and efflux of Na^+ and K^+.

The membrane is at resting phase (-70 to - 60 mV) at (0) based on Fig. 2.1. During this phase, Na-K ATPase pump channel is opened allowing the influx of 2 potassium ions and efflux of 3 sodium ions across the cellular membrane. Therefore, the concentration of K^+ is higher inside and Na^+ is higher outside. According to Purves, et al. (2012), intracellular membrane potential is also aided by protein anions and is balanced by extracellular Cl^- concentration. When a signal or stimulus is received, it causes the opening of sodium channels, allowing the ions to enter the cellular membrane and causing the

membrane potential to be more positive resulting in the depolarization phase (1). Action potential is triggered when the depolarization overshoots and reaches the peak (2 and 3).

Overshooting also triggered the activation of potassium ion channels, which will allow the efflux of K^+ across cellular membrane into the extracellular environment. However, when the membrane potential reaches the peak (3), it signals the closing of the sodium ion channels. Continuous efflux of K^+ and blocking of Na^+ influx results in decreasing membrane potential (4) which will lead to repolarization. Repolarization is important in inhibiting the neuronal firing and initiating membrane permeability restoration. During this phase, the potassium ion channels start to become inactivated, causing the non-conduction of K^+. Prolonged inactivation and delayed closing of the potassium ion channel eventually lead to undershooting where the membrane potential becomes too low. This is the hyperpolarization stage (5). Ultimately the potassium ion channel will close and the movement of K^+ is blocked signaling for the restoration of resting membrane potential. In general, whilst Na^+ is mainly responsible in bringing the intracellular membrane potential towards positivity (or increasing the potential), Cl^- and K^+ are of the opposite (lowering membrane potential) (Purves, et al. 2012).

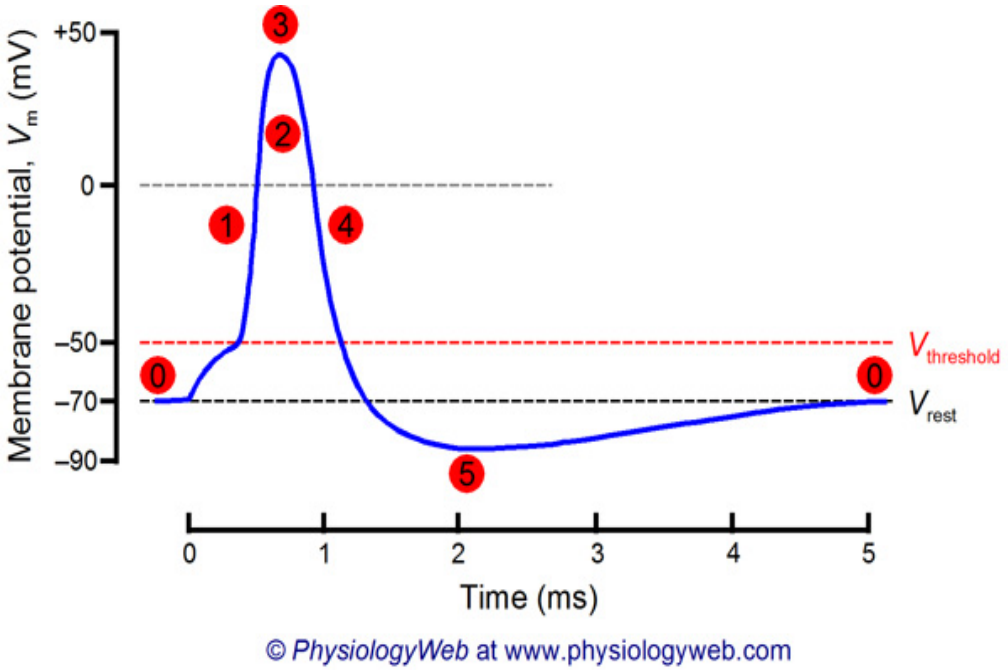


Figure 2.1: The changes of the membrane potential (mV) due to the movement of the ions across cellular membrane. Where 0 = resting membrane potential, 1 = depolarization, 2 = overshooting, 3 = peak, 4 = repolarization and 5 = undershooting or hyperpolarization. From: 'Neuronal Action Potential', Physiology Web, Accessed 6th June 2016 http://www.physiologyweb.com/lecture_notes/neuronal_action_potential/neuronal_action_potential.html

The regulation of membrane potential and the ion channels are extremely important in sustaining normal functioning cells and physiological systems. Abnormal and irregular control of the membrane potential has been revealed to be the main cause of many disorders and diseases such as convulsive, epilepsy, dizziness, depression and even stroke (Wulff, et al. 2009). These disorders occur due to abnormalities and mutations of the ionic channels and are usually referred as 'channelopathies'.

2.2 Voltage-Gated Potassium Channel

Since the past decades, researches have been targeting ion channels as treatment for many diseases and clinical abnormalities (Camargos, et al. 2011). One of these channels is potassium channels which can be classified into 3 structural families according to its amino acid sequence and pore-containing subunit; i) six transmembrane voltage-gated one-pore with S1 – S6 ii) two-transmembrane one-pore inward rectifier K^+ and iii) four transmembrane two-pore (Shieh, et al. 2000). Figure 2.2 shows the structural difference between these 3 potassium channels.

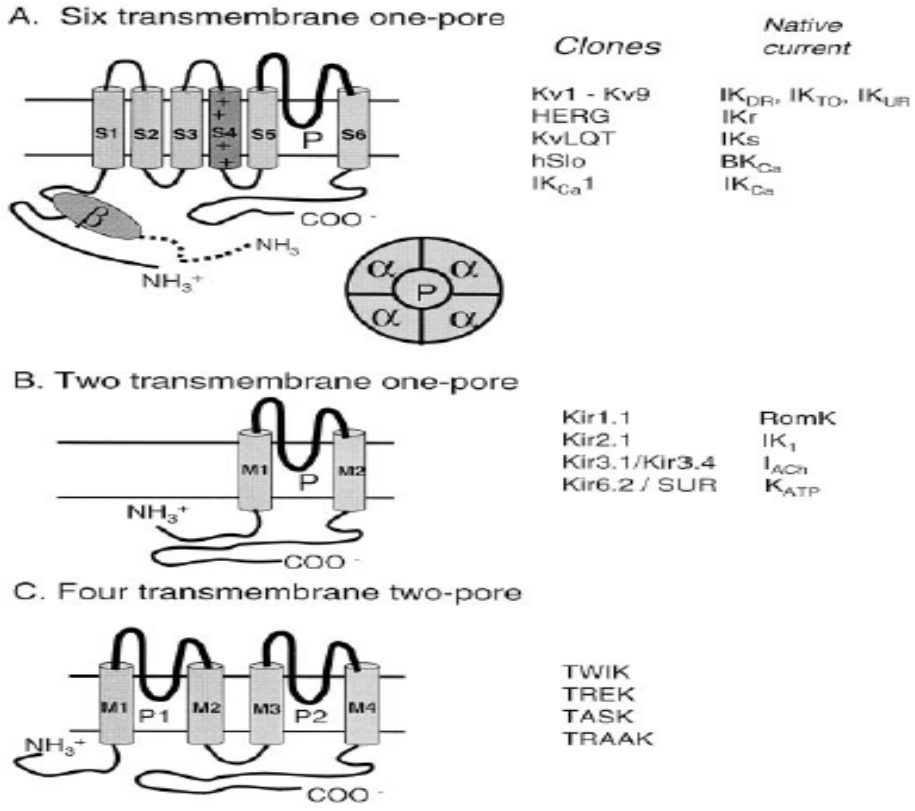


Figure 2.2: Representation of the 3 structural differences of K^+ channels based on the amino acids and pore subunits arrangements. The 6 transmembrane voltage-gated one pore channel (A) is the most abundant K^+ channels in the human body mostly found around active cells such as cardiac, skeletal and neurons. From: "Potassium Channels: Molecular Defects, Diseases and Therapeutic Opportunities" by Shieh, et al. (2000), *Pharmacological Reviews*, 52: 557 – 593.

Voltage dependant or voltage-gated potassium channels are vital in determining the resting membrane potential and membrane excitability of the neurons (Jugloff, et al. 2000). It also plays important roles in inhibiting action potential, potassium channel, neurotransmitter-mediated signaling, regulating Ca^{2+} homeostasis and cell survival (D'Adamo, et al. 2013).

More than 40 voltage-gated potassium channels have been identified and classified into 12 sub-families so far (Kv1 – Kv12). Most of these channels are found in almost every cells of the human body encoded by more than 70 genes which makes voltage-gated potassium channels as the most diverse ion channels. Kv channels appear as either homomeric or heteromeric within groups Kv1 (delayed-rectifier and A-current), Kv2 (delayed rectifier), Kv3 (high-voltage-activated, fast kinetics), Kv4 (somatodendritic A-current) and Kv7 (M-current). Kv1 – 4 was discovered in *Drosophila* and cloning identification of Kv1 *Shaker (Sh)* was followed by Kv2 *Shab (Sb)*, *Kv3 Shaw (Sw)* and Kv4 *Shal (Sl)* (Judge and Bever, 2006). Figure 2.3 shows the subunits of the Kv family with its IUPAC names and structural component.

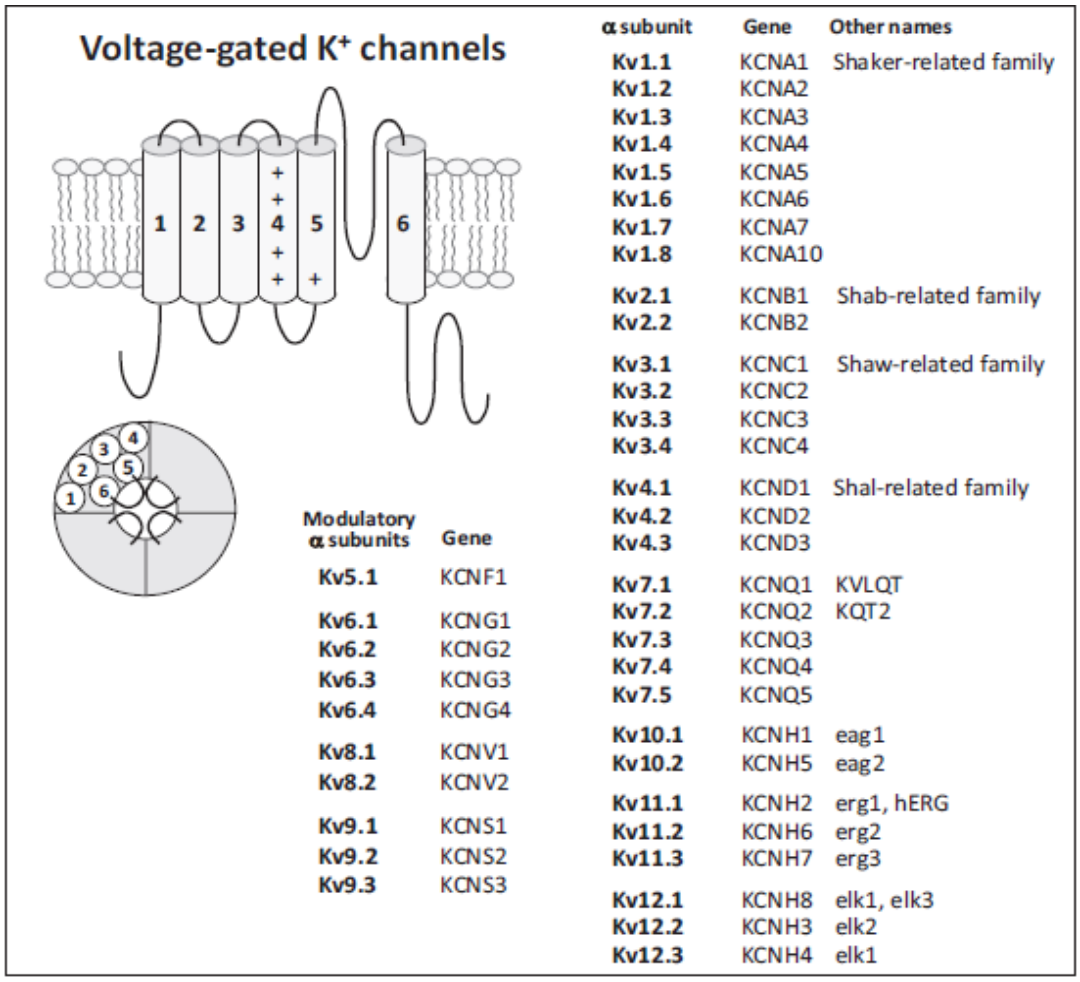


Figure 2.3: Structure of the 6 transmembrane of voltage-gated K^+ channels and its subunits according to International Union of Pharmacology. From: "Neuronal and Cardiovascular Potassium Channels as Therapeutic Drug Targets: Promise and Pitfalls" by Humphries and Dart (2015), *Journal of Biomolecular Screening*, 1 – 19, DOI: 10.1177/1087057115601677.

As mentioned by Shieh, et al. (2000), the ability of the channels to transport K^+ across the cellular membrane is dependent on 3 factors i) permeation pathway which allows K^+ to cross the cellular membrane ii) selectivity filter that recognize K^+ and iii) gating mechanism that changes between open and closed state. Hence, even a small structural difference between the channels can influence the regulation of ionic movement and transport.

According to Ping Li et al. (2013), there are 6 transmembrane segments of voltage-gated potassium or Kv channels that are grouped into voltage-sensor domain or VSD (S1 – S4), a pore domain (S5 – P – S6) and a re-entrant P loop region (Chanda and Bezanilla, 2008). Pore domain or the selectivity filter is responsible in gating or opening and closing the channels. These changes will be detected by VSD in response to membrane potential changes or K^+ permeability (Lee, J.H. et al. 2009). S4 is called the 'voltage sensor' which possesses positively charged arginine residues and is able to influence membrane voltage to exert forces on the gating of the pore (Mann, 2011). Thus, the opening of the pore is regulated by the movement of the voltage sensor by monitoring the ionic current flow (Rodriguez-Menchaca, et al. 2012). It has been reported in a few studies, X-ray structures of S1 – S4 voltage-sensing domains interact with lipids when embedded in the membrane and this helps to reset the sensor to its activated state after depolarization (Long, S.B., et al. 2007; Jiang, Y. et al. 2003; Milescu, M. et al. 2009; Ramu, Y. et al. 2006 and Xu, Y. et al. 2008). Abnormality within the regulation of activation and inactivation of voltage-gated potassium channel due to factors such as mutations could manifest into neuronal disorders.

As shown in Figure 2.4, increased action potential frequency can theoretically be corrected by enhancing K channels so that the positive peak amplitudes can be lowered through efflux of K^+. Prolonged depolarization or delayed repolarization phase causes higher membrane potential which can results in CNS hyperexcitability and also epilepsy. However, CNS depression and

cognition disorders could develop if the membrane potential is too low or prolonged repolarization occurs. This abnormality can be reduced by inhibiting the activation of K channels so that the efflux of K^+ can be prevented and the membrane potential can be increased.

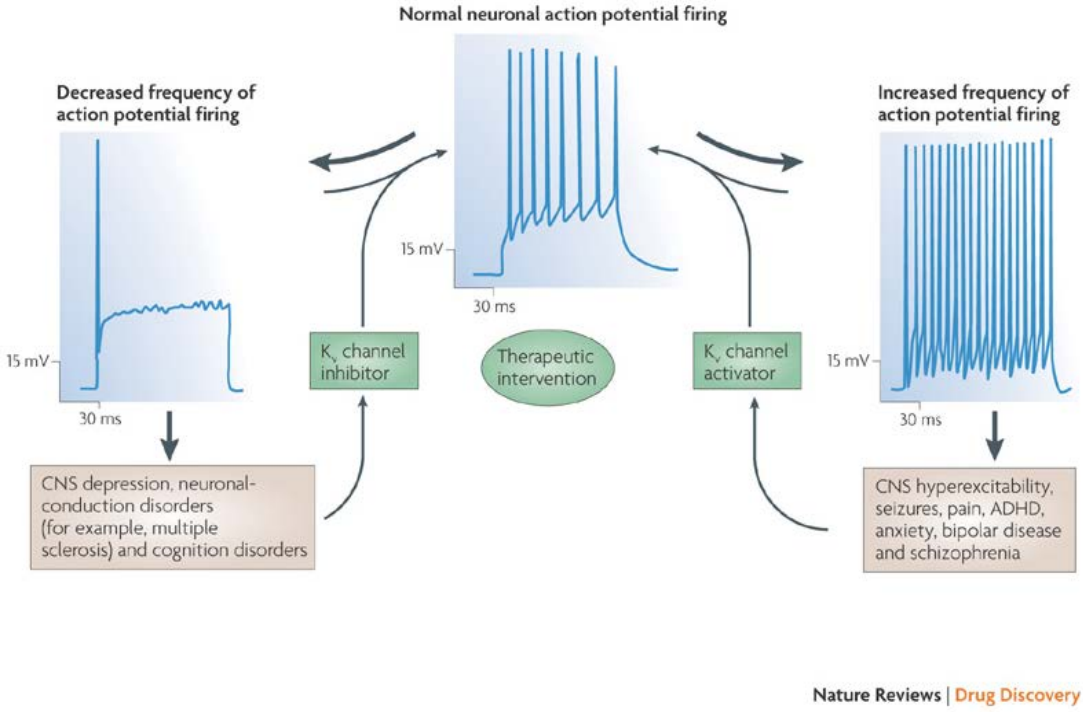


Figure 2.4: The comparison of normal neuronal action potential with depression and epileptic brain waves. These abnormalities are reversed by the action of K channel inhibitors and activators which help to stabilize the membrane potential suited to the disorders. From "Theoretical Effects of Kv Channel Inhibitors and Activators on Pathologically Altered Neuronal Activity" byWulff *et al.*, (2009), *Nature Reviews Drug Discovery*, 8, 982 – 1001, doi: 10.1038/nrd2983.

2.3 Kv1.4 Channel

There are currently a total of 12 Kv families with different anatomical distribution and roles. Each family has its own subfamilies which follows the abbreviations Kv*x.y* for easier identifications (D'Adamo, et al. 2013). According to Gutman, et al. (2005), Kv1 family is mostly distributed around brain, heart, pancreas and kidney. Even though Kv1.2 is the most abundant Kv1 channels around brain structures and anatomy, Kv1.4 exhibits more significant roles in repolarization and hyperpolarization. Table 2.1 explains the details and anatomical importance of Kv1.4.

Channel name	Kv1.4
Description	Voltage-gated potassium channel, A-type, fast-inactivating
Other names	HuK (II), hPCN2, HK1, RCK4, RHK1, RK4, RK8, MK4
Molecular information	Human: 653aa, NM_002233, chr. 11p14.3-15.2, KCNA4, GeneID:3739, PMID: 2263489 Mouse: 654aa, NM_021275, chr.2
Associated subunits	$K_v\beta$, PSD95, synapse-associated protein 97 (SAP97), SAP90, α-actinin-2, KChaP, σ receptor
Functional assays	Voltage-clamp
Current	$K_v1.4/K_v1.2$ heteromultimers may underlie the presynaptic A-type K^+ channel
Conductance	5pS
Ion selectivity	K^+ selective (50 times more selective for K^+ than Na)
Activation	Voltage, V_a = -22mV, -34mV, K_a = 5
Inactivation	N-type inactivation, V_h = -62 mV, τ_h = 47ms (0 mV)
Activators	CaMKII/calcineurin regulation through phosphorylation/dephosphorylation makes inactivation Ca^{2+} - dependent
Gating inhibitors	None
Blockers	4-Aminopyridine (13μM), tetraethylammonium (> 100mM), UK78282 (170 nM), riluzole (70μM), quinidine (10μM – 1mM), nicardipine (0.8μM)
Radioligands	None
Channel distribution	Brain (olfactory bulb, corpus striatum > hippocampus, superior and inferior colliculus > cerebral cortex, midbrain basal ganglia > pons/medulla), lung-carcinoid, skeletal muscle, heart, pancreatic islet
Physiological functions	Neuronal afterhyperpolarization
Mutations and	$K_V1.4$ expression increases in rat ventricular myocytes after

pathophysiology	myocardial infarction and induction of diabetes
Pharmacological significance	Not established
Comments	Can coassemble with other K_V1 family members in heteromultimers but not with members of other K_V families; intronless coding region; mouse $K_V1.4$ mRNA contains an internal ribosome entry site in its 5'-noncoding region and may be translated by cap-independent mechanisms, mammalian *Shaker*-related family.

Table 2.1: The nomenclature, molecular relationship and details of Kv1.4 channel as of 2005. From: Table 5 of 'International Union of Pharmacology. LIII. Nomenclature and Molecular Relationships of Voltage-Gated Potassium Channels' by Gutman, et al. (2005), *Pharmacological Reviews*, 57: 473 – 508.

Current produced by potassium channel is the I_A which allows action potential to reach dendrites (Jefferys, J.G.R. 2010). Generally there are two classes generated by Kv currents, the dominant sustained K-current ($I_{K,V}$) and the fast inactivating transient A-current ($I_{K,A}$) which is elicited by Kv1.4 (Chen, H. et al. 2013). It is estimated that the molecular weight of Kv1.4 is 73 211 and it shares similar membrane topology along with moderate amino acid sequences as Kv1.1. Its rapid inactivating characteristic influence the lowering of membrane potential after action potential and helps to halt the neuronal excitability faster compared to other channels (Figure 2.5). This channel also plays a crucial role in repolarization of cardiac myocytes along with Kv4.2 and Kv4.3 as the molecular bases (Rasmusson, et al. 1998).

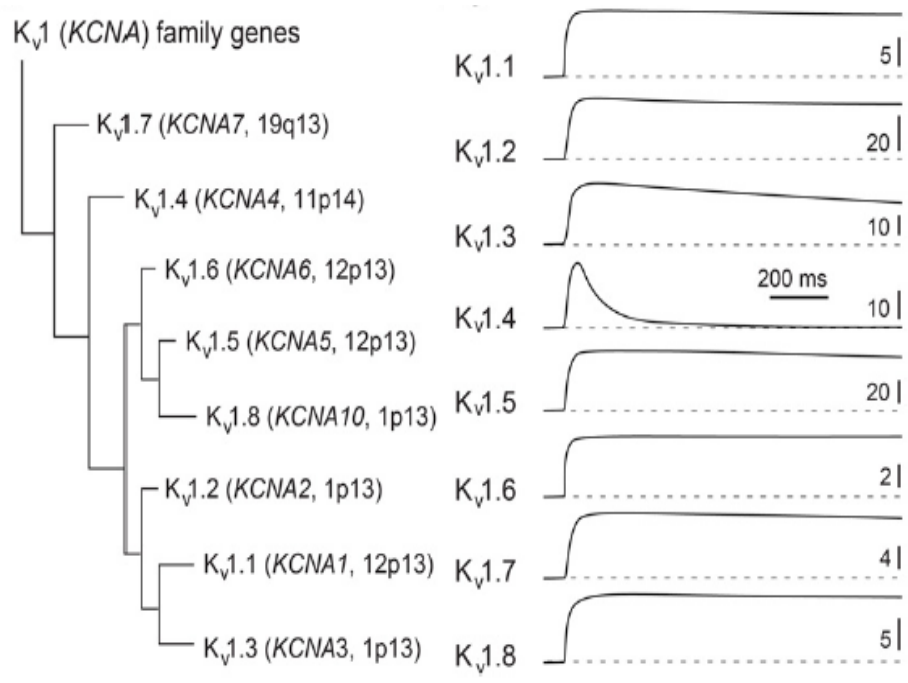


Figure 2.5: *Shaker*-related family of Kv1 channels. The phylogenetic tree of the gene family with IUPHAR and HGNC names shown with localization of the chromomes. Currents produced by each Kv1 families showing comparison of the rapid inactivation rate of Kv1.4 with others. Currents amplitudes in µA. Adapted from Heinemann, et al. 1996; Tian, et al. 2002; Finol-Urdaneta, et al. 2006. From: "Distinctive Role of Kv1.1 Subunit in the Biology and Functions of Low Threshold K^+ Channels with Implications for Neurological Disease" by Ovsepian, et al. (2016), *Pharmacology and Therapeutics*, 159, 93 – 101.

Kv1.4 plays major roles in many physiological processes including the quantal release of neurotransmitters, neuronal excitation, cardiac action potential, muscle contraction, hormonal secretion, transporting electrolytes for epithelial, cell volume and cell proliferation in neuronal and non-neuronal cells (Lee, J.H. et al. 2009). *Shaker* K channels are structurally designed with two types of inactivation; the fast N-type and slow C-type inactivation mechanisms (Oliva, et al. 2005; Gonzales-Perez, et al. 2008).

The N-type inactivation is rapid and characterized by the NH_2 terminal that blocks the current flow of the channel intracelullarly by the 'ball' linked to a 'chain' domain structure as proposed by Armstrong and Bezanilla in 1972 (Lee, J.H. et al. 2009). The exact molecular composition of the ball is roughly composed of 20 amino acids in the amino-terminal followed by 40 more residues which constitutes the chain (Cai, et al. 2007). The ball is made of hydrophobic residues and positive charges which is vital to push the ball towards the pore during depolarization thus, initiating inactivation. Binding of the 'ball and chain' to the pore is voltage insensitive and initiates occlusion of the permeation pathway (Figure 2.6A) and conformational changes (allosteric mechanism) (Figure 2.6B) (Bett and Rasmusson, 2004).

The permeation pathway occurs by blocking of the pore by the 'ball' during N-type inactivation and prevents the movement of K^+ across the cellular membrane (Figure 2.6A). Hence, direct effects of the fast inactivation can be seen immediately. The exact mechanisms of conformational changes are the focus of many ongoing studies involving K channels. It occurs due to the coupling of N-type to C-type. Unlike the N-type, the molecular and structural basis of C-type is still debatable. Yet, it is stipulated to involve the selectivity filter, extracellular conformational changes and permeant ions and intracellular pore closure (Bett and Rasmusson, 2004), intracellular quinidine binding (Wang, et al. 2003), intracellular osmotic pressure (Jiang, et al. 2003), mutations on the extracellular face of the mouth of the pore and mutations on the intracellular side of the pore (Figure2.6B). The inactivation of N-type also

contributes to the development of C-type inactivation. Even though N and C – types collaborate together during inactivation, the recovery from the slow C-type inactivation governs the availability of the channel for initiation of the next action potential (Chen, H. et al. 2013).

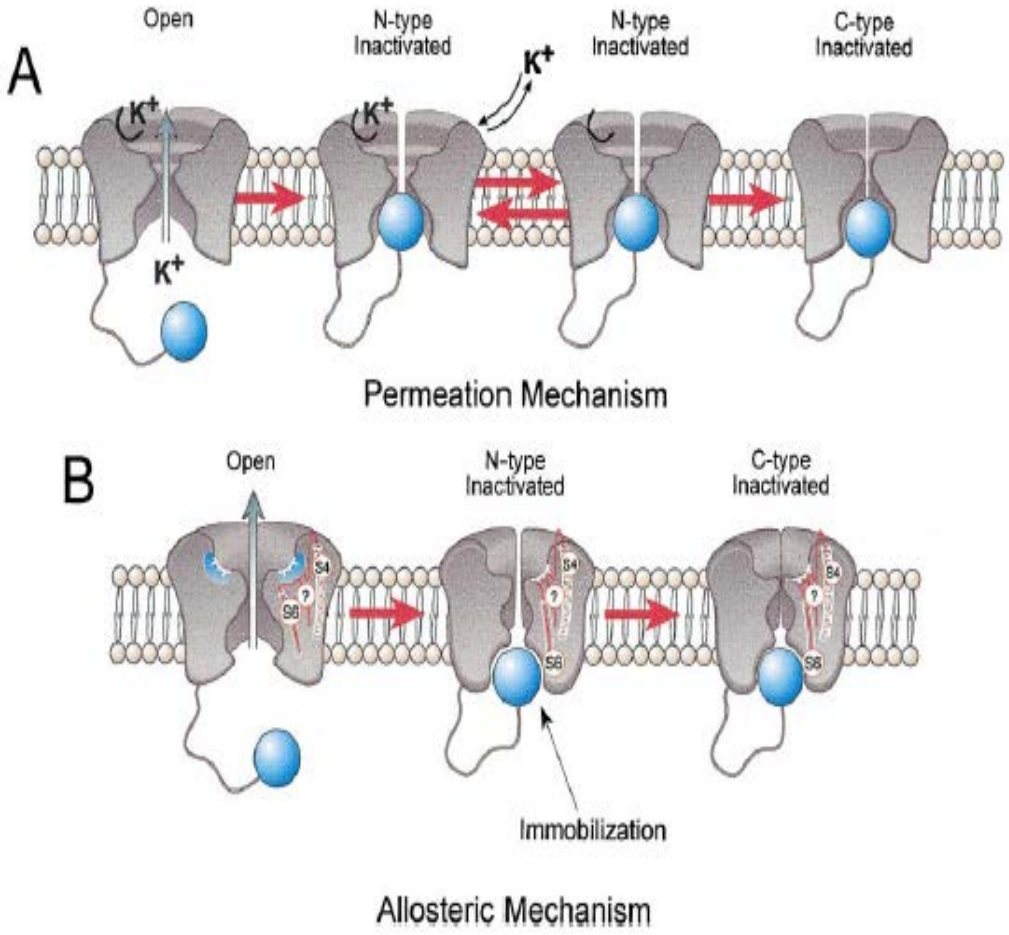


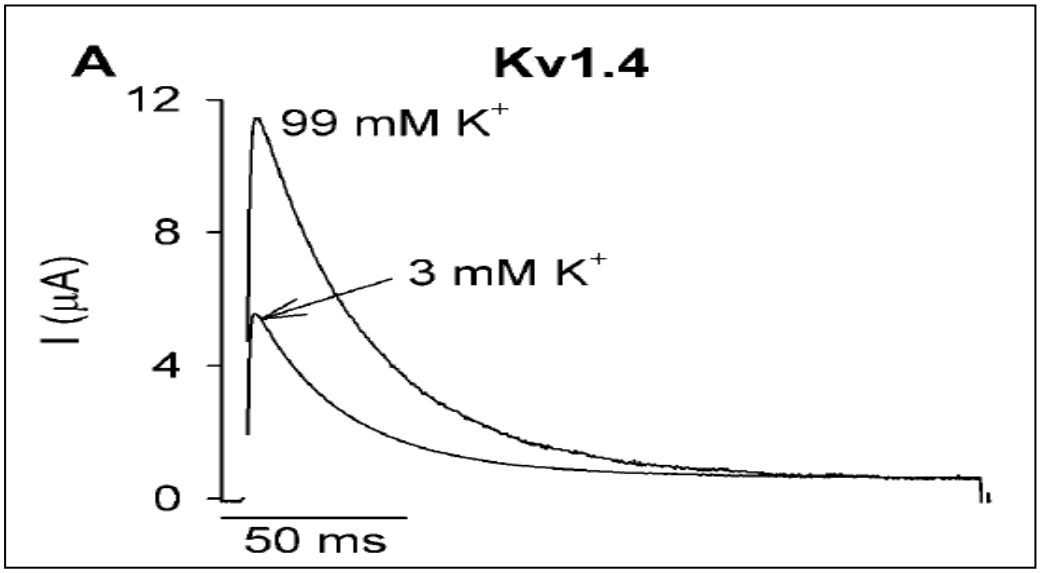
Figure 2.6: The inactivation mechanism of A-type K channels as hypothesized by two mechanism; the permeation and allosteric. A; the permeation mechanism allows the efflux of K^+, increasing the $[K]_o$ and occupying the selectivity filter. N-type inactivation prevents the outflow of K^+ decreasing $[K]_o$ resulting in collapse of the selectivity filter which develops the C-type inactivation. [Adapted from Baukrowitz and Yellen (1995)]. B; allosteric mechanism involves stabilization or immobilization conformation by binding of the N-terminal which is postulated to be associated with the S4 and S6 transmembrane domains and which helps to develop C-type inactivation. From: 'Inactivation of Voltage-gated Cardiac K^+ Channels' by Rasmusson, et al. (1998). *American Heart Association, Inc*.

The activation, inactivation and closing of K^+ channels are influenced and regulated by many factors. Activation of the channels allows conductance of K^+ across the cellular membrane, whether inward or outwardly rectifying (depending on the type of K^+ channels) and is mostly involved during subthreshold depolarization, whilst inactivation mostly occurs during depolarization and results in a state of opened channels but with no conductance of the K^+. Inactivation contributes to repolarization and hyperpolarization which also help in channels recovery (Bahring, et al. 2012). Closed channels blocked the channel gating preventing total flow of K^+ and initiates the restoration of resting membrane potential (Antz and Fakler, 1998).

In 1966, McAllister and Noble proved that extracellular K^+ concentration can activates potassium channel and increases the inwardly rectifying cardiac K^+ current. This effect has since been discovered to be applicable to almost all potassium channels (both inward and outward rectifying currents) (Baukrowitz and Yellen, 1995). Increased efflux of K^+ through the open channel results in accumulation of extracellular $[K]_o$ in the selectivity filter through a modulatory site, which enhances the activation of K channels and increasing the K current (Figure 2.7A). Rapid N-type inactivation causes the occlusion of the pore through the 'ball and chain' permeation mechanism preventing efflux of K^+ and empties the selectivity filter. The selectivity filter has been proven to collapse with low extracellular [K] which will signal the C-type inactivation. Thus, the inactivation of C-type is also modulated and initiated by the inactivation of N-type (Hoshi and Armstrong, 2013; Claydon, et al. 2004;

Lopez-Barneo, et al. 1993). The exact modulation of independent C-type inactivation is also hypothesized to involve S4 and S6 residue which will help in signaling the pore occlusion. The occurrence of double inactivations (N-type and C-type) result in prolonged repolarization and lowering of the membrane potential.

In a study carried by Claydon, et al. (2004) on the activation of Kv1.4 channels by extracellular charges, found that the channel activation and inactivation are also influenced by pH changes. It is postulated that acidic environment releases H^+ with positive charges which may interfere and compete with occupancy of K^+ on the selectivity filter. As the filter is low of K^+, it will collapse and thus signaling the development of C-type inactivation. Therefore, a lower pH or acidic environment enhances Kv1.4 inactivation and could also contribute to prolong repolarization (Figure2.7B). Similar study by Li, et al. (2002) also showed the same conclusions.



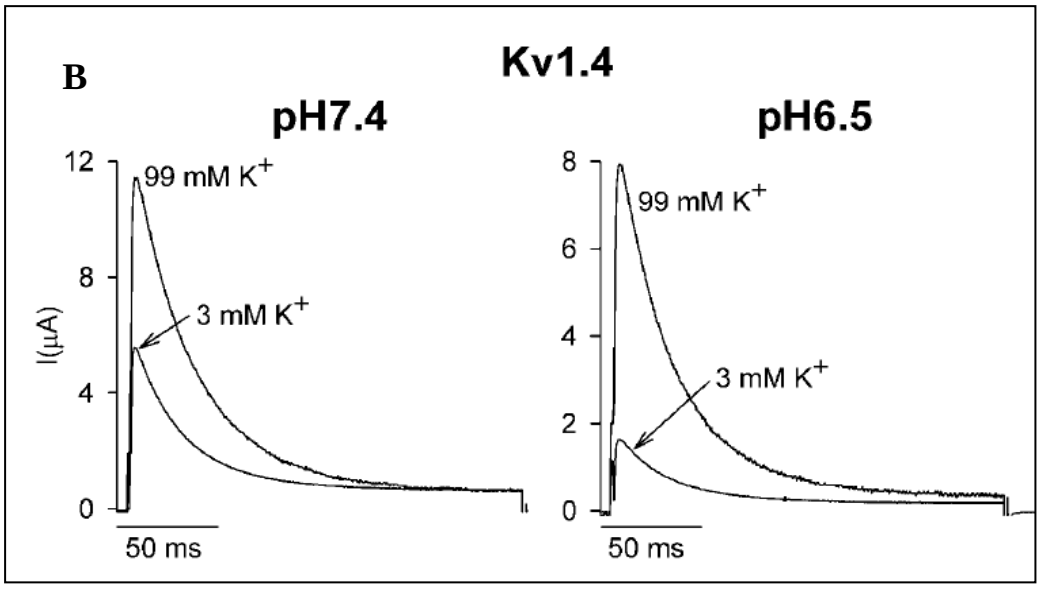


Figure 2.7: A; current reading by lower [K]o 3mM is much lower as compared with higher 9mM [K]o showing the inactivation of Kv1.4 channels is enhanced at lower K^+ concentration as the selectivity filter is emptied and collapsed. B; a lower pH of 6.5 (acidic) results in much lower current reading compared to higher pH 7.4. Thus, the inactivation of Kv1.4 can also be influenced by an acidic environment. From: 'K^+ Activation of Kir3.1/3.4 and Kv1.4 K^+ Channels is Regulated by Extracellular Charges' by Claydon, et al. (2004). *Biophysical Journal*, 2407 – 2418.

2.4 4-Hydroxybenzoic acid (4-Hba)

In light of the increasing demands of natural products constituents, more compounds and extraction of plants and herbs have been carried out. Some of the herbs are well-known and are still used and practiced especially among Asian and African communities. They are reported to have antiepileptic effects and proven to be effective to treat convulsions by direct or indirect pharmacological mechanisms (Zhu, et al. 2014; Ekstein and Schachter, 2010).

4-hba can be found in many plants and fruits such as *Dendrocalamus asper* (bamboo), *Veronica peregrina* (flower) strawberries, apples, mulberries (Juurlink, et al. 2014), *Daucus carota* (carrots), *Elaeis guineensis* (oil palm),

Vitis vinifera (grapes), *Fagara macrophylla* (east african satinwood), *Xanthophyllum rubescens* (yellow leaf tree), and many more (Manuja, et al. 2013). Due to the abundance of hydroxybenzoic acids in many famously consumed foods, further studies on its effective mechanism has been carried on such as on cardiovascular system (Juurlink, et al. 2014), root membrane potential of tobacco plants (Mucciarelli, et al. 2000), mediated lifespan extension on *Caenorhabditis elegans* (Kim, et al. 2013), cucumber seed germination (Crisan, et al. 2007) and cucumber root membrane potential (Camusso, et al. 2008). However, there are not much researches that has been carried out on the effects of 4-hba on the membrane potential of animal models or even terrestrial organisms.

In the extraction of *Dendrocalamus asper* shoots by Universiti Malaysia Terengganu (UMT) in 2014 found 5 major compounds namely 4-hydroxybenzaldehyde, palmitic acid, lauric acid and another two impure major palmitic acid with minor fatty acid attached. However, 4-hydroxybenzaldehyde is easily oxidized into 4-hydroxybenzoic acid due to its excess valence electrons and is less stable (Dobhal, et al. 2010), making it less suitable for further test. Preliminary electrophysiological studies on the enhancement effect of these compounds (synthetic) on GABA (A) receptor found that 4-hydroxybenzoic acid can positively modulates GABA (A) current unlike palmitic and lauric acids which fail to increase the current amplitude of GABA (A). As GABA (A) is inhibiting, its enhancement could potentially reduce the irregularly high membrane potential spikes seen in neuronal disorders such as

epilepsy (Bilal, 2015). Nevertheless, there are no studies carried out on the action of synthetic 4-hba on potassium channels, which also help in lowering the membrane potential.

According to Japan's report (by Ishikawa Kazuhide) for SIDS Initial Assessment for 9th SIAM (France, 1999) on 4-hba, this compound is mostly used as intermediate for pesticide, antiseptics and pharmaceuticals. However, recent studies show that 4-hba is currently being added as potential food additives, as paints and coatings and for personal care products (National Center for Biotechnology Information, 2016). It is also reported to have antifungal, antialgal, antimutagenic, antisickling, extrogenic activity and used as trapping agent on hydroxyl radical generation using cerebral ischemia and reperfusion (Manuja, et al. 2013). With molecular weigh 138.12074 g/mol, it is able to pass through blood vessels, blood brain barriers and also cerebrospinal fluid (CSF). It has a pKa of 4.58 which is a low acid as compared to hydrochloric acid with pKa of -10 (acidity increases with more negative value) but it is more acidic than amines such as lysine with pKa more than +10.

4-Hba is a phenolic compound from benzoic acid derivatives (BADs) along with salicylic acid, gallic acid and vanilic acid (Camusso, et al. 2007). Phenolics compounds exist mostly as secondary metabolites in plant tissues that play important roles as antioxidants that can decrease oxidative stress induced tissue damage from chronic diseases and possess anticancer activities (Khadem and Marles, 2010). 4-Hba is part of the non-flavonoids group of