EVALUATION OF ANTI-HYPERTENSIVE AND VASORELAXANT EFFECTS OF *GYNURA PROCUMBENS* MERR. EXTRACTS

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EVALUATION OF ANTI-HYPERTENSIVE AND VASORELAXANT EFFECTS OF *GYNURA PROCUMBENS* MERR. EXTRACTS

by

ZAFAR IQBAL

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Dedicated
To my Family and Teachers
Whom I love the most

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Zafar Iqbal

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LIST OF ABBREVIATIONS

℃	Degree celsius
%	Percent
±	Plus, minus
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ACEI	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
Ang	Angiotensin
ANOVA	Analysis of variance
AT1	Angiotensin type I receptor
ATP	Adenosine triphosphate
AV	Atrioventricular
BK_{ca}	Big-conductance calcium-sensitive potassium channel
BP	Blood pressure
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHF	Congestive heart failure
Cl^-	Chloride
CNS	Central nervous system
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DMSO	Dimethylsulfoxide

EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxation factor
EP	Epinephrine
eNOS	Endothelial nitric oxide synthase
GC-MS	Gas chromatography mass-spectrometry
G. procumbens	*Gynura procumbens*
GPPE	*Gynura procumbens* petroleum ether extract
GPCE	*Gynura procumbens* chloroform extract
GPME	*Gynura procumbens* methanol extract
GPWE	*Gynura procumbens* water extract
g	Gram
HPLC	High performance liquid chromatography
i.p	Intraperitoneal
i.v	Intravenous
IK_{ca}	Intermediate-conductance calcium-sensitive potassium channel
IP	PGI_2 receptor
IP_3	1,4,5-inositol triphosphate
K^+	Potassium
K_{ATP}	ATP-sensitive potassium channel
K_{ca}	Calcium-activated potassium channel
K_{IR}	Inward rectifier potassium channel
K_V	Voltage-gated K^+ channel
L-NAME	Nω-Nitro-L-arginine methyl ester
µg	Microgram
µL	Microliter
M	Molar

MAP	Mean arterial pressure
mg	Milligram
MI	Myocardial infarction
mL	Milliliter
MLC	Myosin light chain
mM	Millimolar
K_{IR}	Inward rectifier potassium channel
K_V	Voltage-gated K^+ channel
L-NAME	Nω-Nitro-L-arginine methyl ester
µg	Microgram
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M	Molar
MAP	Mean arterial pressure
mg	Milligram
MI	Myocardial infarction
mL	Milliliter
MLC	Myosin light chain
mM	Millimolar
GC-MS	Gas chromatography-Mass spectrometry
Na^+	Sodium
NE	Norepinephrine
NO	Nitric oxide
NOS	Nitric oxide synthase
PEG	Polyethylene glycol
PGI_2	Prostacyclin
PIP_2	Phosphatidyl inositol-(4,5)-bisphosphate
PKC	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
PVR	Peripheral vascular resistance
ROS	Reactive oxygen species
S.E.M	Standard error of mean
SBP	Systolic blood pressure
SD	Sprague Dawley
SNP	Sodium nitroprusside
SNS	Sympathetic nervous system
SVR	Systemic vascular resistance
TEA	Tetraethyl ammonium
TPR	Total peripheral resistance
VSM	Vascular smooth muscle

PENILAIAN KESAN ANTI-HIPERTENSI DAN KESAN VASORELAKSAN EKSTRAK *GYNURA PROCUMBENS* MERR

ABSTRAK

Penyakit kardiovaskular terus meningkat di kedua-dua negara maju dan membangun. Tekanan darah tinggi merupakan "pembunuh senyap" yang sedia diketahui menjadi punca utama kepada komplikasi kardiovaskular. Kawalan tekanan darah yang tidak mencukupi boleh membina komplikasi yang berbeza seperti strok, infarksi miokardium, hipertrofi ventrikel kiri, kegagalan buah pinggang dan jantung. *Gynura procumbens* (G. procumbens) secara tradisinya digunakan untuk rawatan hipertensi. Ia telah dihipotesiskan bahawa, *G. procumbens* mungkin mempunyai ciri-ciri anti-hipertensi, hipotensi dan vasorelaksan yang dihasilkan oleh beberapa molekul aktif, yang akan menurunkan tekanan darah dan vasodilasi. Daun-daun *G. procumbens* kering dikizar menjadi serbuk halus. Serbuk daun ini telah diekstrak secara bersiri menggunakan eter petroleum, kloroform, metanol dan air melalui proses maserasi. Setiap ekstrak telah dikeringkan di bawah tekanan yang dikurangkan. Empat jeniz ekstrak, iaitu eter petroleum (GPPE), kloroform (GPCE), metanol (GPME) dan air (GPWE) daripada *G. procumbens* diperolehi. Kesan setiap ekstrak diperiksa pada cincin aortik tikus yang terpencil dan persediaan tikus yang dibius. Objektif kajian ini adalah untuk mengkaji aktiviti anti-hipertensi secara dalam tikus berhipertensi sponttan, aktiviti vasorelaksan dalam persediaan cincin aortik tikus, aktiviti penuninan tekanan darah dan mekanisme tindakan tikus normotif anestetik bagi ekstrak *G. procumbens*. Pemberian harian oral GPWE mempunyai lebih banyak kesan anti-hipertensi dalam SHR berbanding ekstrak lain. Pemberian intravena GPWE, berbanding ekstrak lain, juga mempunyai lebih banyak kesan

pengurangan tekanan darah dalam tikus normotif anestetik. GPPE dan pecahannya menunjukkan lebih banyak vasorelaksasi dalam persediaan cincin aorta. Penguncupan yang disebabkan oleh kalsium adalah ketara ($p < 0.01$) dan ($p < 0.001$) dihalang oleh GPPE dalam lengkungan tindak balas kalsium bergantung kepekatan cincin aorta. GPPE mempunyai kesan yang sama seperti verapamil pada saluran kalsium bergantung voltan cincin aorta. Saluran kalium sensitif dengan ketara ($p < 0.05$) menghalang vasorelaksasi GPPE. Pra-rawatan dengan propranolol menghalang ketara ($p < 0.01$) kesan-kesan hipotensi GPWE dalam tikus normotif anestetik. Kesan penurunan GPWE pada tikus anestetik mungkin disebabkan oleh kesan β-adrenergik. GPWE boleh bertindak sebagai β_2-agonis. Ekstrak yang lebih polar (GPWE) di dapati telah mengekalkan beberapa aktiviti anti-hipertensi dan penurunan tekanan darah, manakala ekstrak bukan kutub (GPPE) mempunyai lebih banyak aktiviti vasorelaksan. Keputusan ini menunjukkan bahawa *G. procumbens* menginduksi kesan kardiovaskularnya pada reseptor β_2-adrenergik dalam otot licin vaskular secara merangsang laluan adenosine monophosphate kitaran, saluran potassium sensitif ATP dan menghalang saluran kalsium jenis-L. Analisis Kromatografi gas-spektrometri massa dan kromatografi cecair berprestasi tinggi menunjukkan kehadiran spatulenol dan asid ursolik, yang menunjukkan bahawa kesan kardiovaskular mungkin berkaitan dengan kehadiran sebatian-sebatian ini.

EVALUATION OF ANTI-HYPERTENSIVE AND VASORELAXANT EFFECTS OF *GYNURA PROCUMBENS* MERR. EXTRACTS

ABSTRACT

The cardiovascular diseases are continually increasing in both developed and developing countries. High blood pressure is a well-known "silent killer" which is the leading cause of cardiovascular complications. The inadequate control of blood pressure can develop different complications such as stroke, myocardial infarction, left ventricular hypertrophy, renal and heart failure. *Gynura procumbens* (*G. procumbens*) traditionally used for treatment of hypertension. It was hypothesised that, *G. procumbens* may have anti-hypertensive, hypotensive and vasorelaxant properties due to some active molecules that would lower blood pressure and vasodilations. The dried *G. procumbens* leaves were ground into a fine powder. The powdered leaves material was serially extracted with petroleum ether, chloroform, methanol and water by maceration process. Each extract was dried under reduced pressure. Four extracts, petroleum ether (GPPE), chloroform (GPCE), methanol (GPME) and water (GPWE) of *G. procumbens* were obtained. The effect of each extract was examined on isolated rat aortic ring and anesthetized rat preparations. The aims of the present study were to investigate the anti-hypertensive activity in spontaneously hypertensive rats (SHR), vasorelaxant activity in rat aortic ring preparations and blood pressure lowering activity and mechanism of action in anesthetized normotensive rats of *G. procumbens* extracts. Daily oral administration of GPWE has more anti-hypertensive effects in SHR compared to other extracts. The intravenous (i.v.) administration of GPWE among the extracts also has more blood pressure lowering effects in anesthetized normotensive rats. GPPE and its fractions

show more vasorelaxation in aortic ring preparations. Calcium-induced contraction was significantly ($p < 0.01$) and ($p < 0.001$) inhibited with GPPE in concentration dependent calcium response curve of aortic rings. GPPE has similar effects as verapamil on voltage dependent calcium channel (VDCC) of aortic rings. ATP sensitive potassium channel (K_{ATP}) significantly ($p < 0.05$) inhibit the vasorelaxation of GPPE. Pre-treatment with propranolol inhibit significantly ($p < 0.01$) the hypotensive effects of GPWE in anesthetized normotensive rats. The blood pressure lowering effects of GPWE in anesthetized rat may be due to β-adrenergic effects. GPWE may act as β_2-agonist. The more polar extract (GPWE) appeared to retain some anti-hypertensive and blood pressure lowering activities, while non-polar extract (GPPE) has more vasorelaxant activity. The results suggest that *G. procumbens* induced its cardiovascular effects on β_2-adrenergic receptors in the vascular smooth muscle by stimulating cyclic adenosine monophosphate (cAMP) pathway, sensitive potassium channel (K_{ATP}) and inhibiting L-type calcium channels. Gas chromatography mass-spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analysis shows the presence of spathulenol and ursolic acid, which suggest that the cardiovascular effect could be related to the presence of these compounds.

CHAPTER 1

INTRODUCTION

1.1 Background

In 2013, cardiovascular illness was the most common underlying cause of death in the world. According to an assessment, 17.3 million of total deaths in the world, was due to cardiovascular illness (Benjamin, 2017). The cardiovascular diseases are continually increasing in both developed and developing countries (Balakumar et al., 2016). Globally in 2008, the incidence of hypertension among adults aged 25 and above was almost 40 %. In 2009, the total financial load of hypertension in the United States was almost USD 73.4 billion. Even in the presence of clinical practice guidelines (CPGs), optimal hypertension control is not achieved. According to a study, a decrease of 10 mm Hg in systolic blood pressure and 5 mm Hg in diastolic blood pressure, causes a 20 percent decrease of coronary heart disease and a 32 percent decrease of stroke in one year (Al-Ansary et al., 2013). According to the Centres for Disease Control and Prevention (CDC), even a very slight rise in BP increases the risk for cardiovascular disease. Appropriate treatment of hypertension can decrease the chances of heart attacks and strokes.

1.2 Problem statement

In spite of there are many antihypertensive drugs available, but there are many research publications on their quite little effectiveness in the form of mono-therapy as well as long-lasting side effects (Jarari et al., 2016, Guerrero-García and Rubio-Guerra, 2018). Therefore, the finding of novel antihypertensive drugs is still a hot issue. Agents that can act on vascular tone in addition to lower blood pressure

may lead to a good approach in the treatment of hypertension and prevention of cardiovascular morbidities (Yannoutsos et al., 2016). Therefore, the discovery of new molecules is highly desired.

The importance of *G. procumbens* in the cardiovascular studies has been reported (Abrika et al., 2013, Kaur et al., 2012, Kaur et al., 2013, Hoe et al., 2011). Previous studies mostly have investigated the cardiovascular activity of the polar portion of *G. procumbens* extracts, still non-polar part of the plant for its cardiovascular effects have not been validated in the laboratory. This background offers an opportunity to study the plant both pharmacologically and phytochemically.

1.3 Hypothesis

It was hypothesised that, *G. procumbens* may have anti-hypertensive, hypotensive and vasorelaxant properties due to some active molecules that would lower blood pressure and vasodilations. The tail cuff experimental model was proposed to explore the anti-hypertensive effect of different extracts of leaves of *G. procumbens* in spontaneously hypertensive rats (SHR). The anesthetized rat experimental model was suggested for the possible blood pressure lowering mechanism of action for the most active *G. procumbens* extract. The isolated rat aortic ring model was proposed to find the vasorelaxant activity and the possible mechanism of action of different extracts of *G. procumbens*.

1.4 Objectives

1. To investigate the vasorelaxant activity of different extracts obtained from the leaves of *G. procumbens* and to explore the possible mechanism of

action of the most active *G. procumbens* extract using isolated rat aortic ring model.

2. To evaluate the possible blood pressure lowering mechanism of action for the most active *G. procumbens* water extract using anesthetized rat experimental model in normotensive rats.
3. To determine the anti-hypertensive effect of different extracts of leaves of *G. procumbens* in spontaneously hypertensive rats (SHR) using tail cuff experimental model.
4. To characterize, the main possible components responsible for the cardiovascular effects of *G. procumbens* by using Gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC).

CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

Hypertension can be well-defined as if a person experiences systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg or taking the medication for hypertension (CDC, 2012). The Seventh Report of the Joint National Committee (JNC) USA, classified hypertension as below:

1. Normal blood pressure (SBP <120 mmHg, DBP < 80mmHg)
2. Prehypertension (SBP 120 to 139 mmHg, DBP 80 to 89 mmHg)
3. Stage 1 hypertension (SBP 140 to 159 mmHg, DBP 90 to 99 mmHg)
4. Stage 2 hypertension (SBP 160 mmHg, DBP 100 mmHg) (Holm et al., 2006).

High blood pressure is a well-known "silent killer" which is the leading cause of cardiovascular complications. Approximately, 13 percent of world population experiences high blood pressure, and mostly belong to developing countries. In 2001, higher than 32 percent population in Malaysia, aged 18 years and above, and 43 percent, aged 30 years and above, were hypertensive (Loh et al., 2018). High blood pressure is one of the deadly causes of mortality in the world, due to its symptomless behavior and complications which can cause accompanying illnesses, for example, stroke (Loh et al., 2016).

The inadequate control of BP can develop different complications such as stroke, myocardial infarction, left ventricular hypertrophy, renal and heart failure.

The blood pressure depends upon:

1. The force of received blood, by which, the heart muscles are stretched.
2. The resistance to blood flow in the blood vessels.
3. The volume of blood in the blood vessels.
4. The autonomic nervous system.
5. The kidney increases the blood pressure by:
    - Inducing the contraction of the blood vessels.
    - Increasing the volume of blood in the blood vessels (Lionakis et al., 2012).

2.2 The Cardiovascular system

2.2.1 The Heart

The heart works as a pump, which supplies blood throughout the body by constant and rhythmical contractions.

The heart wall comprises three layers:

1. Pericardium (the outermost layer)
2. Myocardium (the middle layer)
3. Endocardium (the innermost layer).

Between the two ventricles an interventricular septum, and between the atrium and ventricle, the atrioventricular (AV) valves are present, which permit only one-way blood flow. The right AV valve is tricuspid, and the left AV valve is bicuspid (Weinhaus and Roberts, 2009).

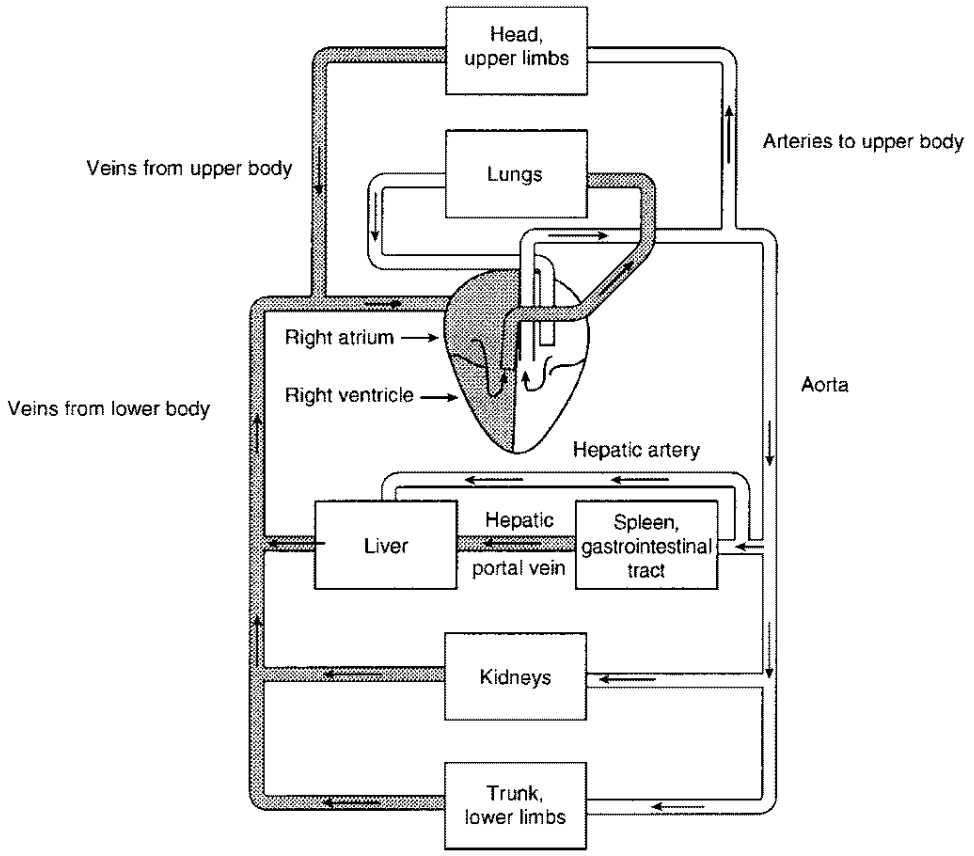

Figure 2.1 The cardiovascular system.

The deoxygenated blood accumulates in the right atrium via the superior venae cavae and inferior venae cavae, and then pushed into the right ventricle. The right ventricle pumps the deoxygenated blood to the lungs, via the pulmonary artery. The oxygenated blood comes to the left atrium, through pulmonary veins. The left atrium pushes the blood into the left ventricle. The left ventricle supplies blood, to the body. The exchange, of oxygen and carbon dioxide, takes place via capillaries. The cardiac muscle is an involuntary striated muscle, like skeletal and smooth muscles. The cardiac muscles are highly resistant, to the tiredness, because of a large number of mitochondria, myoglobin and adequate blood supply (Weinhaus and Roberts, 2009).

2.2.2 Arteries and arterioles

Arterial architecture is increasingly recognized as an essential determinant in the pathophysiology of hypertension and cardiovascular diseases (Mayet and Hughes, 2003). Agents that can act to modulate arterial wall structure and function, in addition to lower blood pressure may lead to a novel approach in the treatment of hypertension and prevention of cardiovascular morbidities (Yannoutsos et al., 2016).

There are three different layers, in the wall of an artery:

1. Tunica intima
2. Tunica media
3. Tunica adventitia

Tunica intima is inner layer, which comprises a single layer of endothelial cells (Figure 2.2). Tunica media is the middle layer and tunica adventitia is the outer layer. There is an internal elastic lamina of the connective tissue between the tunica media and the tunica intima (Loh et al., 2018). Tunica adventitia comprises fibroblasts, collagen, and sympathetic nerves. The arterial resistance depends upon the diameter of the lumen. In diastole phase, the occurrence of elastic tissues in the vessels, decrease resistance (Widmaier et al., 2006). In the walls of the arteriole, there is less elastic tissue so causes more resistance to the blood flow. The slight changes, in the diameter of the arteriole, can cause a significant change in the total peripheral resistance (TPR) (Mayet and Hughes, 2003).

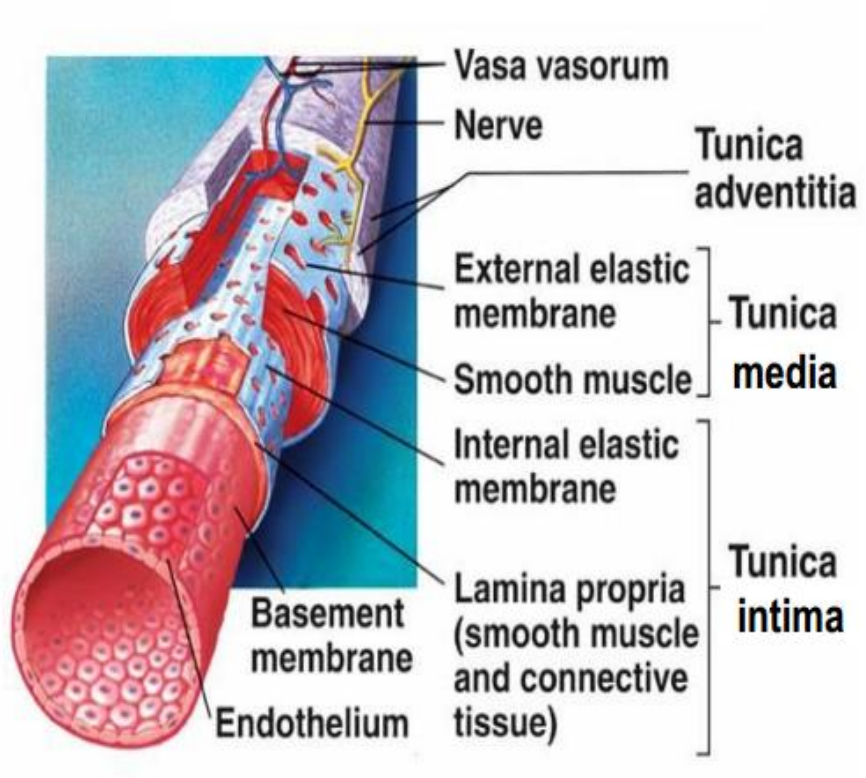

Figure 2.2 Structure and layers of a typical blood vessel in the circulatory system. (Diagram adapted from McGraw-Hill companies.inc).

2.3 Vascular Smooth Muscle Contraction and Relaxation

The vascular smooth muscle cell (VSM) forms an integral structural element of the blood vessels and involve in the regulatory processes of the vascular system (Saddouk et al., 2017). It is necessary to understand the signaling mechanism pathways before the start of vasculature-associated research. The smooth muscle contraction takes place, due to increased intracellular calcium. There are numerous signal transduction mechanisms such as G-protein-coupled pathway, nitric oxide-cGMP pathway, voltage-dependent Ca^{2+} channels (VDCC), and receptor-operated Ca^{2+} channels (ROCC) which control intracellular calcium concentration and consequently the state of vascular tone. These signal transduction mechanisms take place in the vascular endothelium and the VSM. The relaxation and contraction of

the blood vessels occurred by these signal transduction mechanisms and finally maintained by the actin and the myosin filament. The vascular contraction occurs by the sliding of the actin and myosin filaments over one another. The contraction in the VSM takes place by the opening of voltage-dependent calcium channels (L-type calcium channels); the electrical depolarization takes place, which increases intracellular calcium concentration (Figure 2.3). Many chemicals act as a stimulant, for instance, epinephrine, norepinephrine, vasopressin, endothelin-1, thromboxane A2, and angiotensin II can induce contraction. The receptors are present on the endothelium and vascular smooth muscle, by which these chemicals bind and induce contraction in the blood vessel (Webb, 2003).

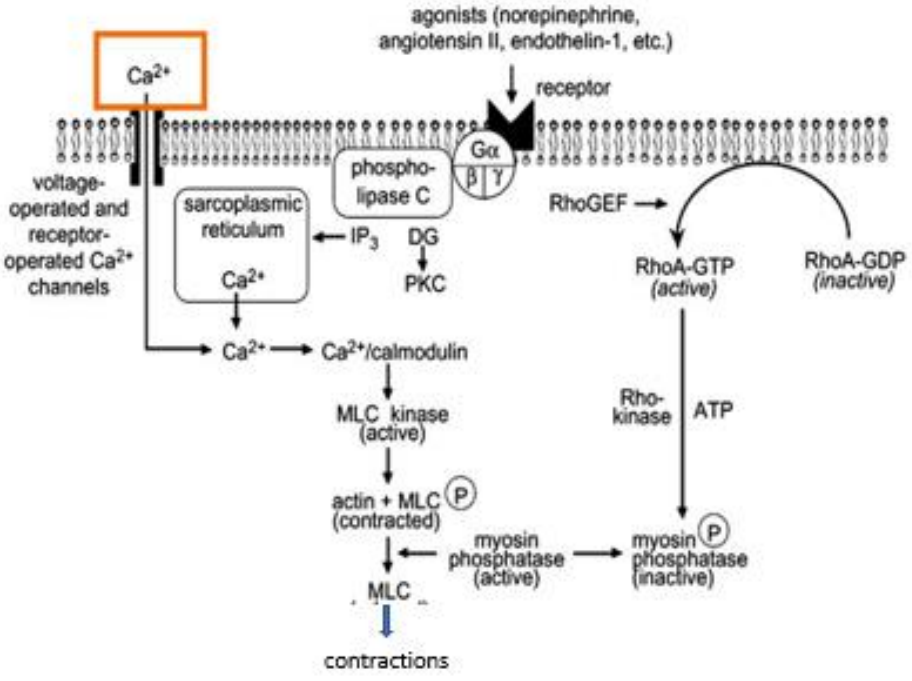

Figure 2.3 Mechanisms, by which contraction of vascular smooth muscle takes place. (Webb, 2003).

The free intracellular calcium binds with the specific protein calmodulin. This calcium-calmodulin complex activates an enzyme myosin light chain kinase (MLCK). The phosphorylation of myosin light chain (MLC) takes place in the presence of ATP. The removal of calcium takes place by the ATP-dependent calcium pump and the sodium-calcium exchanger. The chemical mechanism which modifying the Ca^{2+} metabolism is very important, in the stability of vascular smooth muscle tone (Somlyo et al., 1999).

A myosin light chain phosphatase (MLCP) enzyme is present in myosin light chain, which catalysis Ca^{2+}, consequently intracellular Ca^{2+} level comes down to the resting stage, and dephosphorylation takes place, and relaxation of vascular smooth muscle occurs. The small G protein RhoA and its downstream target Rho kinase play an important role in the regulation of MLC phosphatase activity. Rho kinase phosphorylates the myosin binding subunit of MLC phosphatase, inhibiting its activity and thus promoting the phosphorylated state of MLC (Somlyo et al., 1999, Webb, 2003). For the elimination of cytosolic Ca^{2+}, there are several mechanisms. The relaxation of smooth muscle cells occurs, because of the inhibition of Ca^{2+} / Mg^{2+} - ATPase action, in the sarcoplasmic reticulum, which diminishes cytosolic Ca^{2+}. Furthermore, the blockage of receptor-operated and voltage-operated Ca^{2+} channels, causes the relaxation of smooth muscle, because of a reduction in intracellular Ca^{2+} (Webb, 2003).

2.3.1 Endothelium

The endothelium is present in the whole vascular system. The endothelium regulates the vascular function by neurotransmitters, hormones, and vasoactive factors (Sandoo et al., 2010). For the protection of the blood vessels, an optimum

level of these factors is required, while imbalance of these factors causes endothelial dysfunction. and atherosclerosis (Lerman and Zeiher, 2005, Sandoo et al., 2010). The endothelium releases many vasoactive factors, for example, nitric oxide (NO) and prostacyclin (PGI_2), which induce vasorelaxation and endothelin-1 (ET-1) and thromboxane (TXA_2), induce contraction (Figure 2.4 and Figure 2.5) (Loh et al., 2018).

Figure 2.4 describes the production, of the endothelial nitric oxide and its effects in the cells of vascular smooth muscle. Acetylcholine (ACh), adenosine di-phosphate (ADP), bradykinin (BK) and adenosine triphosphate (ATP) are examples of NO synthesis, by the depletion of intracellular Ca^{2+}stores (Lambert et al., 1986, Schilling and Elliott, 1992, Schilling et al., 1992, Moncada and Higgs, 2006). When intracellular levels of Ca^{2+} increase, eNOS detaches from caveolin and is activated. Ca^{2+} attaches to the protein calmodulin in the cytoplasm of the cell, after which it undergoes structural changes which allows it to bind to eNOS (Fleming and Busse, 1999). Consequently, the eNOS changes L-arginine to NO (Palmer et al., 1988). When the Ca^{2+} level decreases, then break down of calcium-calmodulin complex takes place and deactivated by binding with caveolin (Sandoo et al., 2010).

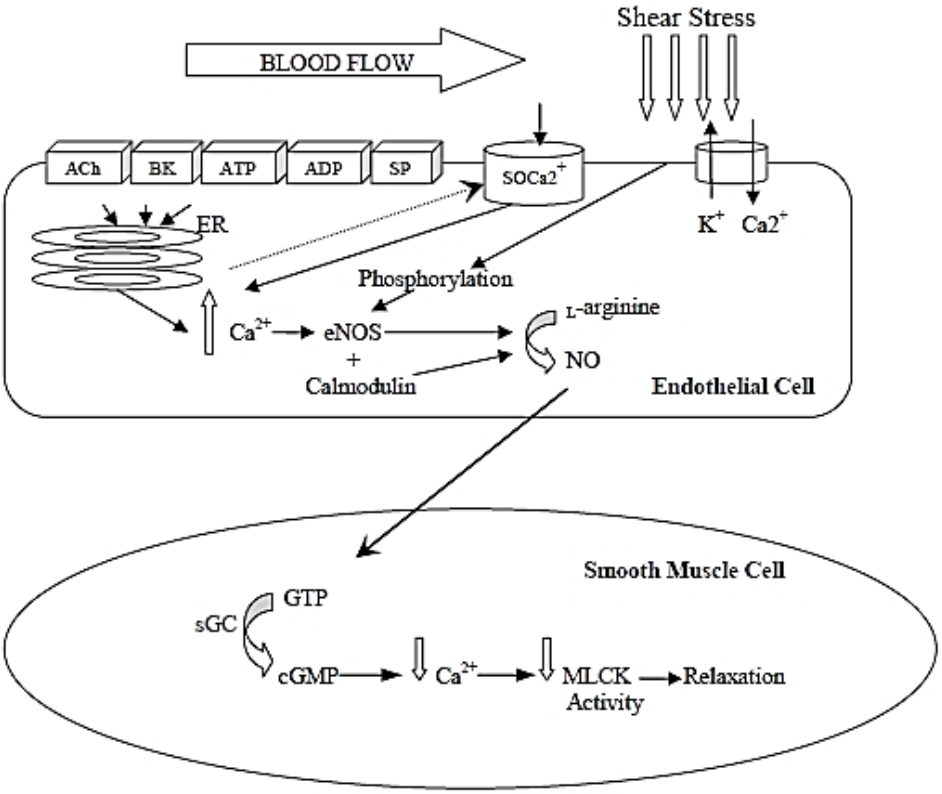

Figure 2.4 The production, of the endothelial nitric oxide and its effects in the cells of vascular smooth muscle (Sandoo et al., 2010).

Abbreviations: ACh= acetylcholine; BK= bradykinin; ATP= adenosine triphosphate; ADP= adenosine diphosphate; SP= substance P; $SOCa^{2+}$= store-operated Ca^{2+} channel; ER= endoplasmic reticulum; NO= nitric oxide; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5-monophosphate; MLCK= myosin light chain kinase. When Ca^{2+} stores of the endoplasmic reticulum are depleted a signal is sent to $SOCa^{2+}$ channel which allows extracellular Ca^{2+} into the endothelial cell.

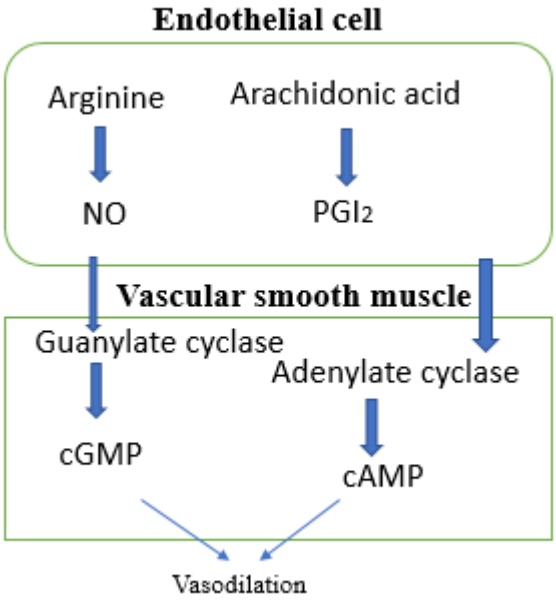

Figure 2.5 Flow chart diagram describe the vasodilation by the production of PGI_2 and NO in the endothelial cell.

A high blood pressure, in the blood vessel, induces shear stress. This shear stress causes the production of NO, by the process of phosphorylation (Figure 2.4). The extent of the shear stress is, directly proportional to the production of the NO. Whenever, the shear stress is for the short period, then the intracellular Ca^{2+} is released, while if shear stress is for a longer period (>30 min), then the production of the NO takes place due to the phosphorylation of the eNOS (Pittner et al., 2005, Sandoo et al., 2010, Mount et al., 2007).

From the endothelial cell, the NO penetrates in to the smooth muscle. Then the NO attaches with the enzyme soluble guanylyl cyclase (sGC) and stimulates the enzyme. The conversion of guanosine triphosphate (GTP) to cGMP increases by the stimulated enzyme. Consequently, the contraction of the smooth muscle decreases. From the sarcoplasmic reticulum, the secretion of the Ca^{2+} decreases and Ca^{2+} restores in the sarcoplasmic reticulum. Thus, the relaxation of smooth muscle cells occurs (Davignon and Ganz, 2004, Sandoo et al., 2010).

With a continuous production of the NO, the vasodilator tone is maintained (Gladwin et al., 2004). The Nω-nitro-l-arginine methyl ester (L-NAME) is a nitric oxide synthase (NOS) inhibitor (Dawes et al., 2001). Disturbance of vascular homeostasis can lead to the development of endothelial dysfunction. The steady production of the endothelin (vasoconstrictors) and the NO (vasodilator) maintains vascular tone (Sandoo et al., 2010). One of the leading causes of endothelial dysfunction is the decreased level of NO in the blood. There is an association, between increased age, the endothelium dysfunction, and cholesterol (Gimbrone and García-Cardeña, 2016).

There are many elements, which regulate the tone of the blood vessels, which are given below in **Error! Reference source not found.**.

Table 2.1 Endothelium-derived vasoactive factors. Summary of major vasoactive factors (dilators and constrictors) found in endothelium, their synthesizing enzyme, target, the effect on tone and mechanism of action.

Vasoactive Factors	**Synthesizing Enzyme**	**Target**	**Effect on Vascular tone**	**Mechanism ofAction**
NO	eNOS	sGC/cGMP	↓↓↓	↓ Ca^{2+}
EDHF	CYP2C9	K^{+}- Ca^{2+}	↓↓	Hyperpolarizes smooth muscle cells
PGH_2	COX-1	Endoperoxide receptor	↑	↑ Ca^{2+}
ET	Endothelin converting enzyme	ETA receptor ETB_1 receptor ETB_2 receptor	↑ ↓ ↑	↑ Ca^{2+} NO release ↑ Ca^{2+}
Ang II	Angiotensin converting enzyme	AT_1 receptor AT_2 receptor	↑	Aldosterone release
PGI_2	Prostacyclin synthase	IP receptor	↓	cAMP/AC
TXA_2	Thromboxane synthase	Thromboxane receptor	↑	↑ Ca^{2+}

Abbreviations: NO - Nitric oxide, EDHF - Endothelium-derived hyperpolarizing factor, PGH2 - Prostaglandin H2 (endoperoxide), ET - Endothelin, Ang II - Angiotension II, PGI - Prostacyclin, TXA2 - Thromboxane A2, ↑ or ↓ - increase or decrease.

The interaction among endothelium and VSM is closely related. Both act synergistically in a complex manner to maintain the normal arterial tone.

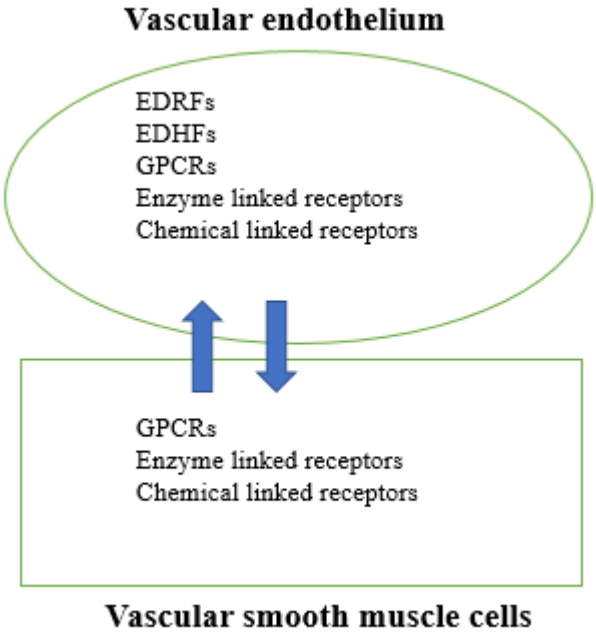

Figure 2.6 The signalling pathways in vascular endothelium and vascular smooth muscle acts synergistically to maintain the vasocontractions and vasorelaxations in the blood vessels.

2.4 Enzyme-Linked Receptors

Enzyme-Linked Receptors are situated on the membrane and known as catalytic receptors, which are stimulated by catalytic enzymes and ligand-receptors. The guanylyl cyclase is essential enzyme-linked receptors that play a key role in the maintenance of the vascular tone. The NO diffuses into the VSM after its synthesis in the endothelium and attaches with soluble guanylyl cyclase (sGC). Activation of sGC by NO in VSM leads to the conversion of guanosine 5' – triphosphate (GTP) to cyclic guanosine 3', 5' -monophosphate (cGMP) as shown in Figure 2.7. The cGMP activates the protein kinase G (PKG) which reduces the intracellular Ca^{2+} release

from sarcoplasmic reticulum store and causes vascular smooth muscle relaxation (Loh et al., 2018, Fellner and Arendshorst, 2002).

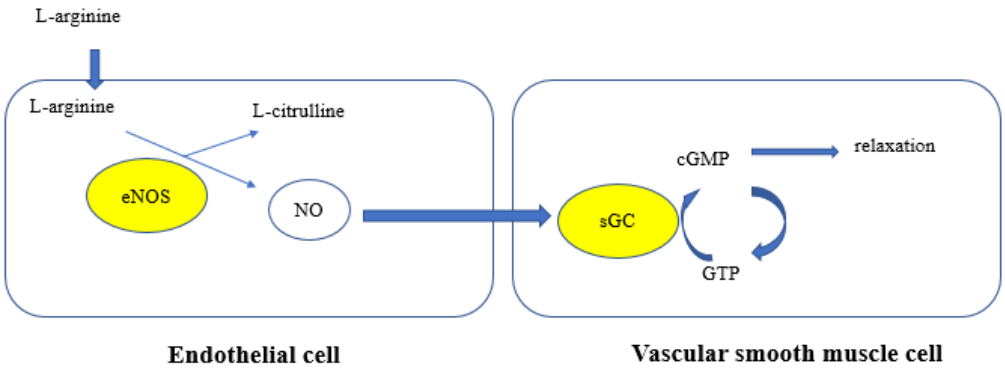

Figure 2.7 The role of Enzyme-Linked Receptors in vasorelaxation.

2.4.1 Soluble Guanylyl Cyclase pathway

In the cytosol of the VSMCs, the sGC is freely available. The NO attaches, with the heme group of the sGC. On the activation of the sGC, the conversion of the GTP into the cGMP takes place, which stimulates the PKG. As a result, vasodilation occurs (Ko et al., 2008, Loh et al., 2018). The 1H- [1,2,4] oxadiazole [4,3-a] quinoxaline-1-one (ODQ) is dissolved in dimethylsulfoxide (DMSO) and block the sGC, by oxidizing the heme group of the sGC (Loh et al., 2018). The methylene blue (MB) is another cGMP blocker (Kontos and Wei, 1993, Evora, 2016).

2.4.2 Serine-Threonine Protein Kinases

These are kinase enzymes, which are triggered when attached to their second messenger, and phosphorylation of their hydroxyl (OH) group takes place. The protein kinase A (PKA), protein kinase C (PKC), and protein kinase G (PKG) is involved in the functioning of the blood vessels. The PKA is a cAMP-dependent and is activated when attaches with cAMP. The vasodilation occurs, when its

phosphorylation takes place. By phosphodiesterase 3 (PDE_3), the breakdown of cAMP takes place, into adenosine monophosphate (Loh et al., 2018, Bouschet et al., 2003). In the endothelium of the blood vessels and VSMCs, the PKC is triggered by DAG and attaches with Ca^{2+} ions at C_1 and C_2 domain respectively. Phosphorylation of the serine or threonine sites takes place when the PKC is activated. Then, contraction of the blood vessels takes place (Huang, 1989). The PKG is activated, on the attachment of the cGMP. Then dilation of the blood vessels takes place (Wall et al., 2003, Paul and Snyder, 2012).

2.5 G-Protein-Coupled Receptors (GPCRs)

G-Protein-Coupled Receptors are present on intracellular surface of cell membranes and known as the seven-transmembrane domain receptors, which are activated when attached to their ligand and transmit signals into the cell. Three types of guanine nucleotide binding protein (G-protein) are present on both endothelium and VSMC. Gα-, Gβ-, and Gγ-proteins are subunits of G-protein. Gα performs the main role in the blood vessels, which is further sub-divided into three types—Gqα, Giα, and Gsα. The activation of the G-protein is started, when GPCRs attach to a ligand and makes the G-protein perform as guanine nucleotide exchange factor (GEF) and replace its guanosine diphosphate (GDP) into GTP. When attached with GTP, the G-protein trimer detaches into Gα-GTP monomer and Gβγ-dimer. Gα-GTP monomer initiates to act with intracellular proteins for signal transduction, and then Gβγ-dimer tends to activate numerous types of signaling molecules, together with ion channels, phospholipases, lipid kinases, and its particular signaling cascades (Walaas et al., 1992, Dorsam and Gutkind, 2007, Yuen et al., 2010).

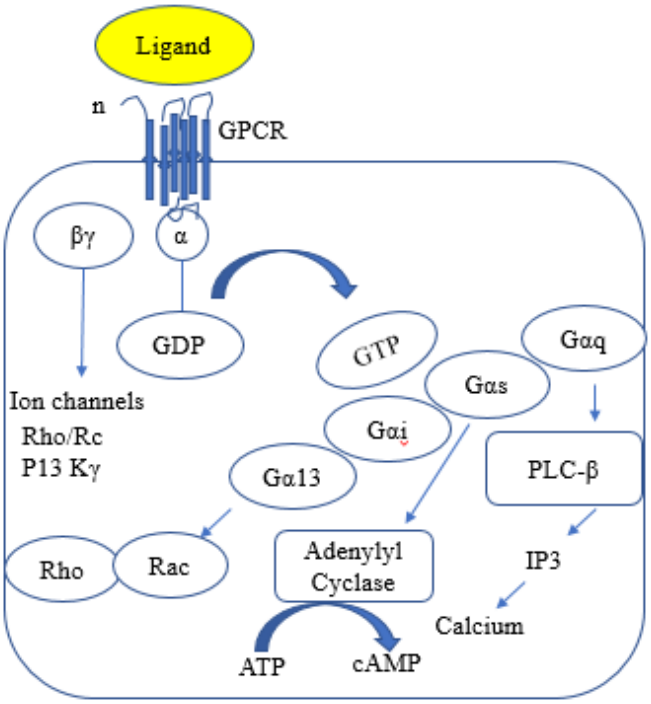

Figure 2.8 G protein-coupled receptor signalling (Lynch and Wang, 2016).

2.5.1 Gqα-Protein-Coupled Receptors

The stimulated Gqα-protein splits the phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers, inositol triphosphate (IP_3) and DAG by binding its Gqα-subunit to the phospholipase C (PLC). The IP_3 binds to the intracellular receptor, IP_3 receptor (IP_3R), on the SR to activate the intracellular release of the Ca^{2+} ions from the SR into the cytosol. While the DAG activates the PKC. As a result, the Ca^{2+} concentration in the cytosol increases. In the blood vasculature, the Gqα-protein-coupled receptors are present in endothelium, which contains angiotensin-2 receptor (AT_2), serotonin receptor ($5\text{-}HT_{1D}$), bradykinin receptor (B_2), muscarinic-3 receptor (M_3), endothelin-B receptor (ET_BR), and calcitonin receptor-like receptor (CALCRL), while there are α1-adrenergic receptor (α1), M_3-muscarinic receptor, angiotensin-1 receptor (AT_1), endothelin receptors (ET_AR and ET_BR),

serotonin receptor (5-HT_2), and TXA_2 receptor in VSMCs (Jakala et al., 2009, Loh et al., 2018, Bockaert et al., 2006, Ishii and Kurachi, 2006).

2.5.2 Giα-Protein-Coupled Receptors

The Giα-protein-coupled receptors are present in the VSMCs, for instance, a α2-adrenergic receptor (α2). When the receptor is stimulated, the activity to convert ATP into cAMP is inhibited, as a result, vasoconstriction occurs (Qin et al., 2008).

2.5.3 Gsα-Protein-Coupled Receptors

The Gsα-protein-coupled receptors are opposite, to the Giα-protein-coupled receptors. When the receptor is stimulated, it activates AC to produce cAMP from ATP, the cAMP will increase, the activation of PKA. As a result, vasodilation occurs. There are two important Gsα-protein-coupled receptors in VSMCs, for instance, the β2-adrenergic receptor (β_2) and PGI_2 receptor (IP) (Jakala et al., 2009).

2.6 Channel-Linked Receptors

The Channel-Linked Receptors are also known as ion channel-linked receptors or ionotropic receptors or ligand-gated receptors. These are triggered when attached to their ligand and allowing ions, for example, Na^+, K^+, Cl^-, and Ca^{2+} to pass through the membrane. The action potential in VSMCs is controlled, by these receptors through depolarization or hyperpolarization. There are two important channel-linked receptors, playing critical roles in vascular tone regulation, which are K^+ and Ca^{2+} channels (Loh et al., 2018).

2.6.1 Potassium Channels

The Potassium channel is widely distributed, in living organisms. There are four types of K^+ channels in the blood vessels:

1. Calcium-activated K^+ channel (Kca)
2. ATP-sensitive K^+ channel (K_{ATP})
3. Inwardly-rectifying K^+ channel (Kir)
4. Voltage-gated K^+ channel (Kv)

In the human blood vessels, Kca channel is sub-divided into three types:

a. Big-conductance (BKca)
b. Intermediate-conductance (IKca)
c. Small-conductance (SKca)

BKca channel is widely spread in VSMCs, while IKca and SKca channels are mostly present, in the endothelium (Jakala et al., 2009, Chen et al., 2012, Eichler et al., 2003). The electric conductance is 2–25 Pico Siemens (Ps) for SKca, 25–100 Pico Siemens (Ps) for IKca and 100–300 Pico Siemens (Ps) for BKca channels. In the VSMCs, the BKca channels are Ca^{2+} and voltage-dependent. The high level of Ca^{2+} in the cells, trigger these channels and allows the efflux of K^+ ions, at that time hyperpolarization and the closing of the Ca^{2+} channels occurred. As a result, vasodilation takes place (Gautam et al., 2006). Furthermore, the BKca channels are indirectly activated by the PKA and PKG (Loh et al., 2018). SKca and IKca channels are not voltage-dependent (Burnham et al., 2006, Barfod et al., 2001). These channels are susceptible, to the calmodulin and Ca^{2+} (García-Pascual et al., 1995, Schumacher et al., 2001). The stimulation of the Kv channels, in the blood vessels, is voltage dependent and linked with the voltage-operated Ca^{2+} channel (VOCC). The

steady state of the membrane potential is reversed by Kv channel. The 4-aminopyridine (4-AP) is a Kv channel blocker (Nelson and Quayle, 1995, Loh et al., 2018). By the attachment with PIP_2, the Kir channel is activated, and the inwards movement of the K^+ ions takes place. Consequently, recovery of the resting membrane potential occurs (Tucker and Baukrowitz, 2008). The Kir channel is activated when the hyperpolarization state occurs, and the influx of K^+ ions takes place. The barium chloride ($BaCl_2$) is the only selective blocker, for the Kir channel (Edwards and Weston, 1995, Loh et al., 2018). The K_{ATP} channel acts as a weak inwardly rectifying K^+ channel, in resting membrane potential, because of that it is included in the classification of Kir channel family. On the activation, of K_{ATP} channel, the K^+ efflux takes place, to keep a negative resting potential and vasorelaxation occurs. The glibenclamide is a selective, K_{ATP} channel inhibitor (Jakala et al., 2009).

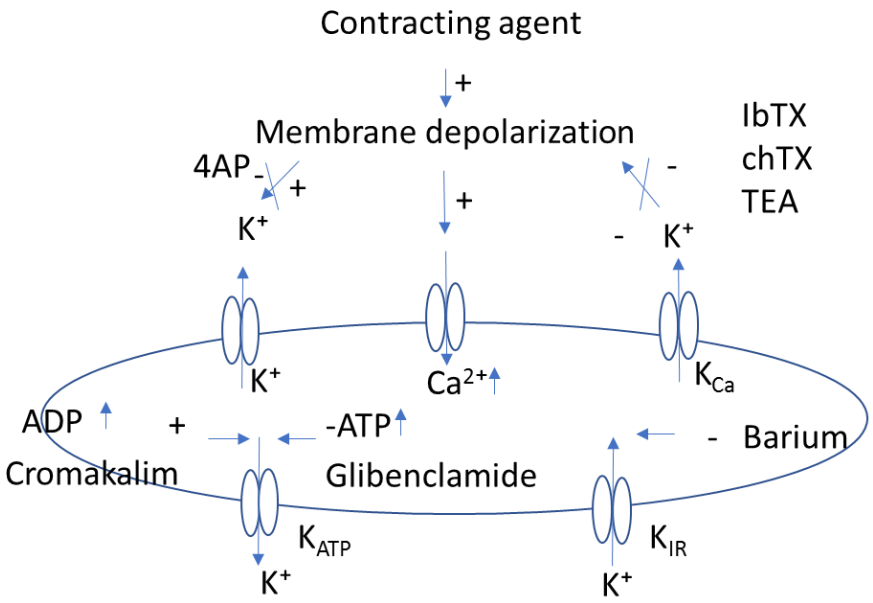

Figure 2.9 Potassium channels are controlling the tone of the vascular smooth muscle cells (Loh et al., 2018).

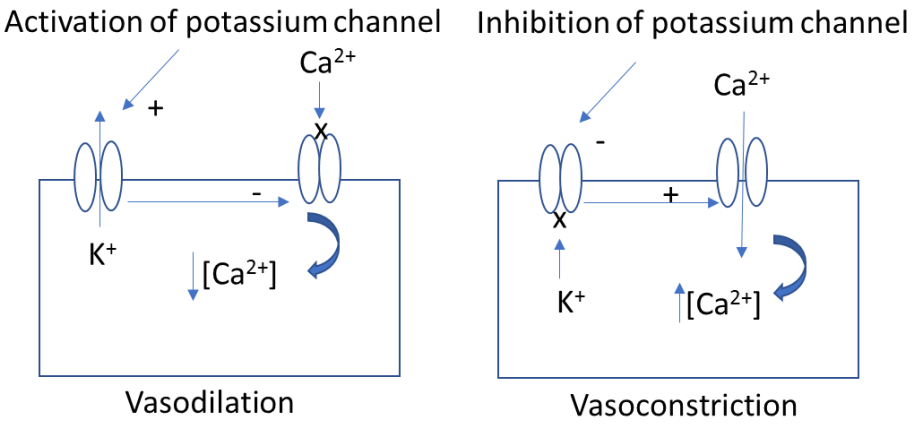

Figure 2.10 Function of the potassium channel in the blood vessels (Sobey, 2001).

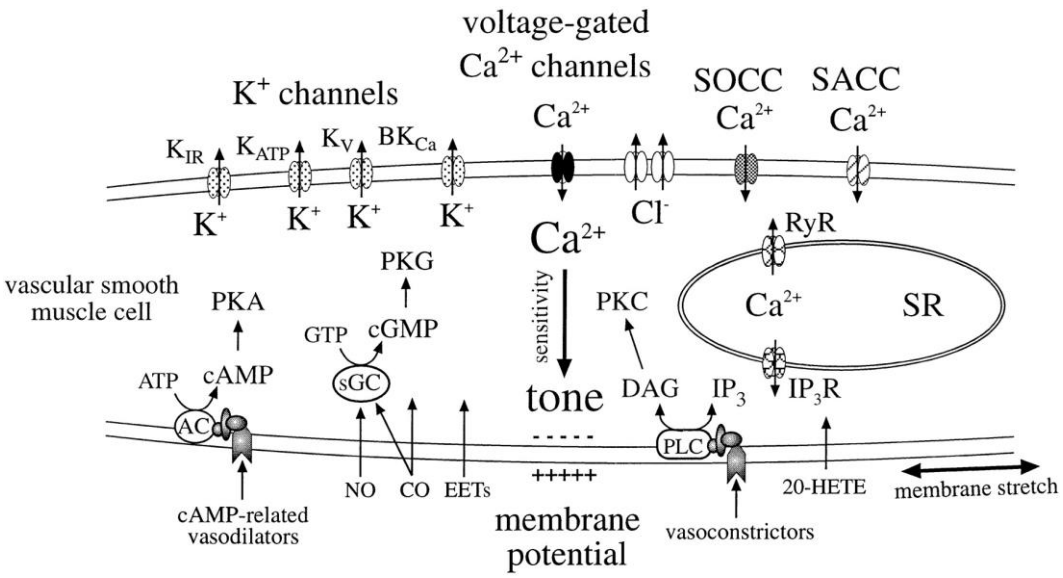

Figure 2.11 Ion channels and the blood vessel.

In a vascular smooth muscle cell, the K_{IR}, K_{ATP}, K_V, and BK_{Ca} are shown on the top. The voltage-gated Ca^{2+} channels are also present, two types of Cl^- channels, SOC channels (SOCC), and SAC channels (SACC). In the sarcoplasmic reticulum (SR) there are ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate receptors (IP_3R). The adenylate cyclase (AC), PKA, cAMP-dependent protein kinase, soluble guanylate cyclase (sGC), PKG, cGMP-dependent protein kinase, epoxyeicostetraenoic acid (EETs), phospholipase (PLC), diacylglycerol (DAG), protein kinase C (PKC) on the bottom (Jackson, 2000).

2.6.2 Calcium Channels

There are Ca^{2+} Channels, in the vascular smooth muscle cell, which are selectively permeable for Ca^{2+} ions and allows Ca^{2+} ions to enter the cytosol, depolarization occurs and causing vasoconstriction. There are three main types, of Ca^{2+} channel:

1. Voltage-operated Ca^{2+} channels (VOCC)
2. Receptor-operated Ca^{2+} channels (ROCC)
3. Store-operated Ca^{2+} channels (SOCC)

Usually, the cytosolic Ca^{2+} concentration is raised by two ways

1. The influx of Ca^{2+} ions from the outside
2. The intracellular release of Ca^{2+} from the SR store

The Ca^{2+} ions are the most important second messenger, in the blood vessels. The membrane depolarization occurs by the increased level of Ca^{2+} ions, in the cells and the up-regulation of Ca^{2+}- calmodulin complexes takes place. The activated calmodulin trigger, the MLC kinases (MLCK) to phosphorylate the MLC. As a result, the formation of actin-myosin protein (AMP) occurs, and the contraction of VSMCs takes place, via the mechanism of filament sliding (Jakala et al., 2009, Marchenko and Sage, 1996, Gao et al., 2003, Webb, 2003). The membrane potential, of the VSMCs, is maintained by the VOCC. Generally, the VOCC is known as an L-type Ca^{2+} channel. The concentration of the Ca^{2+} ions outside of the cell is thousand-time higher when compared to inside the cell, in normal physiological condition (McFadzean and Gibson, 2002). During the depolarization phase, the Ca^{2+} ions from the outside move into the cytosol via VOCC, as a result, vasoconstriction takes place (Patrick, 2002).

Other than the VOCC, there is another way for calcium, in the VSMCs, via the ROCC. The intracellular Ca^{2+} release via ROCC causes the membrane depolarization. The ROCC can induce the intracellular release of Ca^{2+} ions from the SR store into the cytosol. There are three main types of ROCC receptors such as:

1. IP_3R,
2. RyRs, and
3. Store-operated Ca^{2+} channels (SOCC)

The IP_3R are present on the SR and triggered by the second messenger, IP_3, which is formed by stimulated Gqα-protein-coupled receptors. It is the important site, for the release of intracellular Ca^{2+} from the SR store, which upsurges the formation of Ca^{2+}- calmodulin complexes (McFadzean and Gibson, 2002, Landsberg and Yuan, 2004, Putney et al., 2001). When the concentration of Ca^{2+} ions in the cell increases, the RyRs are activated and releases more Ca^{2+} ions from the SR store, which is necessary, for muscle contraction. The main function of SOCC is to the replacement of Ca^{2+} ions, so it is known as a capacitive-dependent calcium entry channel. In the SR store, the Ca^{2+} ions attach to calsequestrin and reducing the concentration of free Ca^{2+} ions. Consequently, more calcium is stored (McFadzean and Gibson, 2002, Loh et al., 2018, Swietach et al., 2008).