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am Zentrum für Biomedizin und Medizintechnik Mannheim
der Medizinischen Fakultät Mannheim
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Interaction of voltage-gated potassium channels and large-conductance calcium-sensitive potassium channels in vascular smooth muscle

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TABLE OF CONTENTS

Page

ABBREVIATIONS

ATP	adenosine triphosphate
BK channels	large-conductance calcium-sensitive and voltage-dependent potassium channels
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CICR	Ca^{2+}-induced Ca^{2+} release
DAG	diacylglycerol
IBTX	iberiotoxin
IC	internal circumference
IP_3	inositol 1,4,5 -trisphosphate
KCa channels	calcium-activated potassium channels
Kv channels	voltage-dependent potassium channels
K_{IR} channels	inward rectifier potassium channels
K_{ATP} channels	ATP-sensitive potassium channels
MLCK	myosin light chain kinase
MRs	myogenic responses
PD	pore-loop domain
PKA	protein kinase A (cAMP-dependent protein kinase)
PKC	protein kinase C
PKG	protein kinase G (cGMP-dependent protein kinase)
qPCR	real-time quantitative polymerase chain reaction technique
RCK	Ca^{2+}-sensing domain that regulate the conductance of K^+ of BK channles
RyRs	ryanodine receptors
SM	smooth muscle
SR	sarcoplasmic reticulum
STOCs	spontaneous transient outward currents
STTX	stromatoxin-1
VDCCs	voltage-dependent Ca^{2+} channels
Vm	membrane potential
VSD	voltage sensor domain
VSM	vascular smooth muscle
VSMCs	vascular smooth muscle cells

1 INTRODUCTION

The cardiovascular system is an organ system comprising three basic parts: heart, blood and vessels, in which the heart as a pump, blood as a carrier and vessels as elastic tubes, are integrated to transport oxygen, hormones, nutrients etc. to tissues of the body and take waste away from them. Disorders happening in any part of them would impair the health of the body and even threaten life.

Among them, vessels play an essential role in determination of blood pressure and regulation of blood distribution to different organs and tissues in order to meet the demand of the body in different environments. As known, except capillaries, the wall of vessels consists of three distinct layers: tunica intima, tunica media and tunica externa, where endothelial cells are located in the intima and smooth muscle cells in the media. Collagen fibers are the major part of the externa, and elastic fibers exist between each tunica (Fig.1.1).

The contractility of vascular smooth muscle cells (VSMCs) at the media creates vascular tone as a key element of vessel function that determines the diameter of vessels and hence creates the resistance to blood flow.

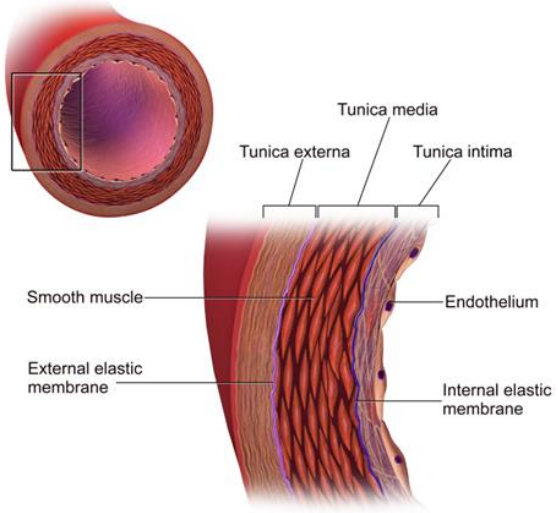

Figure 1.1 Structure of an artery wall
(Blausen.com staff, 2014)

The wall of an artery from the innermost layer to the outermost layer can be divided into tunica intima, tunica media and tunica externa.

1.1 Ion channels in vascular smooth muscle

Like all smooth muscles (SM), the contractile activity of vascular smooth muscle (VSM) is triggered by the increased intracelluar Ca^{2+}. When Ca^{2+} binds to calmodulin (CaM), this Ca^{2+}-CaM complex activates myosin light chain kinase (MLCK), which facilitates phosphorylation of myosin. The phosphorylated myosin sequentially attaches to and slides along actin, forming ‘cross bridges’, by which the muscle fibers shorten and vascular tone is increased (Fig 1.2)(Brozovich et al., 2016).

In VSMCs, the major source of activator Ca^{2+} is influx from the extracelluar space through voltage-dependent Ca^{2+} channels (VDCCs) whose activity is plasma membrane potential determined. As the activity of ion channels expressed within the plasma membrane of VSMCs is the major determinant of the membrane potential, ion channels play a central role in the maintenance of vessel contractile function. Consequently, a number of physiological modulators or stimuli in the circulatory

system, such as neurotransmitters, hormones, endothelium-derived factors and blood pressure itself, can via regulation of the activities of ion channels to mediate membrane potential, alter the entry of Ca^{2+} into VSM and hence regulate vascular tone (Knot and Nelson, 1998).

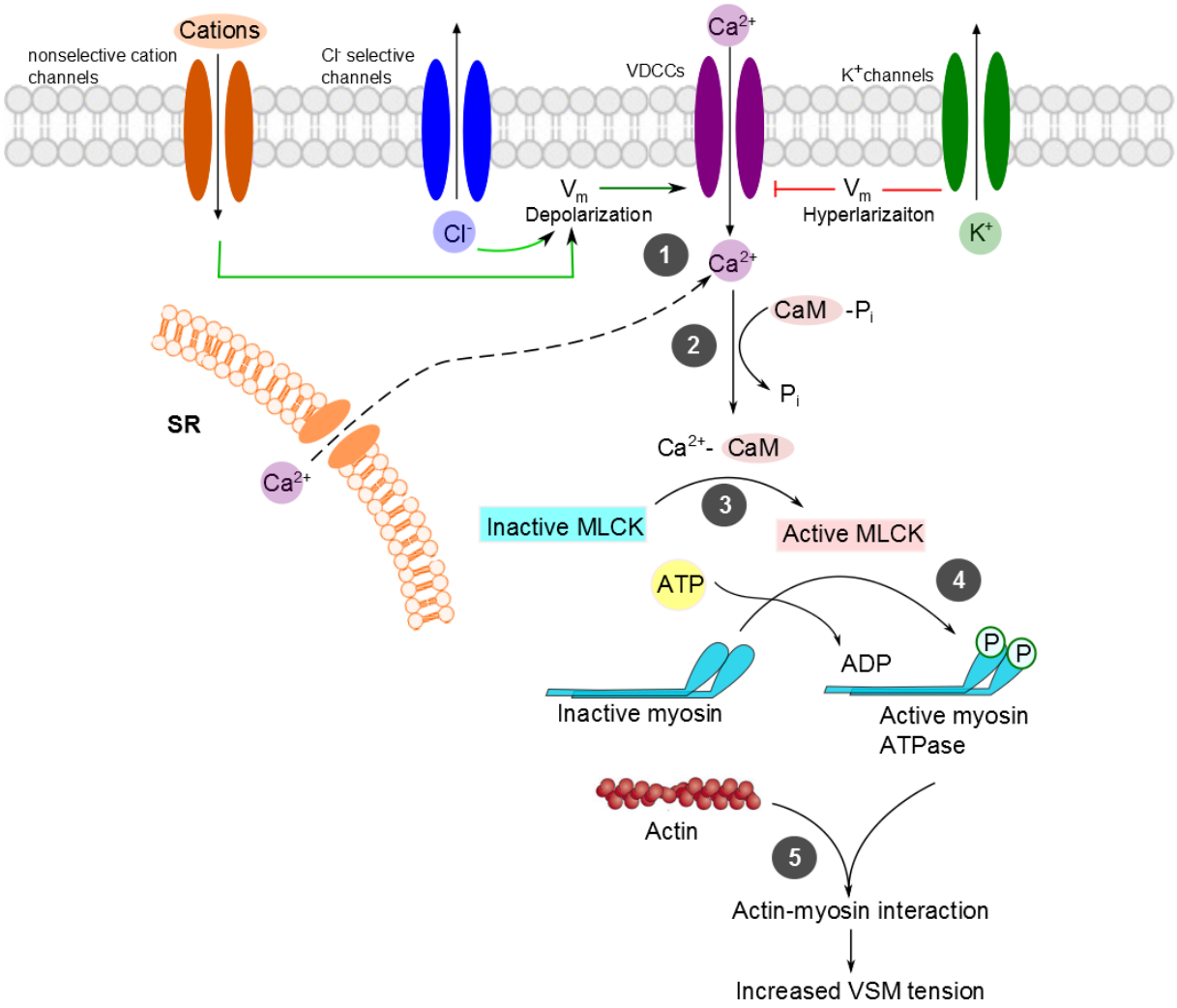

Figure 1.2 Membrane potential in the regulation of arterial smooth muscle tone

There are four major types of ion channels expressed in plasma membranes of VSMCs, which are nonselective cation channels, chloride (Cl^-) selective channels, voltage-dependent Ca^{2+} channels (VDCCs) and potassium (K^+) channels. Their activities determine VSM membrane potential to mediate the entry of Ca^{2+} into VSMCs and hence regulate vascular tension. When the membrane depolarization happened: 1) Intracellular Ca^{2+} concentration increased with Ca^{2+} influx via VDCCs and some Ca^{2+} released from the sarcoplasmic reticulum (SR). 2) Ca^{2+} binds to calmodulin (CaM). 3) Ca^{2+}-CaM activates myosin light chain kinase (MLCK). 4) MLCK phosphorylates light chains at myosin heads and increases myosin ATPase activity. 5) Active myosin cross bridges slide along actin and create VSM tension. V_m, membrane potential

It has been demonstrated that there are four major types of ion channels expressed in plasma membranes of VSMCs: (i) non-selective cation channels including store-operated Ca^{2+} channels and stretch-activated cation channels, (ii) Cl^- selective channels, (iii) voltage-dependent Ca^{2+} channels, and (iv) K^+ channels. Nonselective cation channels and Cl^- selective channels contribute to the depolarization of the membrane potential leading to activation of VDCCs to provide Ca^{2+} entry for VSM contraction, while K^+ channels counteract these vasoconstrictions through facilitation of membrane hyperpolarization and limitation of membrane depolarization resulting in a reduced entry of extracellular Ca^{2+} (Fig 1.2). All these channels may be involved in

the regulation of vascular tone, especially K^+ channels (Jackson 2000; Thorneloe and Nelson 2005).

1.2 Potassium channels

As the major hyperpolarizing ion conductive pathway in VSMCs, potassium channels play a central role in the determination of the membrane potential and in the regulation of vascular tone. It has been established that abnormal expression, downregulation, or dysfunction of potassium channels are involved in the pathogenesis of many cardiovascular pathological conditions: hypotension, excessive vasoconstriction or vasospasm, hypertension as well as ischemia (Nelson and Quayle 1995; Jackson 2000; Thorneloe and Nelson 2005).

There are four major types of potassium channels identified in VSMCs:

1) *Voltage-dependent potassium (Kv) channels* are activated by membrane depolarization.

2) *Large-conductance calcium-sensitive and voltage-dependent potassium (BK, MaxiK, Slo1, KCa1.1) channels* are one subfamily of calcium-activated potassium (KCa) channels characterized by large conductance for potassium ions (250-300pS in symmetrical 150mM KCl) that is 10-20 times greater than the classical voltage-activated K^+ channels. They respond to changes in membrane potential and intracellular Ca^{2+} (Lang and Ritchie, 1990; Pallotta et al., 1981).

3) *Inward rectifier potassium (K_{IR}) channels* respond to changes in extracellular K^+ to regulate the resting membrane potential in small resistance vessels (Bradley et al., 1999; Quayle et al., 1997).

4) *ATP-sensitive potassium (K_{ATP}) channels* are activated by cytoplasmic ATP depletion to sense the metabolic state of VSM.

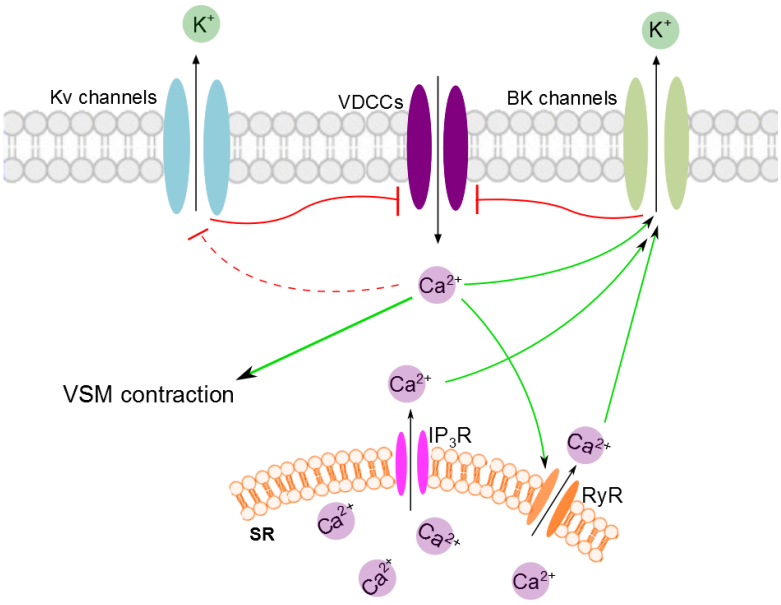

Figure 1.3 Negative feedback mechanisms of potassium channels in VSM contraction

With membrane depolarization, amounts of Ca^{2+} influx through VDCCs, which not only induce VSM contraction, but also evoke Ca^{2+} transiently released from sarcoplasmic reticulum (SR) through RyR and IP_3R. At this time, voltage-dependent (Kv) activated by depolarization and large-conductance, Ca^{2+}-sensitive, voltage-dependent (BK) channels activated by depolarization as well as increased Ca^{2+} provide a hyperpolarization conductance, thereby braking depolarization-mediated VSM contraction by inhibition of Ca^{2+} entry through VDCCs.

Among them, Kv and BK channels are most ubiquitously expressed in VSM, and they have been attracting increasing attention to investigate their specific roles as negative feedback regulators in the regulation of vascular tension and the

possibilities of regulation of their function for treatment of relevant vascular diseases (Fig 1.3).

1.3 Kv7 (KCNQ) channels

To date, 12 subfamilies of Kv channels (Kv1– Kv12) have been identified (Wulff et al., 2009). Among them, Kv7 channels (Kv7.1–Kv7.5) encoded by the KCNQ (KCNQ1–5) genes have emerged as a spotlight in VSM research in the recent ten years. Originally, KCNQ channels were thought to be distributed strictly in particular tissues with distinct functions (Robbins, 2001), such as KCNQ1 discovered in cardiac myocytes contributing to action potential repolarization, KCNQ2, KCNQ3 and KCNQ5 detected in the nervous system contributing to the M-current and the resting potential of neurons, and KCNQ4 in inner ear hair cells relating to the resting membrane potential and the submembrane Ca^{2+} concentration. However, in recent years, an increasing number of reports indicated that KCNQ channels are not only expressed in various VSMCs, but also fulfill an important role in the regulation of contractile and relaxant functions of arteries. Moreover, they might become a novel therapeutic target for the treatment of disorders of the vascular system (Ohya et al., 2003; Yeung et al., 2007).

Structurally, like other Kv channels, Kv7 channels are a complex assembled by four pore-forming α-subunits encoded by KCNQ genes. Each α-subunit contains six trans-membrane spanning domains (S1–S6) with cytoplasmic NH_2- and COOH-terminal regions. The S1-S4 regions form the voltage sensor domain (VSD) where S4 contains positively charged amino acids (arginines) as voltage-sensing elements. A single K^+-selective pore-loop domain (PD) electromechanically coupled to the VSD is located between the S5 and S6 regions. At the COOH-terminal region, α subunits can interact with accessory proteins, e.g., β-subunits encoded by KCNE 1-5 genes, to modulate the biophysical properties of Kv7 channels (Fig 1.4) (Pongs et al. 1999; Bähring et al. 2001; Robbins, 2001; Thorneloe et al. 2001; Korovkina and England, 2002; Pongs and Schwarz 2010; Soldovieri et al., 2011).

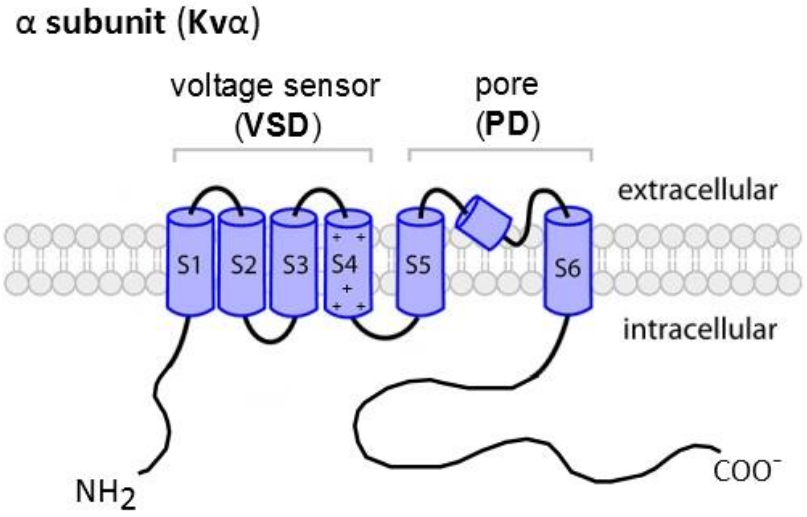

Figure 1.4 Schematic structure of Kv channel α-subunits

The Kv channel α subunit consists of six transmembrane spanning domains (S1–S6) with cytoplasmic NH_2- and COOH- terminal segments, S1-S4 is the voltage sensor domain (VSD) with positively charged arginines in S4, and the single K^+-selective pore-loop domain (PD) is located between S5 and S6.

Functionally, Kv7 channels are directly activated by membrane depolarization. The activation threshold is relatively negative at approximately -60 mV. When membrane depolarization stimulated the VSD into the active state, the VSD can attach to the PD and converts the electrical energy in the membrane into mechanical force to alter the conformation of the K^+ selective pore from the closed to the open state (González et

al. 2012; Stott et al. 2014). As soon as the Kv7 channels open, the increased K^+ efflux driven by the K^+ ions electrochemical gradient promotes hyperpolarization of the plasma membrane. Thus, Kv7 channels provide an important K^+ conductance for the maintenance of the membrane potential of VSMCs in the physiological range (-40mV~ -70mV), to limit membrane depolarization, brake the activation of VDCCs, and hence maintain vascular tone(Sperelakis and Ohya, 1989; Nelson and Quayle 1995; Stott et al. 2014). Notably, an agonist-induced increase of intracellular Ca^{2+} following membrane depolarization has been reported to inhibit Kv channel activity (Tykocki et al., 2017), but the molecular mechanism of this interaction is unclear.

Several selective Kv7 channel-specific pharmacological agents have been developed and became very effective tools to investigate and determine the specific functions of Kv7 channels in various organs. Specifically, XE991 and linopirdine selectively inhibit Kv7 channels and increase vascular tone, while retigabine and flupirtine can selectively activate Kv7 channels except homomeric KCNQ1 and lead to the relaxation of preconstricted arteries (Tatulian et al., 2001; Joshi et al. 2009; Ng et al. 2011; Morales-Cano et al. 2015).

In addition, some studies indicate that Kv7 channels act as effectors for some vasodilators and vasoconstrictors. Thus, Kv7 channels can be activated by cyclic AMP (cAMP)-dependent vasodilators (e.g., isoproterenol) and cyclic GMP (cGMP)-dependent vasodilators (e.g., sodium nitroprusside), whereas vasoconstrictors (e.g., vasopressin) might inhibit Kv7 channels by PKC activation (Stott et al., 2014, 2015).

1.4 BK channels

The BK channel, one of the most ubiquitously expressed channels in various VSMCs, has been investigated intensively in the recent three decades (Nelson and Quayle 1995; Ko et al. 2008). Because of its high expression level and large single channel conductance, BK channels are one of the central determinants in the regulation of the plasma membrane potential (Nelson and Quayle 1995; Jackson 2005).

The structure of BK channels is similar to Kv7 channels (Fig 1.5). It is also a tetramer of pore-forming α-subunits accompanied with tissue specific regulatory β subunits. The α-subunits, encoded by a single gene (KCNMA1), confer the voltage-dependent and Ca^{2+}-sensitive abilities of the BK channel. Each α-subunit is composed of seven trans-membrane spanning domains (S0–S6) with an extracellular NH_2-terminal segment and a large cytoplasmic COOH-terminal tail (Wallner et al., 1996). Likewise, the voltage sensor domain (VSD) of BK channels is formed by S1-S4 where the S4 region also contains positively charged arginines. The S5–S6 domain forms the K^+ selectivity pore-loop domain (PD). The S0 domain with an extracellular NH_2-terminal segment can directly interact with the VSD as well as with regulator β-subunits. The COOH-terminal tail is sensitive to intracellular Ca^{2+}, and is thought to include two tandem Ca^{2+}-sensing domains that regulate the conductance of K^+ (RCK): RCK1 and RCK2. These two RCK domains include at least three Ca^{2+} binding sites: the first high-affinity Ca^{2+} site is the voltage independent Ca^{2+} bowl located in RCK2 which functions at low Ca^{2+} concentrations; the second high-affinity Ca^{2+} site stays at the upstream RCK1, is voltage dependent and modulates channel activity during both activation and deactivation at Ca^{2+} concentrations higher than 10 μmol/L; a third lower affinity divalent cation sensing domain in the RCK1 is able to bind Mg^{2+} and Ca^{2+} to affect VSD function (Schreiber and Salkoff 1997; Toro et al., 1998; Wu and Marx, 2010; González et al. 2012). The biophysical properties of the BK channel,

especially its Ca^{2+} sensitivity, can be modified and enhanced by association with β-subunits that consist of two transmembrane spanning domains leaving an intracellular NH_2- segment and COOH-terminal tail. There are four isofoms of β-subunits encoded by the KCNMB1–4 genes (Uebele et al. 2000; Brenner et al. 2000; Cox and Aldrich 2000). Among them, β1 is abundant in VSMCs(Jiang et al. 1999; Sausbier et al. 2005; Brenner et al. 2000). Besides β-subunits, γ-subunits are also detected to affect BK channel regulation (Zhang and Yan, 2014).

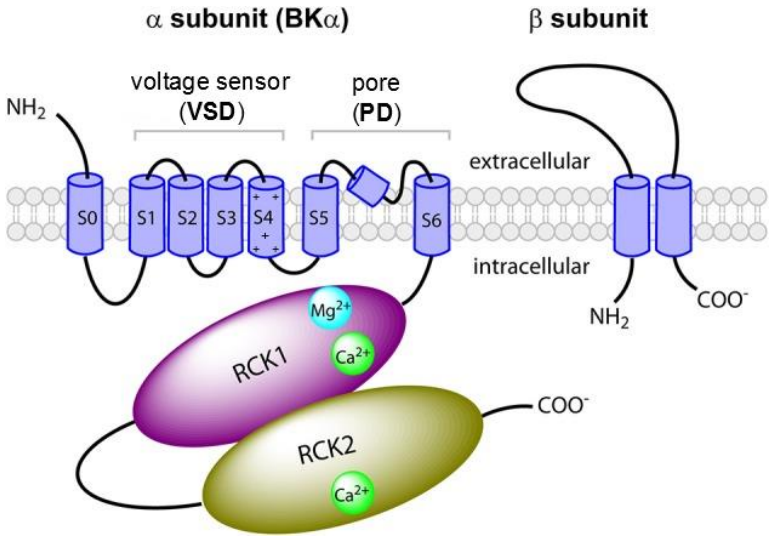

Figure 1.5 Schematic structure of BK channel α and β subunits
(Zhang and Yan, 2014), modified

The BK channel α subunit consists of seven transmembrane spanning domains (S0–S6) with extracellular NH_2- and cytoplasmic COOH-terminal segments, S1-S4 is the voltage sensor domain (VSD) with positively charged arginines in S4, and the single K^+-selective pore-loop domain (PD) is located between S5 and S6;
Accessory β-subunit associate with BK channels, which is composed of two transmembrane spanning domains leaving intracellular NH_2- and COOH-terminal segments.

The BK channel is activated synergistically by membrane depolarization and the increased intracellular localized Ca^{2+}. Generally, when the Ca^{2+} entry through VDCCs stimulates Ryanodine receptors (RyRs) on the SR, Ca^{2+}-induced Ca^{2+} release (CICR) events can be initiated, in which Ca^{2+} is released from the SR in the form of Ca^{2+} sparks; meanwhile, Ca^{2+} is also released in the form of puffs from SR when IP_3 receptors on the SR are activated by Ca^{2+} influx through VDCCs (Jaggar et al., 2000). With these transient localized Ca^{2+} released from the SR as well as with some of the Ca^{2+} entering the cells via VDCCs, binding to the RCK on the BK channel decreases the necessary energy required to open the BK channel by shifting the voltage-dependent activation to negative voltages, so that the BK channel can be opened within a physiological range of membrane potentials and generate spontaneous transient outward currents (STOCs) (Cox and Aldrich 2000; Perez et al., 2001; Hill et al. 2010; Hu and Zhang 2012). Therefore, the activity of BK channels is fundamental for the control of vascular tone by discouraging membrane depolarization and braking further Ca^{2+} entry through VDCCs (Klockner and Isenberg 1985; Brayden and Nelson 1992; Nelson and Quayle 1995; Guia et al. 1999; Herrera and Nelson 2002). Meanwhile, BK channels also provide a bridge between the ionic metabolism of the cell and plasma membrane conductance with its ability to sense changes both in membrane potential and in the intracellular Ca^{2+} concentration.

BK channel selective agents, either activators or blockers, are both useful tools for the exploration of the particular roles of the BK channel. In VSM, BK channels can be blocked by external tetraethylammonium (TEA, half-inhibition concentration ~200 μM), charybdotoxin (~10 nM) or iberiotoxin (IBTX, <10 nM) leading to membrane potential depolarization and vasoconstriction. Among them, IBTX is the most selective blocker

(Galvez et al. 1990; Nelson and Quayle, 1995). However, till now, regarding BK channel activators, although several candidates have emerged, there is no confirmed agent with high selectivity for the BK channel. In 2014, B. Nausch with his colleagues proposed that NS19504 is a positive modulator of BK channels in urinary bladder SM (Nausch et al., 2014), but there is no report describing how NS19504 acts on VSM. In this study, NS19504, as a promising candidate, was tested.

Similar to Kv7 channels, cAMP-dependent and cGMP-dependent vasodilators might also activate the BK channel through PKA and PKG, respectively. Conversely, vasoconstrictors might inhibit BK channels by PKC activation (Ko et al., 2008).

1.5 Questions and aims

1) The role of Kv7 and BK channels in the contractility of skeletal muscle arteries

To date, most investigations on the role of Kv7 channels or BK channels in pressure-induced constrictions were performed on cerebral arteries, while in agonist-induced contractions it was preferred to employ mesenteric arteries. There are only a few studies to show the expression and function of Kv7 or BK channels in skeletal muscle arteries.

Thus, in this study we examined Kv7 and BK channels in two skeletal muscle arteries: the Saphenous artery (A. Saphena), a conduit artery responsible for the blood supply to the skeletal muscles of rat hind limb, and the Gracilis artery (A. Gracilis), a resistance artery responsible for the blood supply to Gracilis muscle (M. gracilis) on the rat hind limb. The role of Kv7 and BK channels in the contractility of A. saphena and A. Gracilis was studied by methoxamine-induced contractions in wire myograph experiment and by pressure-induced myogenic responses in pressure myograph experiments, respectively.

2) The interaction between Kv7 channels and BK channels

As far as we know, although there are an increasing number of investigations focusing on the function of Kv7 channels or BK channels in VSM, there is no report to describe and clarify whether there is functional interaction between both of them.

As mentioned above, Kv7 channels are voltage-dependent, while BK channels are voltage-dependent as well as calcium-sensitive. Both of them can respectively maintain vascular tone through limiting membrane depolarization and regulating Ca^{2+} entry. Theoretically, Kv7 channels could alter the membrane potential and Ca^{2+} influx through VDCCs and thereby affect the activity of BK channels. In turn, BK channels also can regulate the activity of Kv7 channels by inducing changes in membrane potential. For example, inhibition of Kv7 channel activity leads to membrane potential depolarization. This depolarization may activate BK channels directly as well as indirectly by the subsequent increase of intracellular Ca^{2+} via the depolarization-induced opening of VDCCs. On the other hand, inhibition of BK channels also leads to membrane potential depolarization that may directly activate Kv7 channels, but with depolarization, the subsequent increase of intracellular Ca^{2+} might contribute to a deactivation of Kv7 channels, in which the joint effect of activation and deactivation is unclear (Fig 1.6).

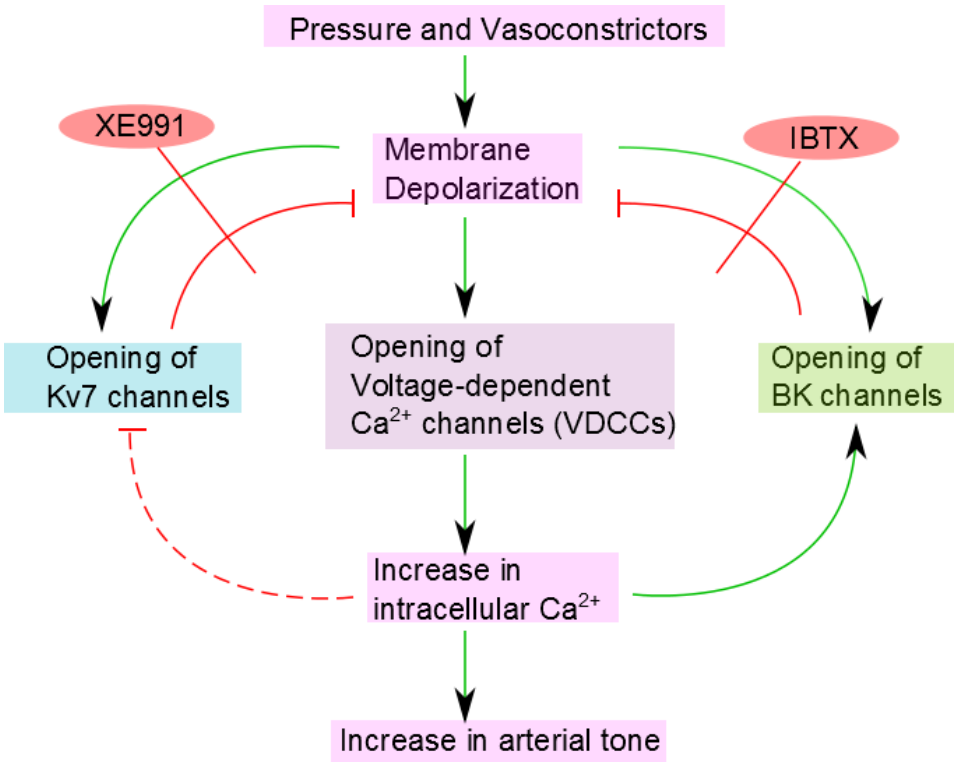

Figure 1.6 Flow diagram of the proposed roles of Kv7 and BK channels
(Nelson & Quayle 1995), modified

Therefore, theoretically an interaction between Kv7 and BK channels exists, but experimental supporting evidence is lacking. In this study, selective activators and blockers of Kv7 and BK channels were employed to examine the hypothesis that there is functional interaction between Kv7 and BK channels under normal physiological conditions, which contributes to the maintenance of skeletal muscle arterial contractility.

2 MATERIAL AND METHODS

2.1 Matertials

2.1.1 Animals

Wistar rats (male, 8-12 weeks old) were decapitated after carbon dioxide (CO_2) anesthetization. The experimental design was approved by the local authority responsible for animal experimentation. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Eighth edition, National Academy of Sciences, 2011).

2.1.2 Arteries

The gracilis artery (A. Gracilis, resistance artery) and the saphenous artery (A. Saphena, conduit artery) both located on the rat hind limb were used to perform the investigation of the present study (Fig. 2.1).

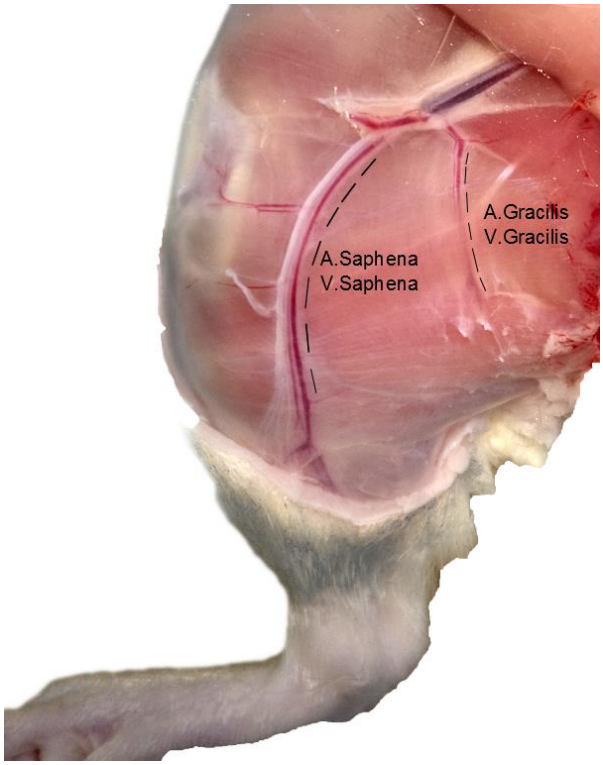

Figure 2.1 Vessel anatomy of the rat left hind limb after removal of the skin

The label *A. Gracilis* represents the Gracilis artery that is one branch of the femoral artery;
The label *A. Saphena* represents the Saphenous artery that arises from the femoral artery
Both of them are accompanied by their corresponding veins (V.) and nerves, respectively.

2.1.3 Reagents

In myograph experiments:

Name	Catalogue No.	Manufacturer
$CaCl_2$*$2H_2O$	A862982 824	Merck
D(+)-Glucose	455236245	Carl Roth
EDTA	485234480	Carl Roth
EGTA	465235141	Carl Roth
HEPES	A1069,1000	Applichem
KCl	475237252	Carl Roth

$MgSO_4*7H_2O$	A0478986321 411	Merck
NaCl	486252534	Carl Roth
$NaH_2PO_4*H_2O$	A0314746 131	Merck
$NaHCO_3$	K45372729 421	Merck
Acetylcholine chloride	A6625-25G	Sigma-Aldrich
DPO-1	2533	Tocris Bioscience
HMR1556	5011	Tocris Bioscience
Iberiotoxin	STI-400	Alomone Labs
Methoxamine hydrochlorid	M6524-500MG	Sigma-Aldrich
NS19504	5276	Tocris Bioscience
Retigabine	Lot. 0005005	Valeant research and development
Stromatoxin-1	STS-350	Alomone Labs
XE991 dihydrochloride	2000	Tocris Bioscience

- Specific function of essential reagents

Name	Function
XE 991	potent and selective Kv7 channel blocker
Retigabine	Kv7 channel activator, especially for Kv7.2-Kv7.5
Iberiotoxin (IBTX)	selective and reversible BK channel blocker
NS19504	BK channel activator
HMR1556	potent and selective Kv7.1 channel blocker
DPO-1	selective Kv1.5 channel blocker
Stromatoxin-1(STTX)	selective Kv2.1 channel blocker
Acetylcholine	endogenous neurotransmitter active at acetylcholine receptors
Methoxamine	α1-adrenergic receptor agonist

In real-time PCR experiments:

Name	Catalogue No.	Manufacturer
RNeasy Mini Kit (50)	74104	QIAGEN
Qiazol Lysis Reagent	5,346,994	QIAGEN Sciences
RNase-Free DNase Set (50)	79254	QIAGEN
Ethanol (90%)	T9132	Roth
5×Reaction Buffer	#EP0452	Thermo scientific
dNTP Mix, 10 nM each	#R0192	Thermo scientific
RevertAid H Minus ReverseTranscriptase	#EP0451	Thermo scientific
Random Primers	48190-011	Invitrogen
Water, nuclease-free	#R0581	Thermo scientific
SensiFAST™ SYBR No-ROX Kit, 2×	BIO-98020	Bioline
Primers *	-	Eurofins

*- Specific primers in qPCR

Gene	Forward Primer Reverse Primer	Primer Sequence 5´→ 3´	Size (bp)	Dilution
KCNQ1	ratfor1int ratrev3	GGCTCTGGGTTTGCACTG (18) CATAGCACCTCCATGCAGTC (20)	105	1:100 1:100
KCNQ2	for_1 rev_1	ACACAGACTCAGACCTCTGCAC (22) AGCCCAACCCAGAATCACTTCC (22)	115	1:100 1:100
KCNQ3	rat for new rat rev new	GCTAGGGACCGGAGCCGACA (20) CCCCTCGGTCTCTCCAGGGC (20)	192	1:100 1:100
KCNQ4	ratfor1int ratrev2	CCCCGCTGCTCTACTGAG (18) ATGACATCATCCACCGTGAG (20)	86	1:100 1:100
KCNQ5	ratfor2out ratrev2	GATGCCAGTGTGACGTGTCCGTGG (24) CCTTTCCGAGGACCTGCTGGTAG (23)	393	1:100 1:100
rBKalpha	for Yan rev Yan	AAACAAGTAATTCCATCAAGCTGGTG(26) CGTAAGTGCCTGGTTGTTTTGG(22)	137	1:100 1:100
rBKbeta1	for3 rev3	ACCAATCTCTTCTGCACAGCAGC(23) AGAGCTGTGACTGGCAGTTCCTT(23)	378	1:100 1:100
eNOS	rat for1 rat rev1	GGATTCTGGCAAGACCGATTAC (22) GGTGAGGACTTGTCCAAACACT (22)	159	1:100 1:100
Hmbs	for_1 rev_1	GCGGAAGAAAACGGCTCAATG (21) AGCATCGCTACCACAGTGTC (20)	95	1:100 1:100

2.1.4 Essential buffers

Experimental solution (pH 7.4, 37°C)

(To simulate the in vivo environment of vessels in wire myograph experiments)

Reagent	MW (g/mol)	Concentration (mmol/L)
NaCl	58,44	120
$NaHCO_3$	84,01	26
KCl	74,55	4.5
$NaH_2PO_4*H_2O$	137,99	1.2
$MgSO_4*7H_2O$	246,48	1
$CaCl_2*2H_2O$	147,02	1.6
EDTA	292,25	0.025
Glucose	180,16	5.5
Hepes	238,31	5

Experimental solution without $NaHCO_3$ (pH 7.4, 37°C)

(To simulate the in vivo environment of vessels in pressure myograph experiments)

Reagent	MW (g/mol)	Concentration (mmol/L)
NaCl	58,44	146
$NaHCO_3$	84,01	-
KCl	74,55	4.5
$NaH_2PO_4*H_2O$	137,99	1.2
$MgSO_4*7H_2O$	246,48	1

$CaCl_2*2H_2O$	147,02	1.6
EDTA	292,25	0.025
Glucose	180,16	5.5
Hepes	238,31	5

Preparation solution (pH 7.4, 37°C)

(To keep vessel viable during isolation, mounting on wire myograph and normalization processes)

Reagent	MW (g/mol)	Concentration (mmol/L)
NaCl	58,44	145
KCl	74,55	4.5
$NaH_2PO_4*H_2O$	137,99	1.2
$MgSO_4*7H_2O$	246,48	1
$CaCl_2*2H_2O$	147,02	0.1
EDTA	292,25	0.025
Hepes	238,31	5

Calcium-free solution (pH 7.4, 37°C)

(To determine the passive diameter of the vessels at the end of pressure myograph experiments)

Reagent	MW (g/mol)	Concentration (mmol/L)
NaCl	58,44	120
$NaHCO_3$	84,01	26
KCl	74,55	4.5
$NaH_2PO_4*H_2O$	137,99	1.2
$MgSO_4*7H_2O$	246,48	1
EGTA	380,35	1
Hepes	238,31	5
Glucose	180,16	5.5

2.1.5 Instruments

In myograph experiments:

Name	Type	Manufacturer
Multi Wire Myograph system	620M	Danish Myo Technology A/S (DMT)
Pressure Myograph system		DMT
Nylon suture	100115	DMT
Stainless steel wire	40 µm	DMT
Forceps	5-Inox-H	Fine Science Tools
Vannas Capsulotomy Scissors	G19760	Geuder
Stereo Microscope	Stimmi 2000-C	Zeiss
Laboratory pH meter	Lab 850	SCHOTT instruments

In real-time PCR experiment:

Name	Type	Manufacturer
Tissue Lyser	II	QIAGEN
NanoQuant Plate	Infinite 200 PRO	TECAN
Mastercyler	Gradient 5333	Eppendorf
Light Cycler	480	Roche
Microcentrifuge	CT15RE	VWR

2.1.6 Software

Name	Manufacturer/Designer
LabChart 7 Software model MKIIII	AD Instruments Pty Ltd, Germany
HaSoTec Bloodvessel Analyzer, BVA 300	HaSoTec, Germany
Light Cycler 480 software	Roche
LinRegPCR software	Academic Medical Center, Netherlands
GraphPad Prism 6	GraphPad Software, USA
Inkscape 0.48	

2.2 Methods

2.2.1 Isolation of arteries

- Once the rat was decapitated, the hind limbs were severed from the rat body.
- The leg was placed in a petri dish with cold preparation solution.
- Under the microscope, the arteries (A. Gracilis and A. Saphena) were carefully isolated from the leg muscle and all connective tissue.

2.2.2 Pressure myograph experiments

This method is a newer approach to investigate physiological functions of vessel segments under isobaric conditions, in which a cannulated vessel segment is mounted between a perfusion pipette and a holding pipette. The perfusion pipette is connected to a pressure reservoir. The holding pipette is closed to prevent flow through the vessel lumen. Through the perfusion pipette, the A. Gracilis receives different levels of pressure that can stimulate the artery to perform myogenic responses. The myogenic response, also known as the “Bayliss effect”, is an intrinsic reaction of small arteries to changes of intravascular pressure, in which the artery can constrict further or dilate from an original partially contracted state (myogenic tone) (Bayliss 1902, Osol et al. 2002). These responses can be detected as diameter changes of an artery. In this study, the changes of myogenic responses were observed when the artery was challenged with different activators and inhibitors of potassium channels (Fig. 2.2).

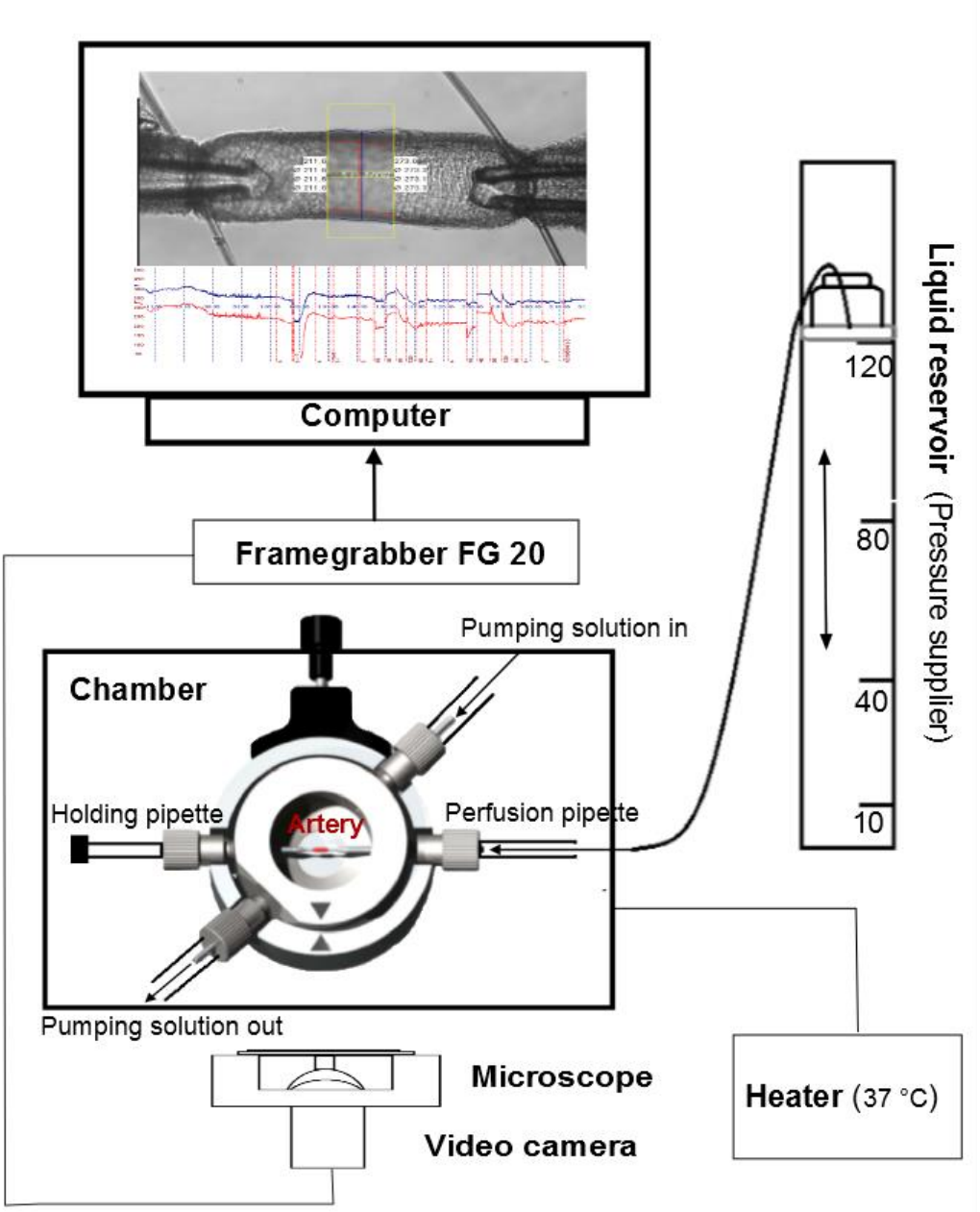

Figure 2.2 Scheme of the arrangement of a pressure myograph experiment

After the artery is mounted, the myograph chamber is placed onto the work stage containing a microscope (objective 10×/0.25) fitted with a video camera, and connected to the heater and the solution perfusion pump. Meanwhile, the perfusion pipette of the chamber is connected to a pressure reservoir, allowing the pressure in the artery to be precisely controlled and altered. The image of the artery is shown on a monitor and the diameter of the artery can be continuously measured throughout the experiment.

2.2.2.1. Mounting of arteries

- An isolated A. Gracilis segment (approx. 2 mm long) was placed into the pressure myograph chamber with fresh experimental solution without $NaHCO_3$ (chamber volume = 1.5 ml).
- Two suture loops were positioned appropriately on each glass pipette (ready for following mounting procedure).
- The isolated A. Gracilis was mounted to the perfusion pipette firstly, and secured onto the pipette by tightening the suture loop around it.

- The endothelium was disrupted by gently pushing a few air bubbles through the vessel lumen from the perfusion pipette with use of a syringe.
- The free end of A. Gracilis was gently fixed on the holding pipette.
- The chamber was placed onto the pressure myograph work stage, and connected to the solution pump for chamber perfusion and the pressure reservoir (at 10mmHg).

2.2.2.2. Initial incubation of arteries

- The chamber was heated to 37°C throughout the experiment.
- The solution pump was switched on to keep the experimental solution without $NaHCO_3$ fresh in the chamber (perfusion rate 1.5ml/min).
- The pressure reservoir was slowly positioned to the level of 80 mmHg.
- After the first 30 minutes, the bath solution mixer was fixed into the chamber beside the artery.
- After the second 30 minutes, the solution pump was switched off for 10min.

2.2.2.3. Viability test

- Application of methoxamine 10^{-5} mol/L, 3 min (to test the contractile activity of the artery).
- Application of acetylcholine 10^{-5} mol/L, 2min (to assess endothelium function).
- Wash out (pump on and off, 10 min each)

2.2.2.4. Myogenic response experiment

- **MRs**, 5min at each pressure level
  - **MRs** represents 'Myogenic Responses' performed at 4 different pressure levels by changing pressure from 80 to 10, 40, 80, 120 mmHg and then back to 80 mmHg

  Wash out (pump on and off, 10 min each)

- **MRs**, 5min at each pressure level, to test stability of MRs

  Wash out (pump on and off, 10 min each)

- Each chamber underwent treatments in the following sequence :

Chamber 1	Chamber 2	Chamber 3	Chamber 4
A***(Table 2.1)**	A	As	As
B	Bs	Bs	B

- **MRs,** 5min at each pressure level

  Wash out (pump on and off, 10 min each)

- **MRs**, 5min at each pressure level

- Calcium-free solution was used to replace the experimental solution in the chamber, 15 min

  MRs, 4min at each pressure level

***Table 2.1 Treatments A, As, B, Bs in different series in pressure myograph experiment**

series	A (mol/L)	As (µl)	Time(min)	B (mol/L)	Bs (µl)	Time(min)
1	NS19504 3×10^{-6}	DMSO 1.5	10	IBTX 10^{-7}	H_2O 5	10
2	Retigabine 3×10^{-6}	DMSO 1.5	10	XE991 3×10^{-6}	H_2O 1.5	10
3	Retigabine 3×10^{-6}	DMSO 1.5	10	NS19504 3×10^{-6}	DMSO 1.5	15
4	NS19504 3×10^{-6}	DMSO 1.5	15	XE991 3×10^{-6}	H_2O 1.5	10
5	XE 991 3×10^{-6}	H_2O 1.5	10	IBTX 10^{-7}	H_2O 5	10
6	Retigabine 3×10^{-6}	DMSO 1.5	10	IBTX 10^{-7}	H_2O 5	10
7	HMR1556 10^{-5}	DMSO 5	10	IBTX 10^{-7}	H_2O 5	10
8	DPO-1 $3*10^{-7}$	DMSO 1.5	10	IBTX 10^{-7}	H_2O 5	10
9	STXX 10^{-7}	H_2O 5	10	IBTX 10^{-7}	H_2O 5	10

2.2.2.5. Analysis of pressure myograph data

The normalized diameter is used to compare the myogenic responses between different arteries treated with different substance, which is the diameter of arteries in MRs (D_x) divided by their diameter at 80 mmHg in calcium-free solution (D_{80max}).

$$\text{Normalized Diameter} = D_x / D_{80max}$$

2.2.3 Wire myograph experiments

Wire myography is another classical method developed in the 1970s to investigate the function of blood vessels under isometric conditions (Bevan and Osher, 1972). This method employs two stainless steel wires that are passed through the lumen of ring vessel segments and hold it at a certain constant internal circumference (IC). As one of the wires is connected to a force transducer, the tension of vessels under different activation conditions can be measured (Fig. 2.3). In this study, the tension of A. Saphena generated by methoxamine was recorded during application of different activators or inhibitors of potassium channels.

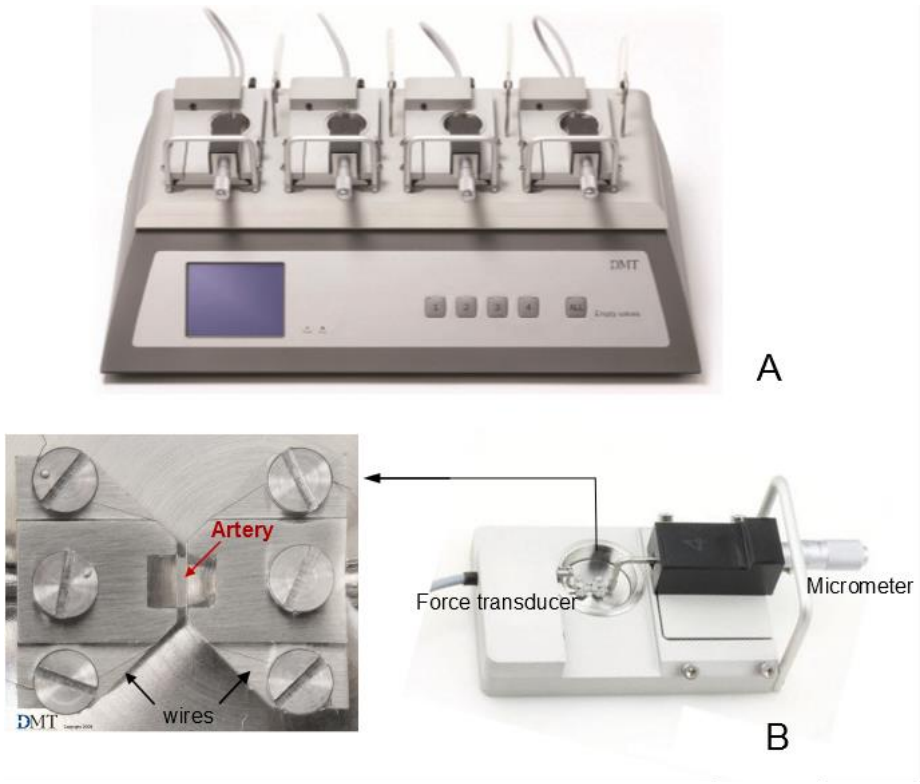

Figure 2.3 Wire myograph system
(www.dmt.dk, Stand 06.04.2017), modified

A) Multi wire myograph system 620M (DMT) with four myograph chambers allowing four vessels to be simultaneously studied. **B)** Each myograph chamber contains two jaws to fix two wires supporting the vessel segment. The left-hand jaw and the right-hand jaw are connected to a force transducer and a micrometer, respectively.

2.2.3.1. Mounting of arteries

- The isolated A. Saphena segment (approx. 2 mm long) was transferred into the wire myograph chamber with fresh cold preparation solution (chamber volume = 5ml).
- One end of a stainless steel wire (approx. 2.5 cm long) was fixed under the upper screw of the left-hand jaw of the chamber (Fig. 2.4 1-2).
- The artery was gently pulled on and then along the wire with fine forceps to feed the wire onto it (Fig. 2.4 3-4).
- When the artery is situated in the gap between the jaws, the free end of the wire was secured under the lower screw of the left-hand jaw (Fig. 2.4 5-6).
- The second wire was gently feeded through the lumen of the vessel after the jaws were moved apart by the micrometer (Fig. 2.4 7-8).
- The jaws were moved together and the second wire was secured under the screws of the right-hand jaw (Fig. 2.4 9-12).
- The jaws were moved apart again to slightly stretch the mounted vessel segment, allowing a piece of a rat whisker to pass through the artery lumen
- The endothelium of the artery was completely rubbed away by turning the whisker around in the lumen.
- The jaws were moved together to position the two wires very close to each other.

- The other three chambers were mounted with vessels subsequently.
- The chambers were placed on the interface of the Wire Myograph set-up, with heating (37°C) and gassing (95% O_2 and 5% CO_2) for 20 min

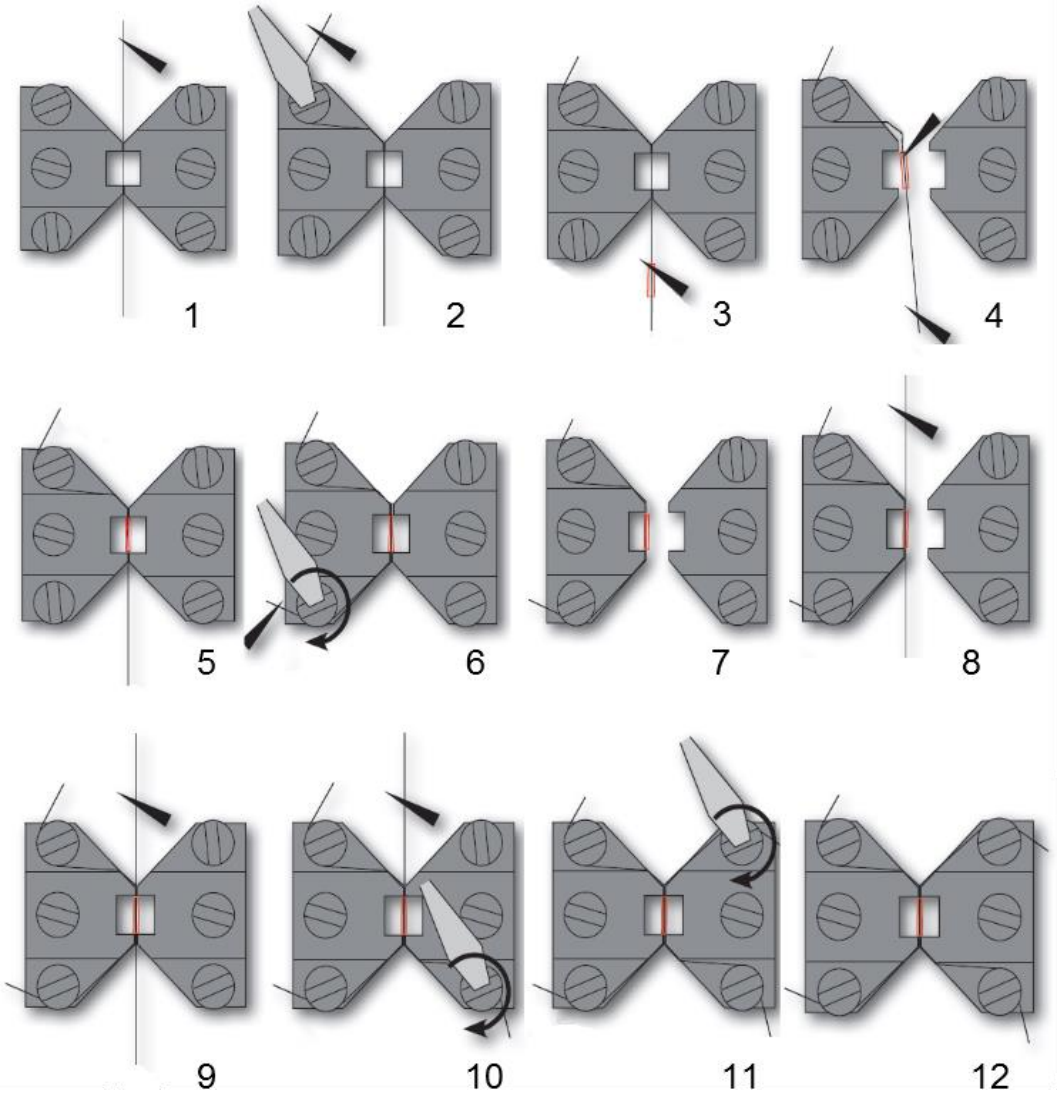

Figure 2.4 wire myograph mounting steps
(www.dmt.dk, Stand 06.04.2017), modified

(1)-(2) Fixation of a stainless steel wire (approx. 2.5 cm long) on the left-hand jaw of the wire myograph chamber; (3)-(6) Mounting of a vessel onto the fixed wire; (7)-(12) Fixation of the second wire passed through the lumen of the vessel on the right-hand jaw.

2.2.3.2. The normalization procedure

This procedure is most critical, because it not only sets the artery to an optimal physiological internal circumference (IC, Fig. 2.5 B) to create a suitable resting tension that ensures that the artery displays optimal responses to vasoactive agents, but also provides standard experimental baseline conditions to perform comparative measurements on different vessels.

The normalization procedure is performed based on the muscular length tension relationship to acquire the value of passive vessel internal circumference at a pressure of 100 mmHg (13.3 kPa) (IC100) and then the vessel is set to the optimal internal circumference (IC1).

As known, the length tension relationship describes the phenomenon that with different length, muscle exhibits different levels of maximum isometric force (Gordon et al., 1966). This relationship comprises passive and active parts:

1) the passive length tension relationship

In the absence of agonists i.e. without the interaction between the myosin and actin filaments, muscle tension is increased with increasing length by stretch.

In the normalization procedure, based on the data of passive tension of the artery and the corresponding applied ICs obtained using the micrometer, this passive length tension relationship is plotted, and the IC100 can be determined according to La Place's Law (Fig. 2.5 C blue line).

La Place's Law

The effective pressure (Pi) = Wall tension / (internal circumference/(2 × π))

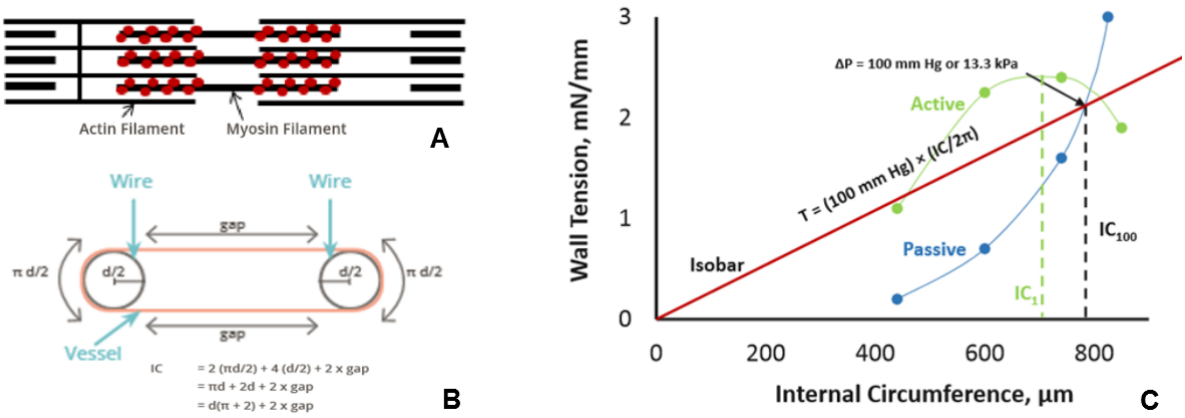

Figure 2.5 Conceptual illustration of wire myograph normalization principles
(www.dmt.dk, Stand 06.04.2017), modified

A) The ideal optimal relative position of myosin and actin filaments can provide the two filaments with maximal extent of contact but still leaves proper space for a maximal constriction. The stretched distance applied in this position corresponds to IC1.
B) In wire myography, the value of the internal circumference of vessels (IC) is related to the diameter of the wires that hold the artery and the distance of the gap between the wires. The vessel is stretched as the gap is getting larger by manipulating the micrometer.
C) The typical separated components of the length/tension graph: ***the passive stretch line (blue)*** represents, in the absence of agonists, vessel tension increases with increasing IC obtained by manipulating the myograph micrometer. The IC100 value is found by plotting this passive line in the normalization procedure. ***The active line (green)*** reflects the changes in vessel tension generated by an agonist at different length conditions (i.e. force contributions only from muscle contraction without passive stretch). The value of IC that corresponds to the maximum agonist response is represented by IC1 (dashed green line).

2) The active length tension relationship:

The length of the muscle reflects the extent of contact between the myosin and actin filaments. The optimal active responses in force can only be developed when the artery stays at an optimal length where the two filaments have maximal contact but still have proper space for contraction (Fig. 2.5 A, C green line).

In the normalization procedure, IC1 represents the optimal length, which can be calculated from IC100.

IC1 = Normalization Factor × IC100

(Normalization Factor depends on the type of vessel and the species used)

The procedure of normalization:

- The Normalization Module in the LabChart software was started.
- The artery was stretched stepwise by manipulating the micrometer (at first two steps of about 300 μm each and then the step size was decreased according to individual vessels to acquire passive tension points.
- When the last passive tension point is above the isobar line of 100 mmHg (13.3 kPa), the normalization is terminated. At this step, the passive IC100 was determined and the optimal IC1 was calculated automatically by this module.
  - There should be at least 4 passive tension points (3 points below the isobar line and 1 point just above) to allow the Normalization Module to calculate an exponential curve fitting to determine the passive IC100, and calculate the optimal IC1.
  - In this study, IC1 = 90% × IC100 (Normalization Factor=0.9) (Mulvany and Halpern, 1977).
- The artery is set to IC1.

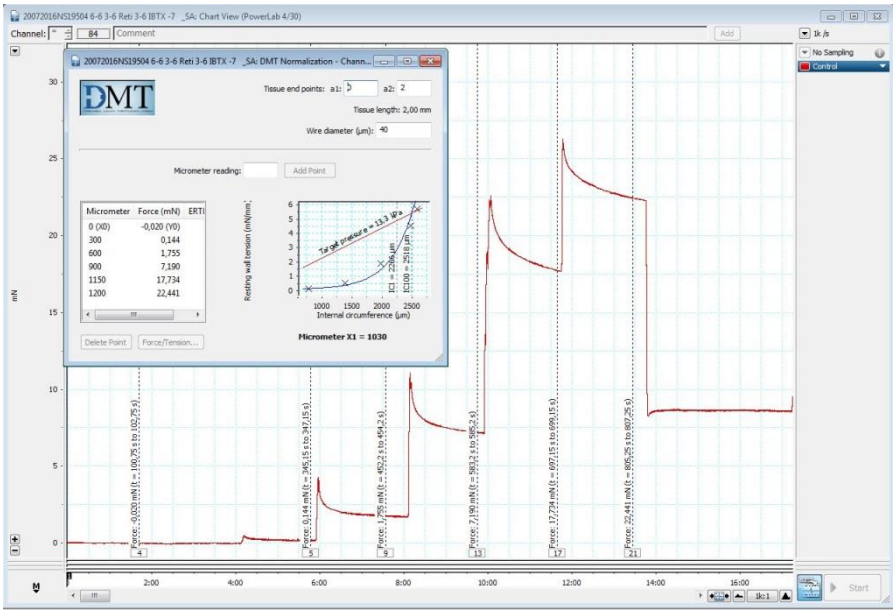

Figure 2.6 Output window of wire myograph normalization

The big window is the main LabChart window displaying the force trace obtained by the stepwise increase in stretch; ***The small window*** automatically plots the passive stretch line dependent on the steps of stretch set by the micrometer and the corresponding artery passive tension, at the end the value of IC1 is generated.

2.2.3.3. Viability test

- The preparation solution in the chambers was changed to experimental solution.
- Application of methoxamine 10^{-5} mol/L, 5 min (to test the contractile activity of arteries).

  Application of acetylcholine 10^{-5} mol/L, 2 min (to assess endothelium function).

  Wash 3×-1×-1×, 5min each

  - i.e., each artery was washed 3 times with 5 minutes interval each. The first time included 3 consecutive washes, the other two only one wash (complete solution change of the chamber).

2.2.3.4. The main experiment

- Application of methoxamine 10^{-5} mol/L, 5 min (to acquire the maximum tension of the artery, T_{max})

  Wash 3×-1×-1×, 5min each

- Application of H_2O(treatments As+Bs*, Table 2.2), 20min

  Methoxamine CRC, 3min at each concentration

  - Methoxamine CRC: Methoxamine Concentration Responses Relationship using 7 different concentrations (10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5} mol/L)

  Wash 3×-1×-1×, 5min each

- Each chamber underwent treatments in the following sequence:

Channel 1	Channel 2	Channel 3	Channel 4
As (**Table 2.2**)			
B	B	Bs	B

- **Methoxamine CRC** 3min at each concentration

  Wash 3×-1×-1× 5min each

A	As	A	As
B	B	Bs	Bs

- **Methoxamine CRC** 3min at each concentration

  Wash 3×-1×-1× 5min each

***Table 2.2 treatments A, As, B and Bs in different series in wire myograph experiment**

series	A (mol/L)		As (µl)		B (mol/L)		Bs (µl)		Time
1	NS19504	3×10^{-6}	DMSO	1.5	IBTX	10^{-7}	H_2O	5	10 min
2	Retigabine	3×10^{-5}	DMSO	1.5	XE991	3×10^{-6}	H_2O	1.5	10 min
3	NS19504	3×10^{-6}	DMSO	1.5	XE991	3×10^{-6}	DMSO	1.5	10 min
4	XE991	3×10^{-6}	H_2O	1.5	IBTX	10^{-7}	H_2O	1.5	10 min
5	Retigabine	3×10^{-5}	DMSO	1.5	NS19504	3×10^{-6}	H_2O	5	10 min
6	Retigabine	3×10^{-6} 10^{-5}	DMSO	1.5	IBTX	10^{-7}	H_2O	5	10 min

2.2.3.5. Analysis of Wire Myograph data

Relative tension is used to indicate the intensity of artery contraction, which is the artery tension generated by methoxamine divided by the maximum tension at methoxamine 10^{-5}M (T_{max}) determined at the beginning of the experiment.

$$\text{Relative Tension} = T_x / T_{max}$$

2.2.4 Real-time PCR

In order to get insight into the relationship between the physiological function of arteries and the potassium channel gene expression profile, the Real-Time quantitative polymerase chain reaction (qPCR) technique was applied in this study. We isolated total RNA from arteries firstly, then reverse transcripted RNA into cDNA, and at the end detected the amount of cDNA templates by use of qPCR.

2.2.4.1. Collection of artery samples

Artery samples were collected in two groups: arteries with endothelium (E+) and arteries without endothelium (E-).

- Once the hind limb of the rat was severed, a whole piece of artery (A. Saphena and A. Gracilis) was isolated immediately in icy preparation solution.
- In the E+ group, the blood in the artery lumen was removed by forceps,

  In the E- group, the endothelium was completely rubbed away by strongly turning a piece of rat whisker around inside the artery lumen (as mentioned in the wire myograph method).
- Artery samples were put into 2.0µl tubes, frozen in liquid nitrogen and stored at -80°C

2.2.4.2. Purification of total RNA

- The tube containing an artery sample was put on ice and 700 µl QIAzol Lysis Reagent and one steel bead were added.
- The TissueLyser II was used to disrupt and homogenize the artery immediately (30Hz, 6min).
- The homogenate was incubated at room temperature (20-25°C) for 5 min.
- 140µl chloroform was added and the tube was shaken vigorously for 15 s.
- The tube was incubated at room temperature for 2-3 min.
- The tube was centrifuged at 12,000xg and 4°C for 15min.
- After centrifugation, the sample was separated into 3 phases. The upper colorless aqueous phase containing RNA (volume approximately 350 µl) was transferred into a new collection tube with 1.5 volumes (525 µl) of 100% ethanol and mixed thoroughly by pipetting up and down several times.
- 700 µl of the sample was pipetted into an RNeasy Mini spin column in a 2 ml collection tube, and centrifuged at≥8000 x g (≥10,000 rpm) for 15 s at room temperature (20-25°C).

  The flow-through was discarded.

- The last step was repeated with the remainder of the sample.

- ***DNase digestion***

- 350 µl of washing buffer RW1 was pipetted to the RNeasy Mini Spin Column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm).

  The flow-through was discarded.

- 10 µl DNase I stock solution was added to 70 µl Buffer RDD and gently mixed.
- The DNase I incubation mix (80µl) was pipetted directly on to the RNeasy Mini Spin Column membrane and incubated at 20–30°C for 15 min.
- 350 µl buffer RW1 was pipetted to the RNeasy Mini Spin Column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm).

  The flow-through was discarded.

- 500 µl buffer RPE was pipetted to the RNeasy Mini spin column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm).

  The flow-through was discarded.

- The last step was repeated, but centrifuged for 2min at ≥8000 x g (≥10,000rpm) to dry the RNeasy Mini spin column membrane.
- The RNeasy Mini spin column was placed into a new 2 ml collection tube, and centrifuged for 1 min at full speed to eliminate any possible carryover of buffer RPE.
- The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube, and pipetted with 30µl RNase-free water directly onto the RNeasy Mini spin column membrane and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
- The last step was repeated with the first eluate and the same collection tube to obtain a higher total RNA concentration.

2.2.4.3. Measurement of RNA concentration

- The RNA sample was measured by TECAN with the Nano Quant plate.
  - The concentration of the RNA should be above 10ng/µL, the ratio (260 / 280 nm) between 1.8 and 2.1.
- The RNA sample was stored at -80°C.

2.2.4.4. Complementary DNA (cDNA) synthesis

- The reverse transcript synthetic solution was prepared following Table 2.3, in which each 20µl synthetic solution contains 100 ng RNA:

Table 2.3 Reverse transcript synthetic solution

Reagent	Volume(µl)
RNA (100 ng) + RNase free H_2O	12
5×Reaction Buffer	4
Random Primers 0.2 µg/µL	1
dNTP Mix, 10 nM each	2
RevertAid H Minus Reverse Transcriptase 200 U/µl	1
Total volume	20

- The reversed transcription was performed by Mastercyler gradient.
  - the synthesis progress was set as:

Table 2.4 Reversed transcription synthesis progress

Temperature	Time
20°C	10 min
50°C	60 min
70°C	10 min
4°C	∞

- The cDNA was stored at -20°C.

2.2.4.5. Real time PCR (qPCR)

The traditional PCR only measures the amount of PCR product at the end point of the amplification, while qPCR can detect the intensity of fluorescence of PCR products generated at the end of each amplification cycle, and then extrapolate back to the initial amount of DNA or cDNA template with higher sensitivity and specificity.

A typical qPCR includes 4 basic phases (Fig. 2.7):

- *Initiation phase:* It is at the first PCR cycles in which the amount of the products is too low to distinguish their fluorescence from the background signal (baseline).

- *Exponential phase:* In this phase, the fluorescence signal of products has reached and is above the detection threshold with an exponential increase. The first fractional PCR cycle above the detection threshold is called threshold cycle (Ct Value). As all of the reagents are fresh and available in this phase, the reaction becomes very specific and precise, and the efficiency indicating how completely the amplicon amplified in each cycle approaches 2. The amount of initial DNA templates is extrapolated from the Ct value and the efficiency in this phase.

- *Linear phase:* with the reaction reagents consumed, the reaction becomes slower, and reaches significant product inhibition.

- *Plateau phase:* with the reagents exhausted, the reaction has stopped, in which there is no more products made and no increase in fluorescence detected.

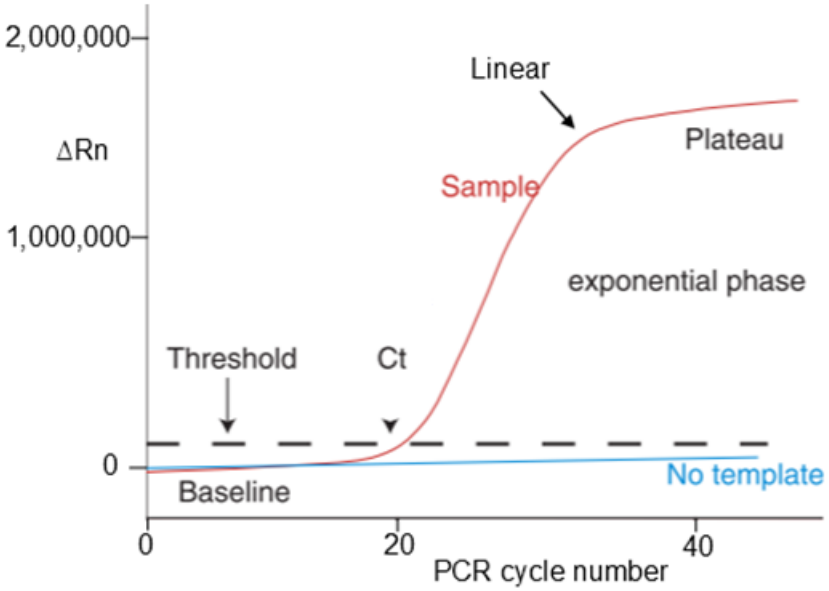

Figure 2.7 Nomenclature in a typical qPCR amplification plot
(Arya et al. 2005), modified

The plot is based on the PCR products fluorescence data collected in each PCR cycle and demonstrates initiation phase (baseline), exponential phase, linear phase and plateau phase.
ΔRn= Fluorescence of the product at each cycle – fluorescence of the baseline
Ct= Threshold cycle

There are some DNA-binding dyes or fluorescent probes that can be employed as fluorescent labels in qPCR. In this study, SYBR Green as a non-sequence-specific DNA-binding dye was used. The unbound SYBR Green exhibits little fluorescence, while the one intercalating into the Minor Groove of double stranded DNA (dsDNA) would emit fluorescence. Therefore, the more dsDNA amplicons are produced, the stronger is the fluorescence emitted by SYBR Green. (Fig. 2.8) However, according to this mechanism, nonspecific PCR products and primer-dimers could decrease its specificity. Since the characteristic melting peak at the melting temperature (Tm) of the amplicon is specific for the corresponding PCR product, the melting curve at the end of qPCR is necessary to distinguish amplification artifacts and to increase the specificity of the reaction (Arya et al., 2005).

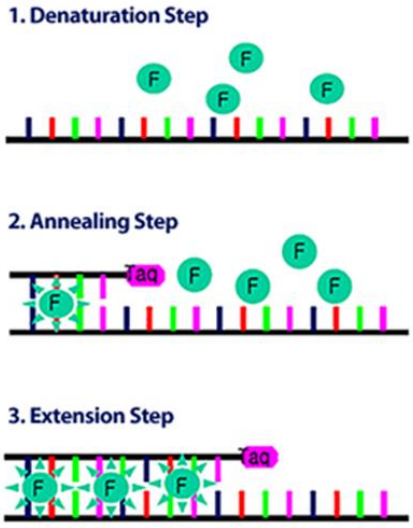

Figure 2.8 Detection mechanism of double-stranded DNA-binding dye SYBR Green
(http://www.bioneercn.com/products_detail014/&productId=ea3d0846-b14c-4a34-8bca-d5e2b3c89cfb.html, Stand 06.04.2017), modified

The unbound SYBR Green exhibits little fluorescence during the denaturation step. From annealing, especially in the extension step as soon as SYBR Green binds to double-stranded DNA products, it emits fluorescence.

In this study, each gene was tested in triplicates for one sample in a qPCR 96-well plate including a reference gene (Hmbs) as control.

- SYBR Mix was prepared according to Table 2.5.

Table 2.5 Composition of SYBR Mix

Regagent	Volume/well (µl)
cDNA Template	2
SensiFAST™ SYBR No-ROX Kit, 2×	10
Nuclease free H_2O	3
Forward primer	2.5
Reverse primer	2.5
Total volume	20

- 18µl of the SYBR mix was added into each well.
- 2µl of corresponding cDNA was added to each well.
- The plate was sealed with self-adhesive sealing foil, centrifuged shortly and loaded into the Light Cycler 480 machine.
- The program of Light Cycler was started, and the amplification progress was set according to Table 2.6.

Table 2.6 reaction parameters of real-time PCR progress

step	Hold	Target(°C)	Acquisition Mode	Ramp-Rate (°C/s)	Acquisitions (°C)	Cycles
Denaturation	2 min	95	None	4.4	-	1
Amplification						47
Annealing	5 s	95	None	4.4	-	
Elongation	30 s	60	Single	2.2	-	
Recording	1 min	95	None	4.4	-	1
melting curve	1 min	40	None	2.2	-	
	1 s	60	None	1	-	
	-	95	Continuous	0.6	10	
Cooling	10 s	40	None	2.2	-	1

2.2.4.6. Analysis of real-time-PCR data

- Melting curves were checked by use of the Light Cycler 480 software to ensure the specificity of qPCR products.
- The threshold cycle number (Ct) and amplification efficiency (E) were calculated using the LinRegPCR program
- The expression of the target gene was normalized against Hmbs (reference gene):

$$\text{Relative Expression} = E^{Ct}(\text{Hmbs}) / E^{Ct}(\text{target})$$

2.2.5 Statistics

All data are presented as mean ± the standard error of the mean (SEM); n is the number of animals. Multiple data from different groups were analyzed using repeated measures ANOVA followed by post-hoc test (Holm-Sidak), single data from two groups were compared by an unpaired t test.

In this study, $p<0.05$ was considered to be statistically significant.

3 RESULTS

3.1 Expression of Kv7 and BK channel genes in rat skeletal muscle arteries

To determine which subunits of KCNQ and BK channels are expressed in the A. Saphena and A. Gracilis, the arteries were studied in two groups, with endothelium (E+) and without endothelium (E-), by use of the qPCR technique.

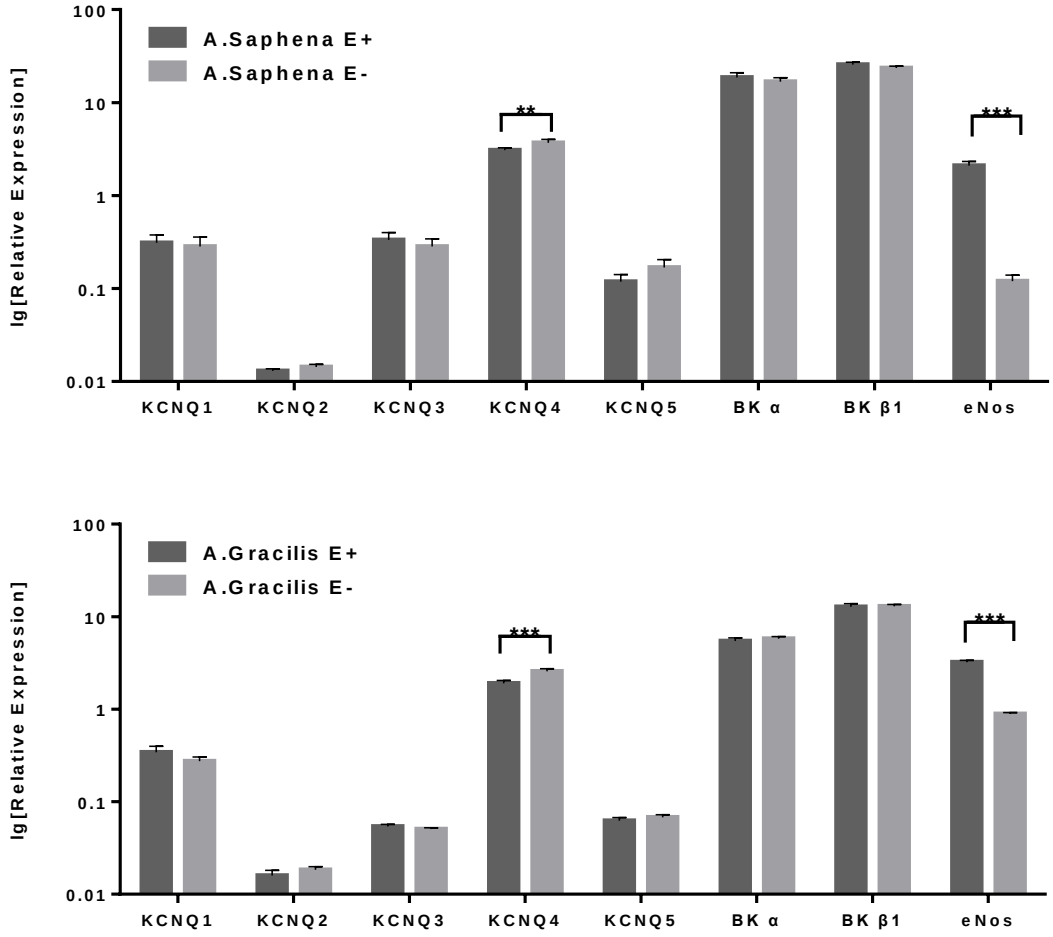

Figure 3.1.1 Expression of KCNQ and BK channel genes

A) Relative expression (normalized by Hmbs) profile of KCNQ and BK channel genes in Saphenous artery (A.Saphena) with endothelium (E+) and without endothelium (E-). **B)** Relative expression profile of KCNQ and BK channel genes in Gracilis artery (A.Gracilis) with endothelium (E+) and without endothelium (E-). n=8;**- p<0.01; *** - p<0.001.

In general, the expression profiles of the potassium channel genes in A. Saphena were similar to those in A. Gracilis: the highest expression was observed for BKα, BKβ1 and KCNQ4, followed by KCNQ1, KCNQ5 and KCNQ3 at a lower expression levels. The lowest expressed gene was KCNQ2 (Fig. 3.1.1).

Comparing the E- group with the E+ group, for A.Saphena (Fig. 3.1.1A), eNOS as a marker for the endothelium, in the E- group represented only 5.7% of that in the E+ group, but the expression of the potassium channel genes was similar, except that KCNQ4 in the E+ group was lower than that in the E- group. For A. Gracilis, (Fig. 3.1.1B), besides the significant difference that there was only 27% eNOS in the E- group compared with the E+ group of A. Gracilis, as with A.Saphena, the data also

indicate that the expression of almost all KCNQ and BK channel genes were similar, except lower KCNQ4 in the E+ group.

3.2 Function of Kv7 and BK channels in rat skeletal muscle arteries

In this study, the function of Kv7 and BK channels was studied by application of their respective blockers and activators. Specifically, XE991 was used as a selective blocker of Kv7 channels (Greenwood and Ohya, 2009), retigabine as a selective activator of Kv7 channels (Wickenden et al., 2000); iberiotoxin (IBTX) was used as a selective blocker of BK channels (Galvez et al., 1990), and NS19504 as a novel activator of BK channels (Nausch et al., 2014).

3.2.1 Respective function of Kv7 and BK channels

3.2.1.1 Effect of Kv7 channel blocker and activator on arterial contractility

Firstly, the actions of the Kv7 channel blocker XE991 and activator retigabine were studied on methoxamine-induced contractions in wire myograph (Fig 3.2.1) and on pressure-induced myogenic responses in pressure myograph (Fig 3.2.3) experiments.

Regarding methoxamine-induced contractions, it was observed that arterial tension was dramatically attenuated by retigabine at $3*10^{-5}$M, but not changed by XE991 alone at $3*10^{-6}$M. The tension in the combined presence of retigabine and XE991 was stronger than that in the presence of retigabine alone, but similar to that in the presence of XE991 alone (Fig 3.2.2 A). Thus, in the presence of XE991, retigabine was not able to affect methoxamine-induced tension. Correspondingly, the anti-contractile effect of retigabine was decreased in the presence of XE991 (Fig 3.2.2 B) while the functional availability of Kv7 channels, expressed as the contractile effect of XE991, was notably increased in the presence of retigabine (Fig 3.2.2 C).

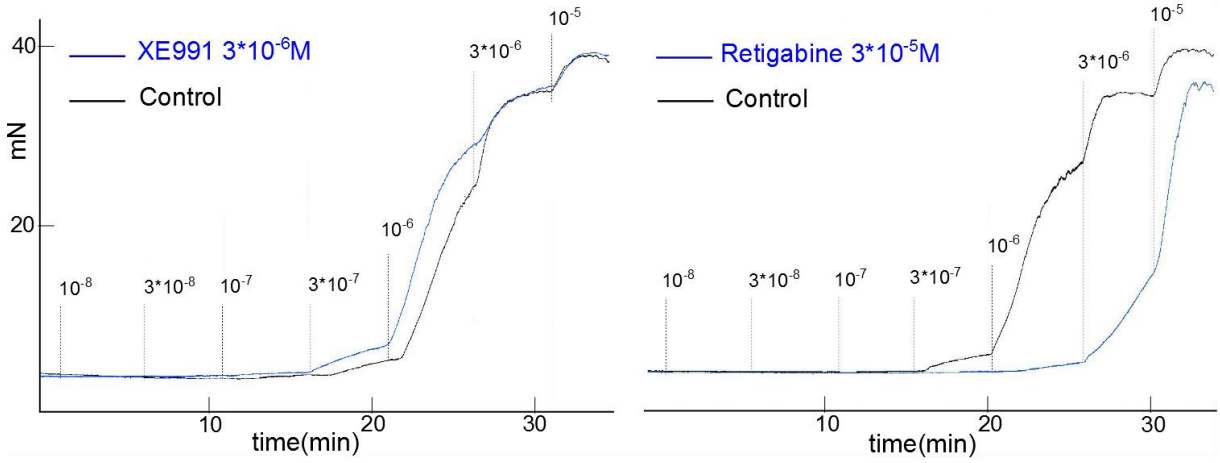

Figure 3.2.1 Original recordings of methoxamine-induced contractions in the presence of XE991 and retigabine in a wire myograph experiment

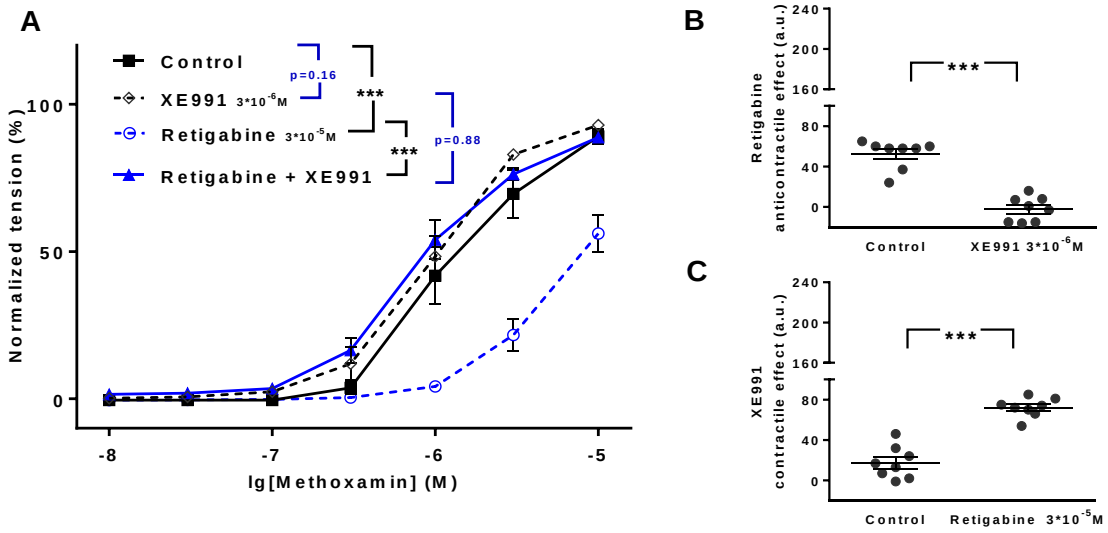

Figure 3.2.2 Effect of retigabine and XE991 on methoxamine-induced contractions

A) Normalized tension of A.Saphena with different methoxamine concentrations in the absence of Kv7 channel active agents (Control), in the presence of XE991 (XE991 $3*10^{-6}$M), in the presence of retigabine (Retigabine $3*10^{-5}$M) and in the combined presence of retigabine and XE991 (Retigabine + XE991). **B)** Anti-contractile effect of retigabine in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). **C)** Contractile effect of XE991 (Kv7 channel functional availability) in the absence (Control) and presence of retigabine (Retigabine $3*10^{-5}$M). n=9; * - p<0.05, **- p<0.01; *** - p<0.001.

Similar results were obtained for myogenic responses: retigabine at $3*10^{-6}$M weakened the myogenic response, while XE991 at $3*10^{-6}$M strengthened it. The myogenic response in the presence of XE991 and retigabine together was as strong as that with XE991 alone (Fig 3.2.4 A). Of note, XE991 attenuated the anti-contractile effect of retigabine (Fig 3.2.4 B), whereas retigabine enhanced the functional availability of Kv7 channels (Fig 3.2.4 C).

The mutual complete inhibition of their respective effects indicates that XE991 and retigabine in the concentrations applied can be employed as a selective Kv7 channel activator and blocker, respectively.

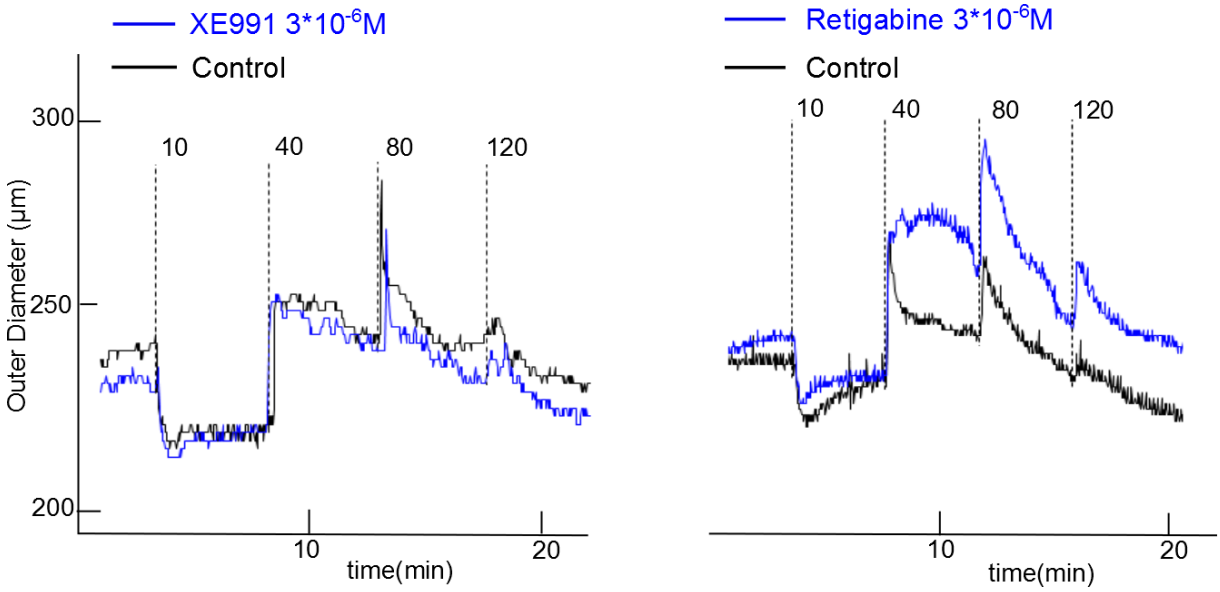

Figure 3.2.3 Original recordings of myogenic responses in the presence of XE991 and retigabine in a pressure myograph experiment

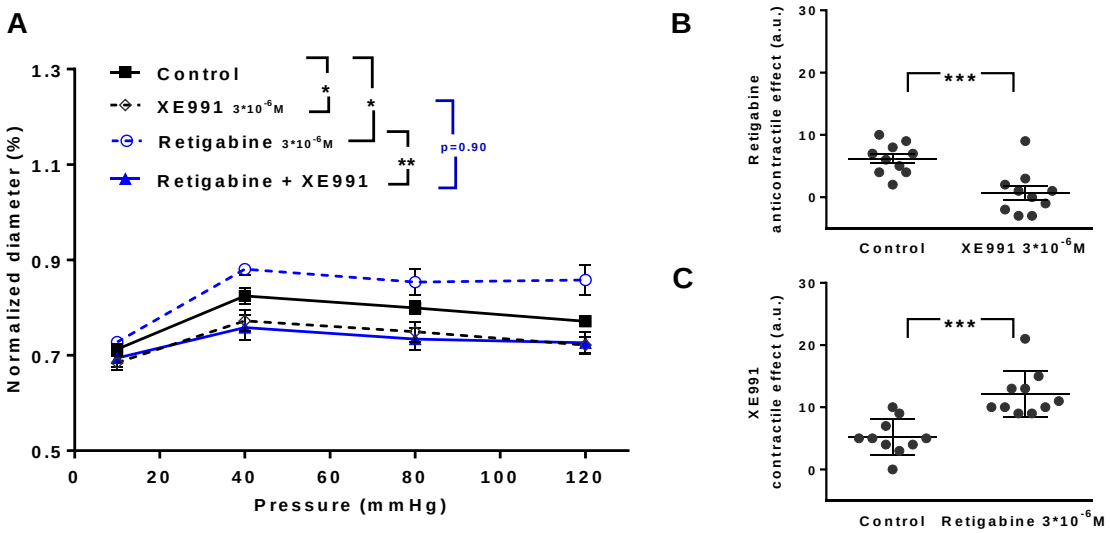

Figure 3.2.4 Effect of retigabine and XE991 on the myogenic response

A) Normalized diameter of A.Gracilis with different intra-luminal pressures in the absence of Kv7 channel active agents (Control), in the presence of XE991 (XE991 $3*10^{-6}$M), in the presence of retigabine (Retigabine $3*10^{-6}$M) and in the combined presence of retigabine and XE991 (Retigabine and XE991). **B)** Anti-contractile effect of retigabine in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). **C)** Contractile effect of XE991 (Kv7 channel functional availability) in the absence (Control) and presence of retigabine (Retigabine 10^{-7}M). n=10; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.1.2 Effect of BK channel blocker and activator on arterial contractility

Meanwhile, the action of the BK channel blocker IBTX and activator NS19504 was also studied on methoxamine-induced contractions in wire myograph (Fig 3.2.5) and on pressure-induced myogenic responses in pressure myograph experiments (Fig 3.2.7).

Because there is no agent commonly recognized and confirmed as a selective activator of BK channels till now, NS19504 as a novel candidate was tested in more detail, i.e. in three concentrations ($3*10^{-6}$M, $6*10^{-6}$M, 10^{-5}M), in wire myograph experiments to detect whether it is a real potent and selective activator of BK channels in arterial smooth muscle.

It was observed that the methoxamine-induced arterial tension was increased by IBTX at 10^{-7}M, while attenuated by NS19504 ($3*10^{-6}$M, $6*10^{-6}$M, 10^{-5}M) in a concentration-dependent manner. Notably, the tension in the presence of IBTX combined with NS19504 ($3*10^{-6}$M, $6*10^{-6}$M) was similar to that in the presence of IBTX alone (Fig 3.2.6 A1, A2). However, with the concentration of NS19504 increased to 10^{-5}M, the tension in the presence of the combination of IBTX and NS19504 was weaker than that in the presence of IBTX alone. Thus, in the presence of IBTX, the lower concentrations of NS19504 were not able to affect methoxamine-induced contractions. Furthermore, the anti-contractile effect of NS19504 was decreased in the presence of IBTX (Fig 3.2.6 B1, B2, B3), whereas the functional availability of BK channels, expressed as the contractile effect of IBTX, was increased in the presence of NS19504 (Fig 3.2.6 C1, C2, C3).

Similar results were obtained for myogenic responses: NS19504 at $3*10^{-6}$ M weakened the myogenic response, while NS19504 combined with IBTX and IBTX alone strengthened the myogenic response to a similar extent (Fig 3.2.8, A). Correspondingly, IBTX attenuated the anti-contractile effect of NS19504 (Fig 3.2.8, B), while NS19504 enhanced the functional availability of BK channels (Fig 3.2.8, C).

The mutual complete inhibition of their respective effects indicates that IBTX and NS19504 (at $6*10^{-6}$ M in A.Saphena and at $3*10^{-6}$ M in A.Gracilis) can be employed as a selective BK channel activator and blocker, respectively.

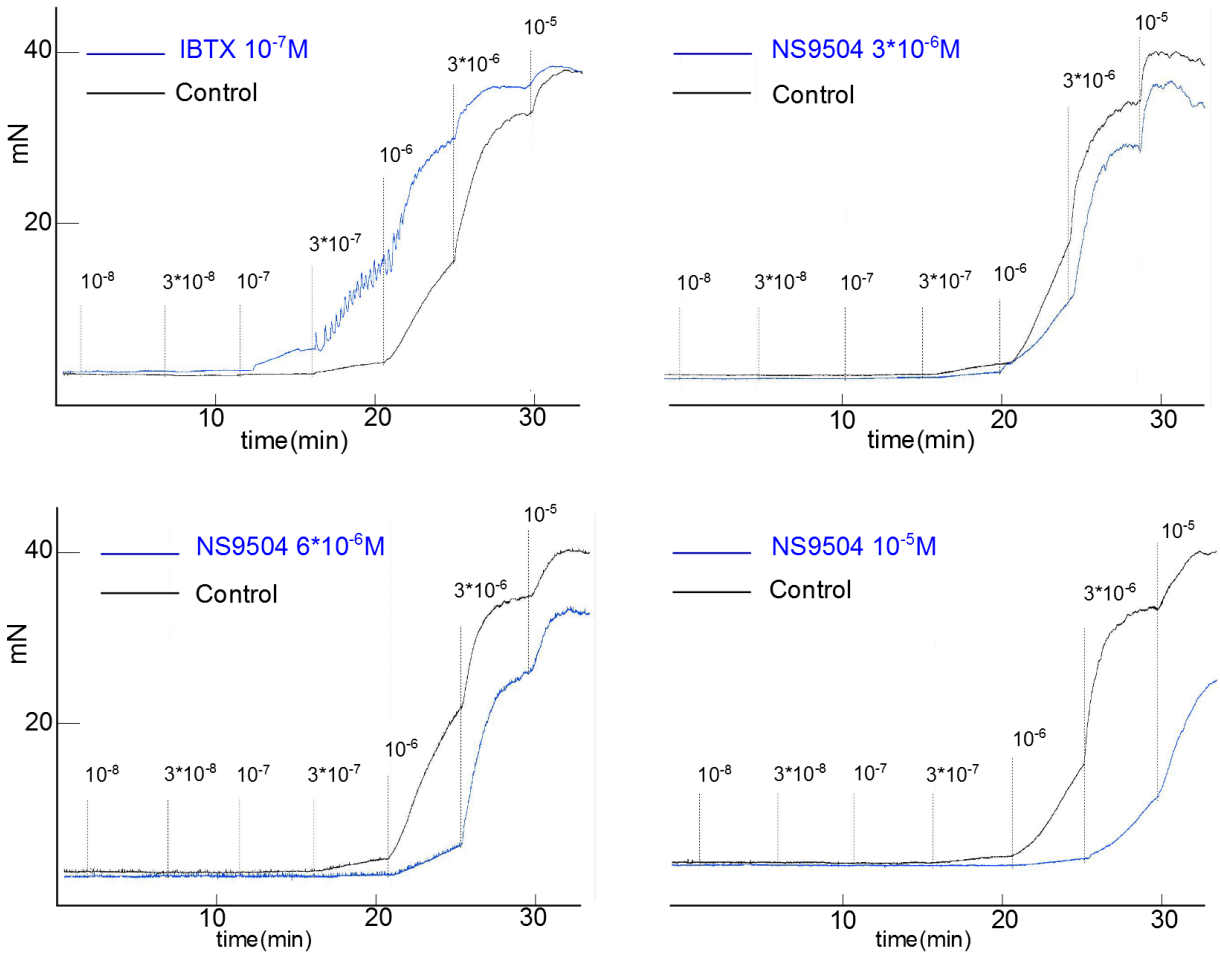

Figure 3.2.5 Original recordings of methoxamine-induced contractions in the presence of IBTX and NS19504 in a wire myograph experiment

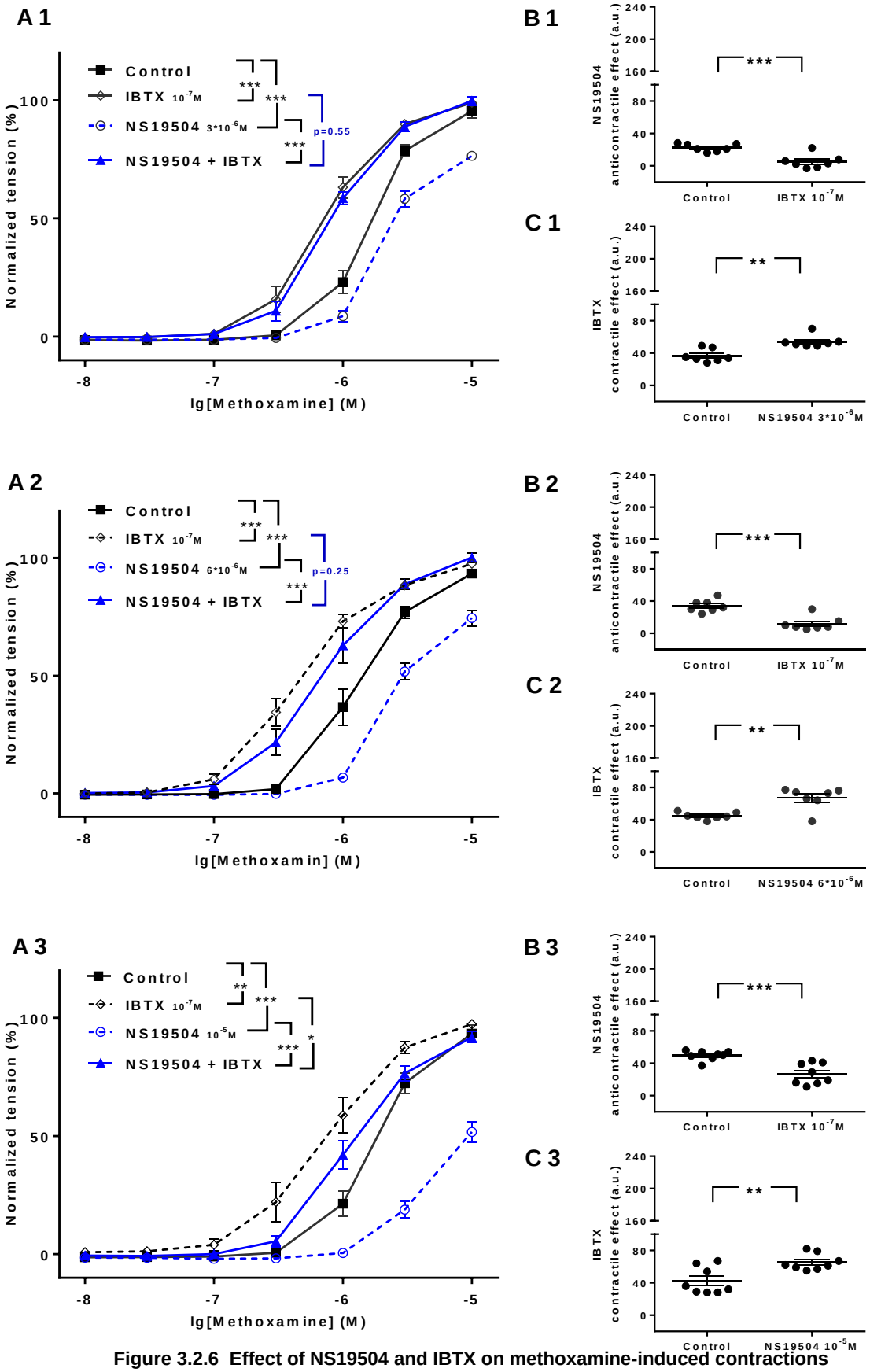

Figure 3.2.6 Effect of NS19504 and IBTX on methoxamine-induced contractions

A1-A3) Normalized A.Saphena tension with different methoxamine concentrations in the absence of BK channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of NS19504 (A1: NS19504 $3*10^{-6}$M; A2: NS19504 $6*10^{-6}$M; A3: NS19504 10^{-5}M) and in the combined presence of NS19504 and IBTX (NS19504 and IBTX). **B1-B3)** NS19504 anti-contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C1-C3)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of NS19504 (C1: NS19504 $3*10^{-6}$M; C2: NS19504 $6*10^{-6}$M; C3: NS19504 10^{-5}M). n1=7; n2=7; n3=8; * - p<0.05, **- p<0.01; *** - p<0.001.

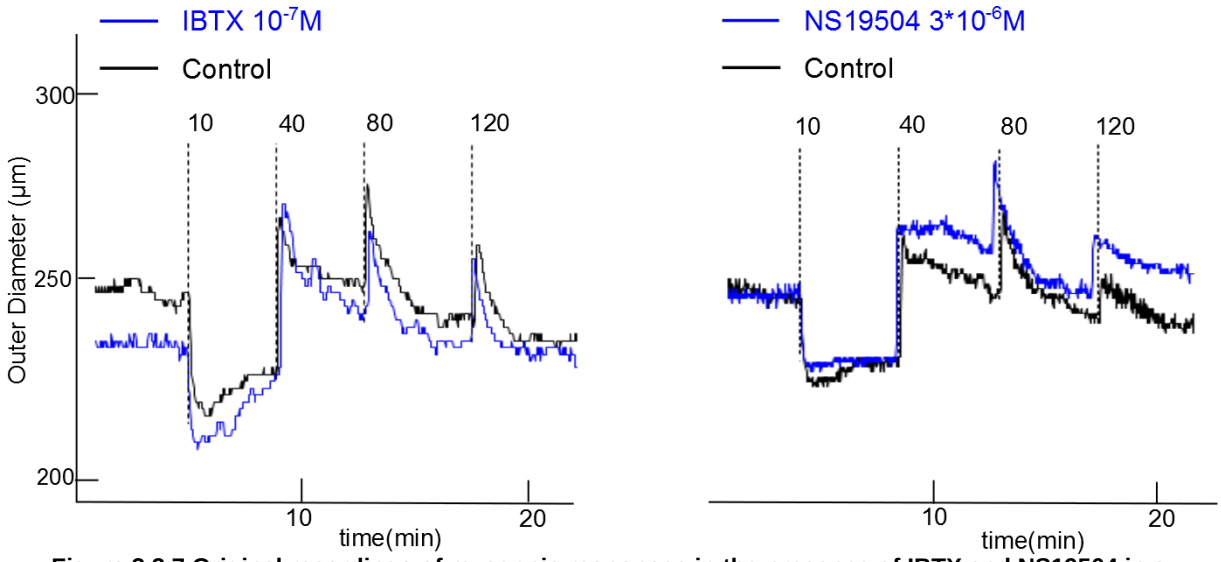

Figure 3.2.7 Original recordings of myogenic responses in the presence of IBTX and NS19504 in a pressure myograph experiment

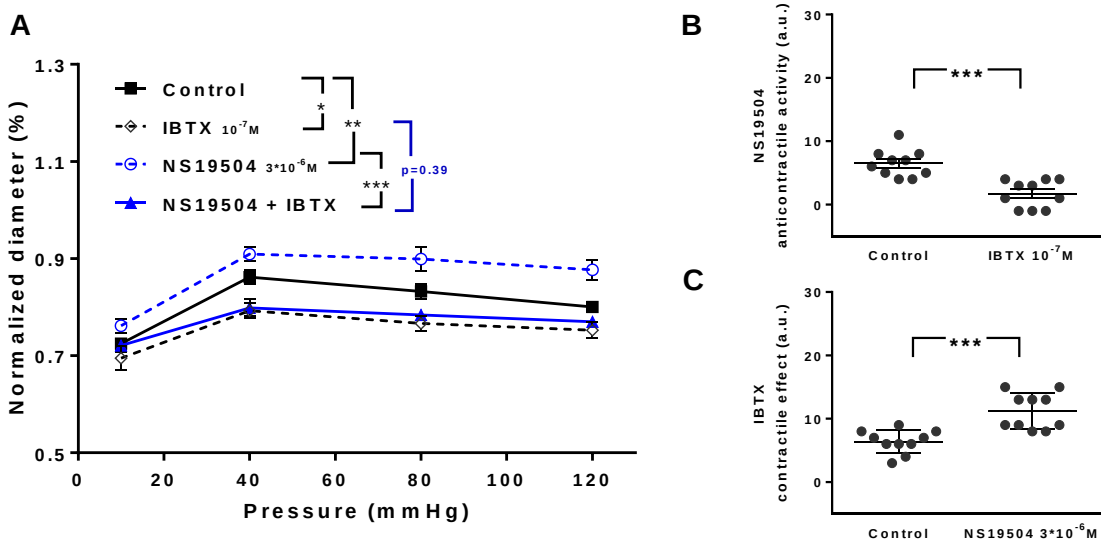

Figure 3.2.8 Effect of NS19504 and IBTX on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of BK channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of NS19504 (NS19504 $3*10^{-6}$M) and in the combined presence of NS19504 and IBTX (NS19504 and IBTX). **B)** NS19504 anti-contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of NS19504 (NS19504 $3*10^{-6}$M). n=10; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.1.3 Effect of NS19504 on arterial contractility

Since the effect of NS19504 at 10^{-5}M cannot be completely blocked by IBTX as shown in the experiments mentioned above (Fig 3.2.6), it was studied whether higher concentrations of NS19504 possess the ability to affect other channels, such as Kv7 channels.

- Effect of NS19504 in the combined presence of IBTX and XE991 on arterial contractility

In this experiment, the methoxamine-induced tension was strongly increased by the combination of IBTX (10^{-7}M) and XE991 ($3*10^{-6}$M) and considerably reduced by 10^{-5}M NS19504 alone. The combination of 10^{-5}M NS19504 together with IBTX and XE991 affected the methoxamine-induced tension to a similar extent as the combination of XE991 and IBTX alone (Fig 3.2.9 A). Thus, in the presence of IBTX and XE991, NS19504 (10^{-5}M) did not affect arterial tension. Beyond that, the contractile effect of IBTX together with XE991 was increased in the presence of NS19504 (Fig 3.2.9 B), whereas the anti-contractile effect of NS19504 was decreased in the presence of IBTX together with XE991 (Fig 3.2.9 C).

Therefore, the data indicate that NS19504 at higher concentrations activates BK channels as well as Kv7 channels without affecting other targets.

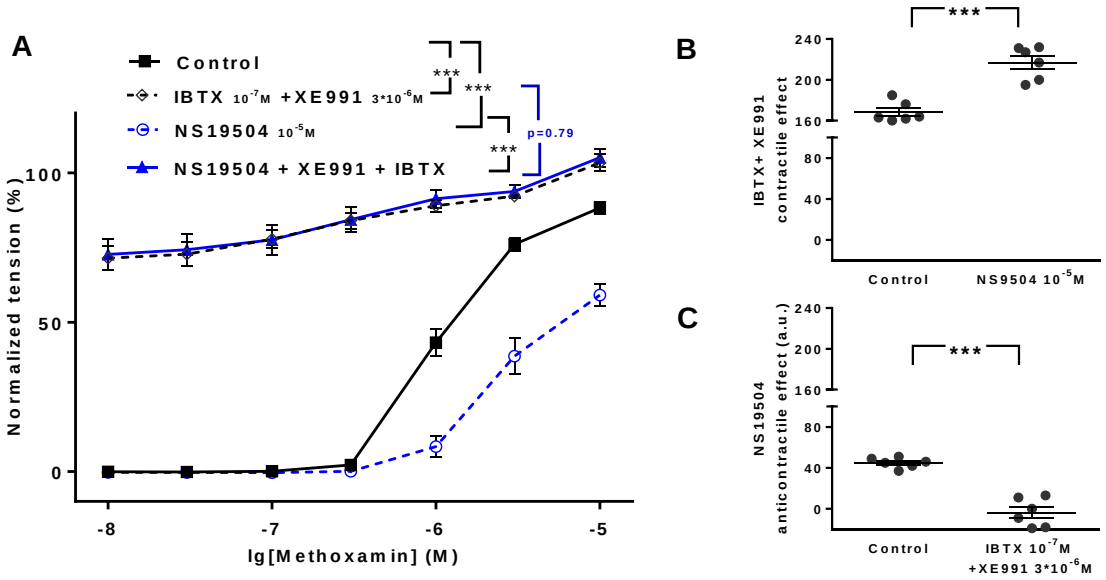

Figure 3.2.9 Effect of NS19504 and IBTX together with XE991 on methoxamine-induced contractions

A) Normalized tension of A.Saphena with different methoxamine concentrations in the absence of potassium channel active agents (Control), in the combined presence of IBTX with XE991 (IBTX 10^{-7}M+XE991 $3*10^{-6}$), in the presence of NS19504 (NS19504 10^{-5}M) and in the combined presence of NS19504 and IBTX with XE991 (NS19504+XE991+IBTX). **B)** IBTX with XE991 contractile effect in the absence (Control) and presence of NS19504 (NS19504 10^{-5}M). **C)** NS19504 anti-contractile effect in the absence (Control) and presence of IBTX with XE991 (IBTX+ XE991). n=6; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.2 Functional interaction of Kv7 channels and BK channels

3.2.2.1 Combined effect of Kv7 channel blocker and BK channel blocker on arterial contractility

After studying the respective effects of Kv7 channel active agents and BK channel active agents on arterial contractility, the combined effects of them were investigated in the subsequent experiments:

Firstly, the combined action of XE991 and IBTX was studied on methoxamine-induced contractions in wire myograph and on pressure-induced myogenic responses in pressure myograph experiments.

Regarding methoxamine-induced contractions, arterial tension was increased in the presence of IBTX at 10^{-7}M alone or XE991 at $3*10^{-6}$M alone, and considerably enhanced in the presence of the combination of both XE991 and IBTX (Fig 3.1.10, A). Correspondingly, the functional availabilities of Kv7 channels and BK channels were increased in the presence of IBTX and XE991, respectively (Fig 3.1.10, B, C).

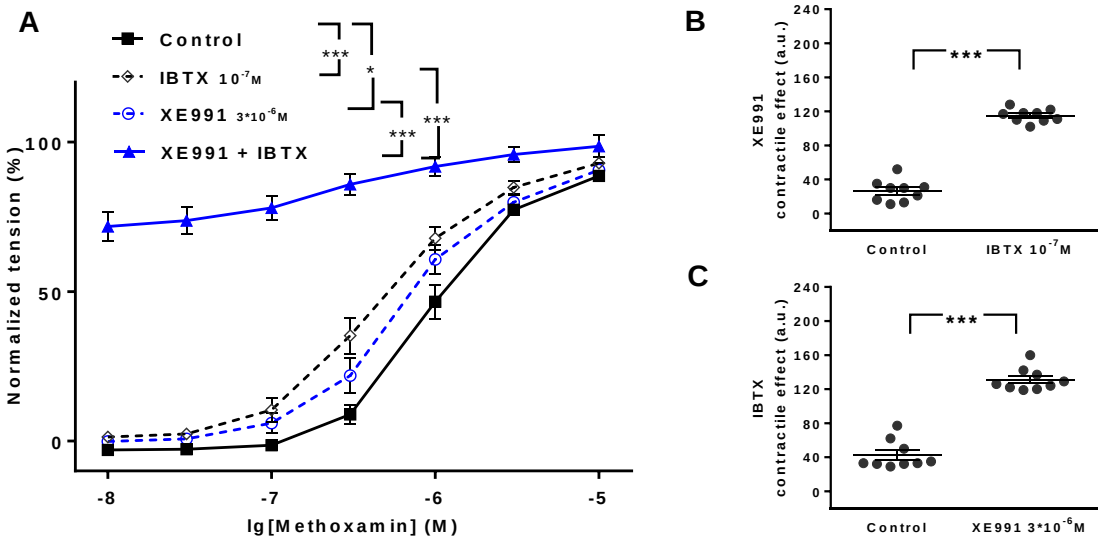

Figure 3.1.10 Effect of XE991 and IBTX on methoxamine-induced contractions

A) Normalized tension of A.Saphena with different methoxamine concentrations in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of XE991 (XE991 $3*10^{-6}$M) and in the combined presence of XE991 and IBTX (XE991 + IBTX). **B)** XE991 contractile effect (Kv7 channel functional availability) in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). n=12; * - p<0.05, **- p<0.01; *** - p<0.001.

When studying myogenic responses, there were two protocols employed to test the combined effect of IBTX and XE991: (i) IBTX was applied at first and XE991 was subsequently added, and (ii) XE991 was applied firstly and then IBTX was added. The results obtained with these two protocols were similar. The myogenic response was strengthened in the presence of either IBTX at 10^{-7}M alone or XE991 at $3*10^{-6}$M alone. A further strengthened myogenic response was observed in the combined presence of IBTX and XE991 (Fig 3.2.11, A1, A2). Meanwhile, the functional availabilities of Kv7 channels and BK channels were enhanced markedly in the presence of IBTX (Fig 3.2.11 B1, B2) and XE991 (Fig 3.2.11, C1, C2), respectively.

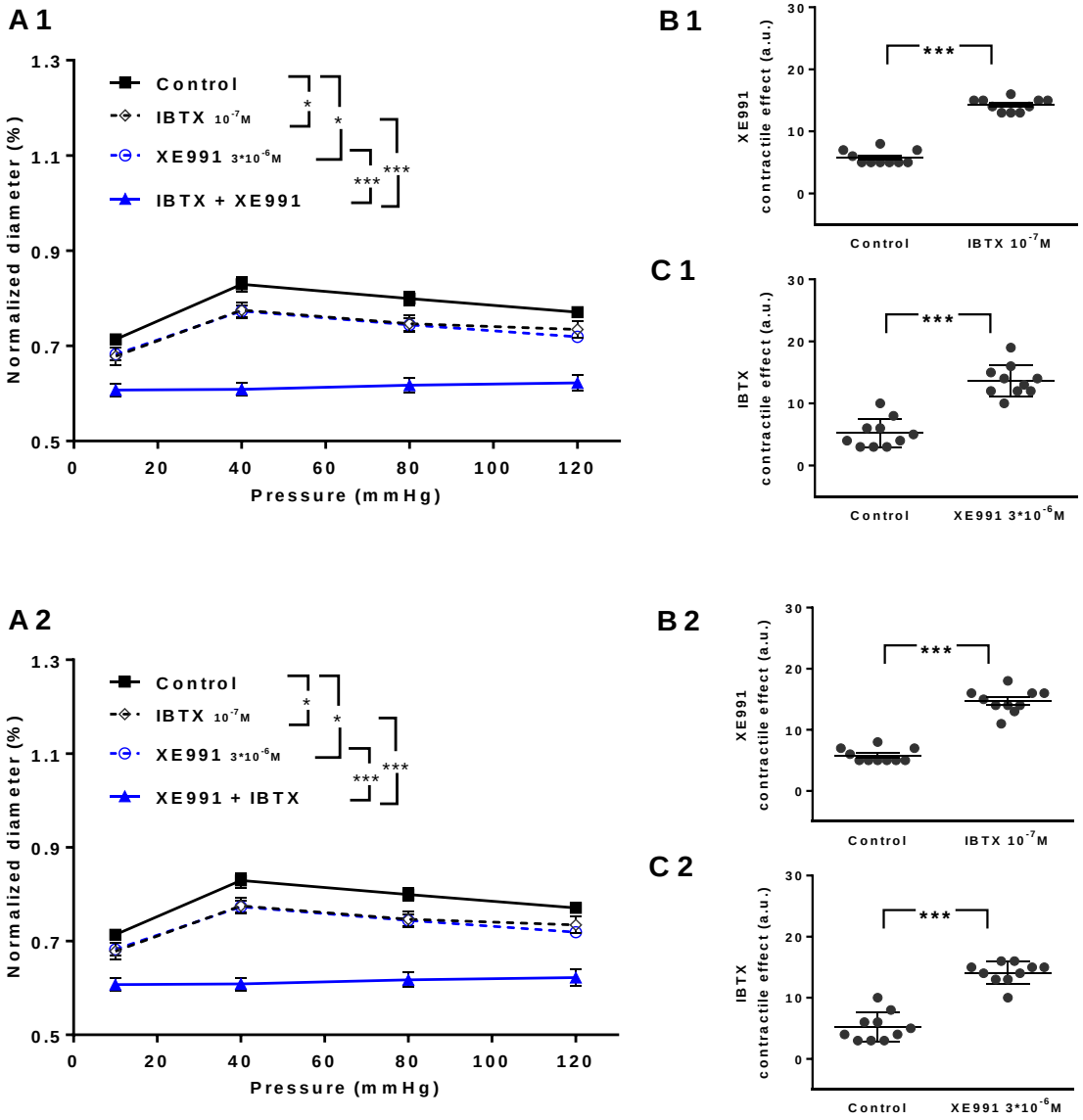

Figure 3.2.11 Effect of XE991 and IBTX on the myogenic response

A1-A2) Normalized diameter of A.Gracilis with different intra-luminal pressures in the absence of potassium channel blockers (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of XE991 (XE991 $3*10^{-6}$M) and in the combined presence of IBTX and XE991 (A1 in sequence XE991+ IBTX, A2 in sequence IBTX+ XE991). **B1-B2**) XE991 contractile effect (Kv7 channel functional availability) in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C1-C2)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). n1=10, n2=10; * - p<0.05, **- p<0.01; *** - p<0.001.

Regarding agents selectively acting on subfamilies of Kv7 channels (Kv7.1-Kv7.5), there is only a selective Kv7.1 blocker, HMR1556 (Gogelein et al., 2000), available. Therefore, the interaction of Kv7.1 channels and BK channels was studied in the myogenic response by using HMR1556 and IBTX. The data show that there was no effect of HMR1556 at 10^{-5}M on the myogenic response, while IBTX at 10^{-7}M alone strengthened the myogenic response. The effect of the combination of IBTX and HMR1556 was similar to the effect of IBTX alone. Meanwhile, HMR1556 did not alter the functional availability of the BK channel. In turn, IBTX also did not change the HMR1556 contractile effect (the functional availability of Kv7.1 channels) (Fig 3.2.12).

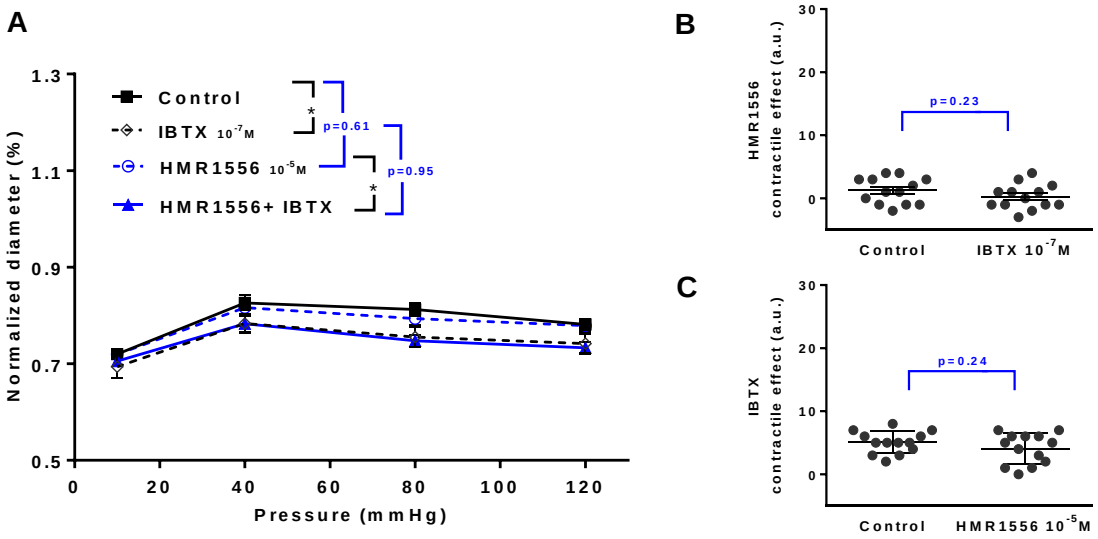

Figure 3.2.12 Effect of HMR1556 and IBTX on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of HMR1556 (HMR1556 10^{-5}M) and in the combined presence of HMR1556 and IBTX (HMR1556 and IBTX). **B)** HMR1556 contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of HMR1556 (HMR1556 10^{-5}M). n=13; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.2.2 Combined effect of Kv7 channel activator and BK channel activator on arterial contractility

The combined action of NS19504 and retigabine was studied on methoxamine-induced contractions in wire myograph and on pressure-induced myogenic responses in pressure myograph experiments.

In methoxamine-induced contractions, NS19504 was studied at two concentrations ($3*10^{-6}$M, $6*10^{-6}$M), both of which decreased the tension of the artery. Moreover, NS19504 at $6*10^{-6}$M and retigabine at 10^{-5}M, respectively weakened arterial tension to a similar extent. The combined presence of NS19504 and retigabine resulted in a further attenuation of artery tension (Fig 3.2.13 A1, A2). At both concentrations of NS19504, the anti-contractile effect of NS19504 was decreased in the presence of retigabine (Fig 3.2.13 B1, B2) and the anti-contractile effect of retigabine was reduced in the presence of NS19504 (Fig 3.2.13 C1, C2).

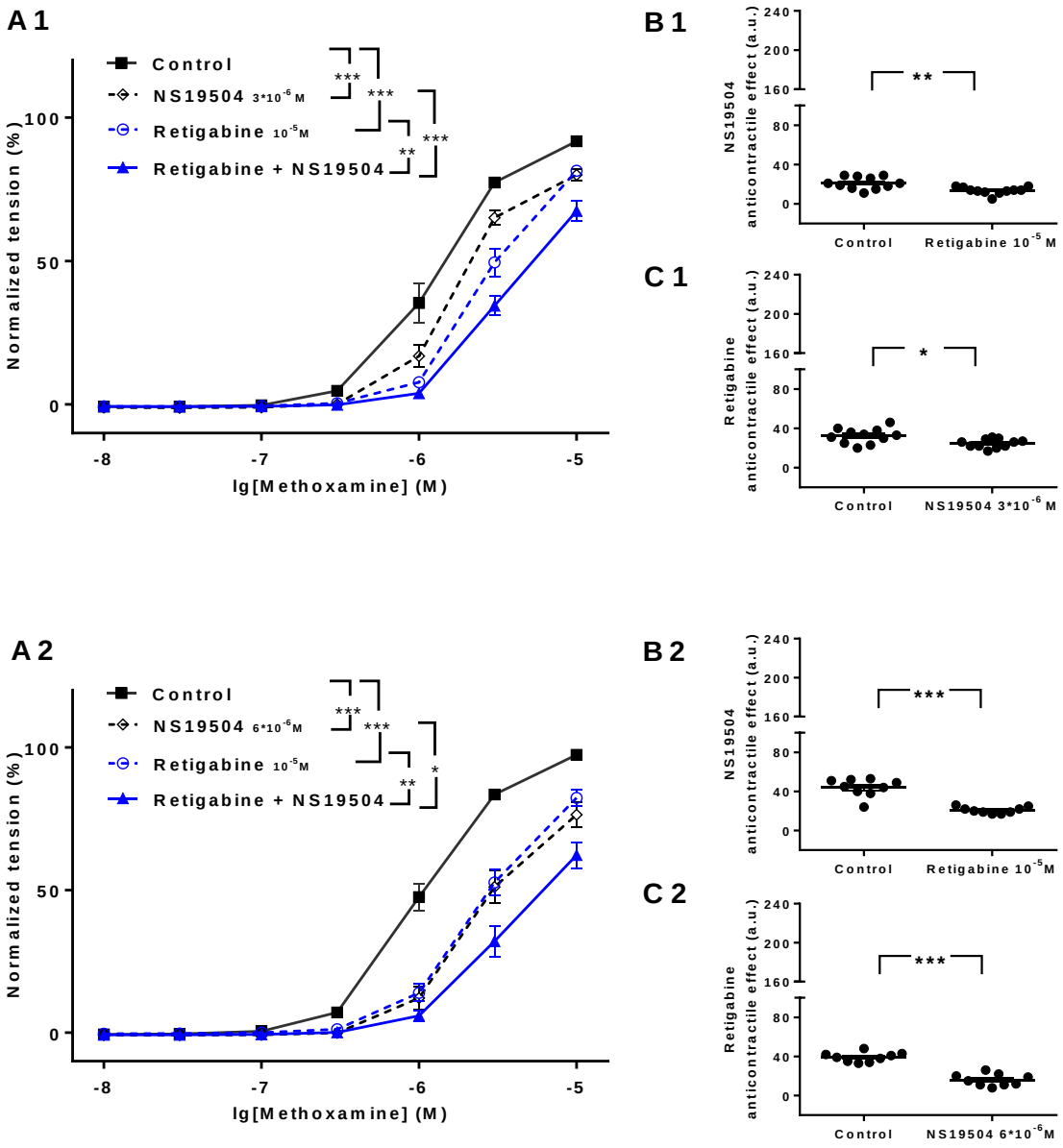

Figure 3.2.13 Effect of retigabine and NS19504 on methoxamine-induced contractions

A1-A2) Normalized A.Saphena tension at different methoxamine concentration in the absence of potassium channel active agents (Control), in the presence of retigabine (Retigabine 10^{-5}M), in the presence of NS19504 (A1: NS19504 $3*10^{-6}$M, A2: NS19504 $6*10^{-6}$M) and in the combined presence of retigabine and NS19504 (Retigabine + NS19504). **B1-B2)** NS19504 anti-contractile effect in the absence (Control) and presence of retigabine (Retigabine 10^{-5}M). **C1-C2)** Retigabine anti-contractile effect in the absence (Control) and presence of NS19504 (C1: NS19504 $3*10^{-6}$M, C2: NS19504 $6*10^{-6}$M). n1=11, n2=9; * - p<0.05, **- p<0.01; *** - p<0.001.

As to the myogenic response, it was weakened respectively by NS19504 at $3*10^{-6}$M and retigabine at $3*10^{-6}$M. However, in the combined presence of NS19504 and retigabine, the myogenic response was without difference compared with that observed in either NS19504 alone or retigabine alone (Fig 3.2.14 A). Correspondingly, the anti-contractile effect of NS19504 (Fig 3.2.14 B) and the anti-contractile effect of retigabine (Fig 3.2.14 C) are decreased in the presence of retigabine or NS19504, respectively.

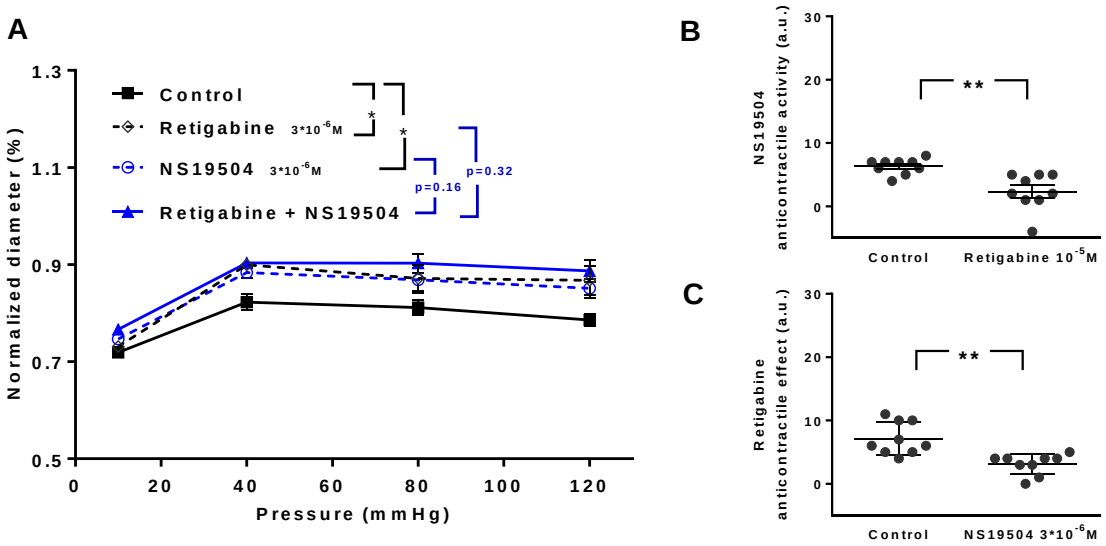

Figure 3.2.14 Effect of retigabine and NS19504 on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of retigabine (Retigabine $3*10^{-6}$M), in the presence of NS19504 (NS19504 $3*10^{-6}$M) and in the combined presence of retigabine and NS19504 (Retigabine + NS19504). **B)** NS19504 anti-contractile effect in the absence of (Control) and presence of retigabine (Retigabine $3*10^{-6}$M). **C)** Retigabine anti-contractile effect in the absence of (Control) and presence of NS19504 (NS19504 $3*10^{-6}$M). n=9; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.2.3 Combined effect of Kv7 channel activator and BK channel blocker on arterial contractility

The combined action of retigabine and IBTX was studied on methoxamine-induced contractions in wire myograph and on pressure-induced myogenic responses in pressure myograph experiments.

In methoxamine-induced contractions, retigabine was studied at two concentrations ($3*10^{-6}$M, 10^{-5}M), both of which decreased methoxamine-induced contractions that were increased by IBTX at 10^{-7}M. When IBTX was combined with retigabine at $3*10^{-6}$M, this combination weakened arterial contraction less than retigabine at $3*10^{-6}$M alone, whereas the combined effect of IBTX and retigabine with 10^{-5}M was similar to the effect of retigabine at 10^{-5}M alone (Fig 3.2.15 A1, A2). In addition, in the presence of IBTX, the anti-contractile effect of retigabine was enhanced (Fig 3.2.15 B1, B2), while the functional availability of the BK channel was declined in the presence of retigabine at both concentrations (Fig 3.2.15 C1, C2).

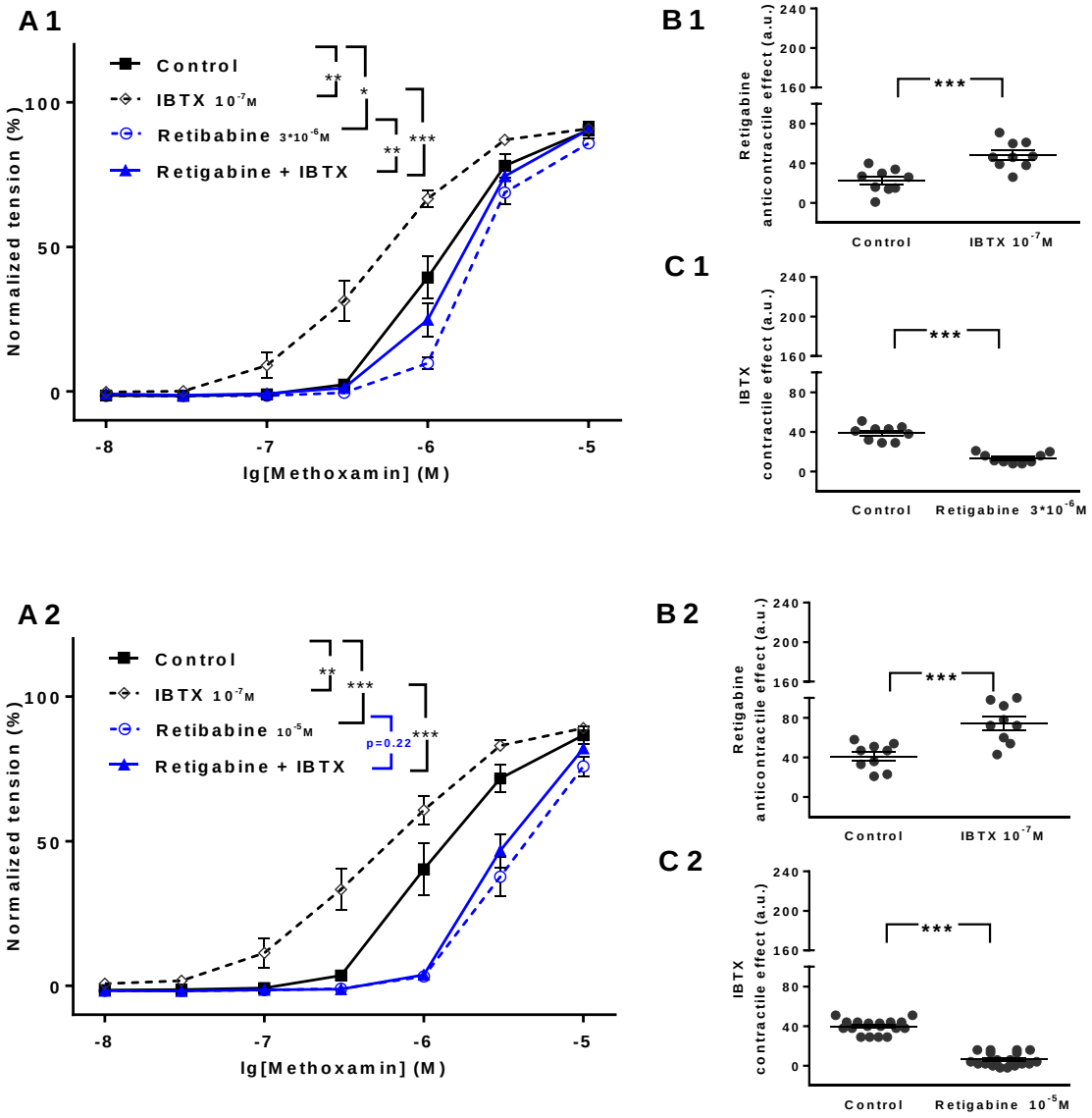

Figure 3.2.15 Effect of retigabine and IBTX on methoxamine-induced contractions

A1-A2) Normalized A.Saphena tension with different methoxamine concentrations in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of retigabine (A1: Retigabine $3*10^{-6}$M; A2: Retigabine 10^{-5}M) and in the combined presence of retigabine and IBTX (Retigabine + IBTX). **B1-B2)** Retigabine anti-contractile effect in the absence of (Control) and presence of IBTX (IBTX 10^{-7}M). **C1-C2)** IBTX contractile effect in the absence of (Control) and presence of retigabine (C1: Retigabine $3*10^{-6}$M, C2: Retigabine 10^{-5}M). n1=9, n2=9; * - p<0.05, **- p<0.01; *** - p<0.001.

In accord with the results above, retigabine at $3*10^{-6}$M weakened the myogenic response, while IBTX at 10^{-7}M strengthened it. Meanwhile, the myogenic response in the combined presence of retigabine and IBTX was similar to that in the presence of retigabine alone (Fig 3.2.16 A). Correspondingly, IBTX increased the anti-contractile effect of retigabine (Fig 3.2.16 B), whereas retigabine attenuated the functional availability of BK channels (Fig3.2.16 C).

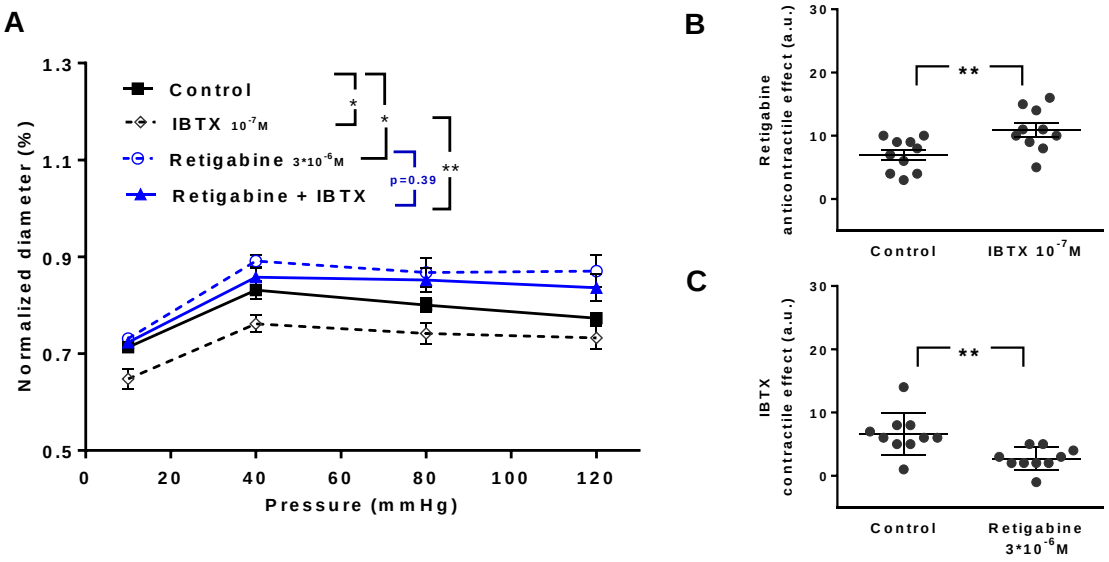

Figure 3.2.16 Effect of retigabine and IBTX on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of retigabine (Retigabine $3*10^{-6}$M) and in the combined presence of retigabine and IBTX (Retigabine and IBTX). **B)** Retigabine anti-contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of retigabine (Retigabine $3*10^{-6}$M). n=10; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.2.4 Combined effect of Kv7 channel blocker and BK channel activator on arterial contractility

The combined action of XE991 and NS19504 was studied on methoxamine-induced contractions in wire myograph and on pressure-induced myogenic responses in pressure myograph experiments.

On methoxamine-induced contractions, NS19504 was studied at two concentrations ($3*10^{-6}$M, $6*10^{-6}$M):

At NS19504 $3*10^{-6}$M, artery tension in the combination of NS19504 and XE991 was weaker than that in the presence of XE991 only, but stronger than that in the presence of NS19504 alone (Fig 3.2.17 A1). Meanwhile, there was no change in the anti-contractile effect of NS19504 and the functional availability of Kv7 channels in the presence of XE991 and NS19504, respectively (Fig 3.2.17 B1, C1).

At NS19504 $6*10^{-6}$M, artery tension in the combined presence of NS19504 and XE991 was similar to that in the presence of NS19504 alone (Fig 3.2.17 A2). Moreover, the anti-contractile effect of NS19504 was notably increased with XE991 (Fig 3.2.17 B2), whereas the functional availability of Kv7channels was decreased considerably in the presence of NS19504 (Fig 3.2.17 C2).

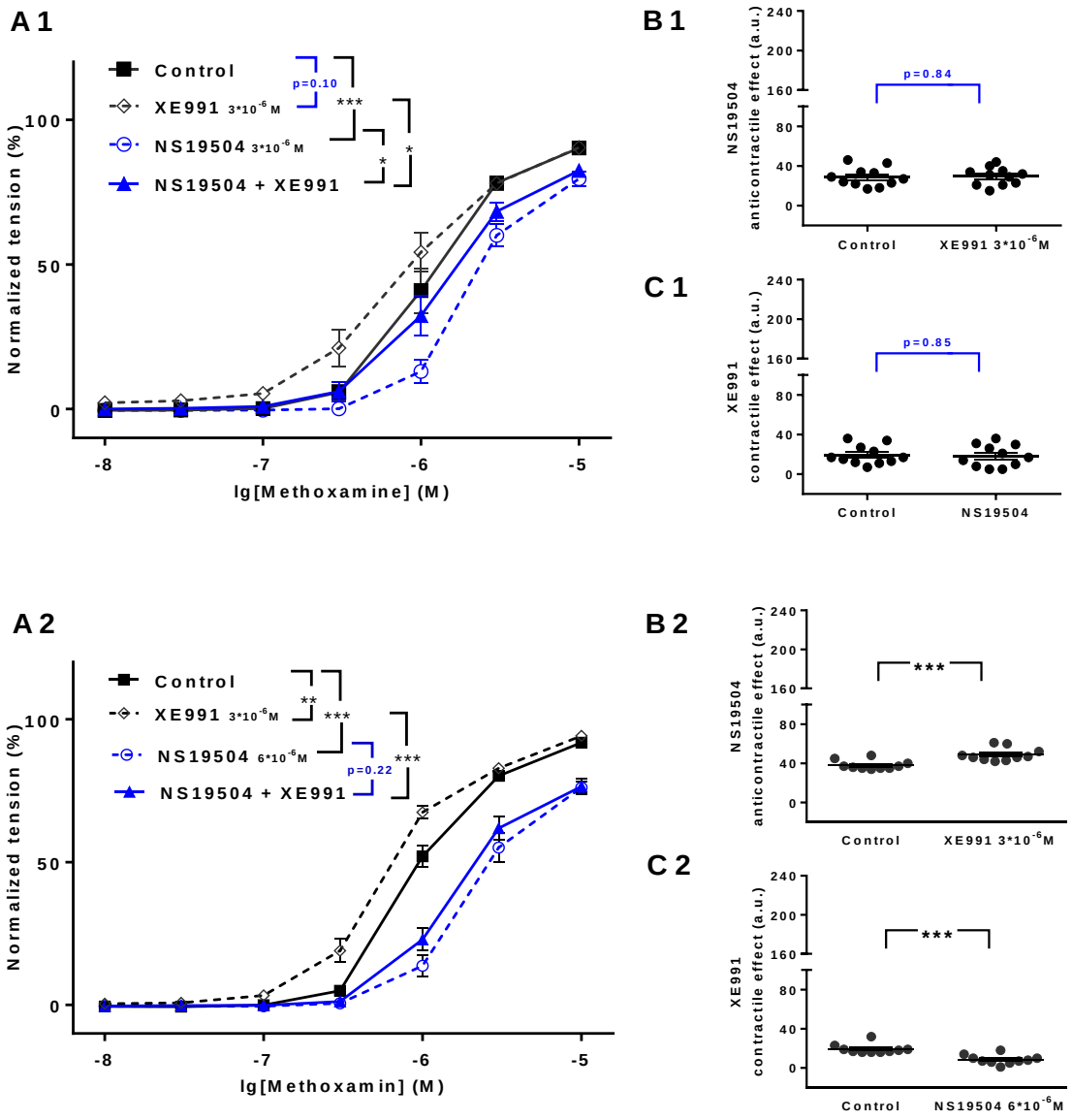

Figure 3.2.17 Effect of NS19504 and XE991 on methoxamine-induced contractions

A1-A2) Normalized tension of A.Saphena with different methoxamine concentrations in the absence of potassium channel active agents (Control), in the presence of XE991 (XE991 $3*10^{-6}$M), in the presence of NS19504 (A1: NS19504 $3*10^{-6}$M; A2: NS19504 $6*10^{-6}$M) and in the combined presence of NS19504 and XE991 (NS19504 + XE991). **B1-B2)** NS19504 anti-contractile effect in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). **C1-C2)** XE991 contractile effect (Kv7 channel functional availability) in the absence (Control) and presence of NS19504 (C1: NS19504 $3*10^{-6}$M; C2: NS19504 $6*10^{-6}$M). n1=11, n2=10; * - p<0.05, **- p<0.01; *** - p<0.001.

Likewise, the myogenic response in the combined presence of NS 19504 at $3*10^{-6}$M and XE991 at $3*10^{-6}$M was weaker than that in the presence of XE991 alone, but stronger than that in the presence of NS19504 alone (Fig 3.2.18 A). There is no difference in the anti-contractile effect of NS19504 with or without XE991, and in the

functional availability of Kv7 channels with or without NS19504 (Fig 3.2.18 B, C).

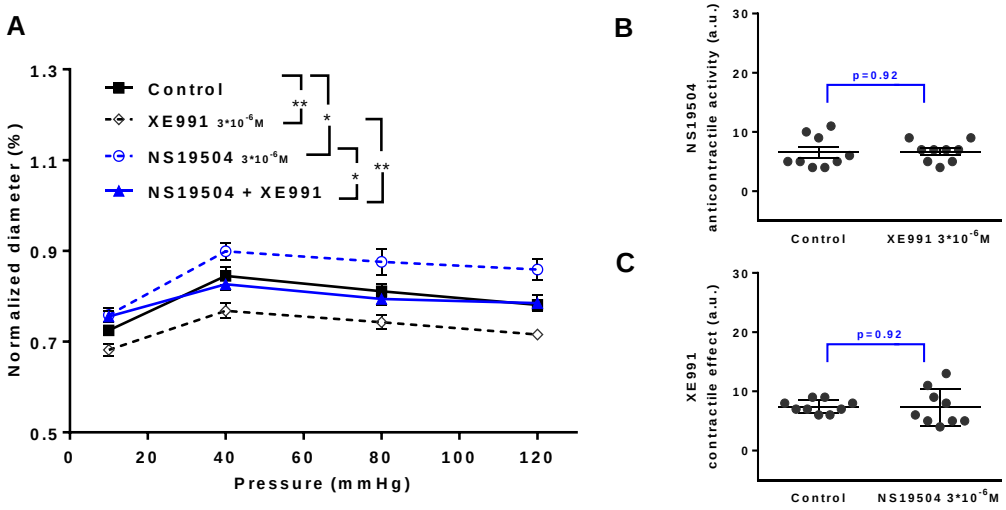

Figure 3.2.18 Effect of NS19504 and XE991 on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of XE991 (XE991 $3*10^{-6}$M), in the presence of NS19504 (NS19504 $3*10^{-6}$M) and in the combined presence of NS19504 and XE991 (NS19504 + XE991). **B)** NS19504 anti-contractile effect in the absence (Control) and presence of XE991 (XE991 $3*10^{-6}$M). **C)** XE991 contractile effect (Kv7 channel functional availability) in the absence (Control) and presence of NS19504 (NS19504 $3*10^{-6}$M). n=9; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.2.5 Combined effect of BK channel blocker and non-Kv7 channel blocker on arterial contractility

- Combined effect of BK channel blocker and Kv1.5 channel blocker on the myogenic response

The interaction of BK channels and Kv1.5 channels in the myogenic response was studied by using the selective Kv1.5 channel blocker DPO-1 (Lagrutta et al., 2006) and IBTX. The results show that the myogenic response was strengthened in the respective presence of IBTX at 10^{-7}M and of DPO-1 at $3*10^{-7}$M. The combined presence of IBTX and DPO-1 resulted in a further strengthening of the myogenic response (Fig 3.2.19 A). Correspondingly, the functional availability of Kv1.5 channels, represented as the contractile effect of DPO-1, as well as the functional availability of the BK channels was increased in the presence of IBTX and DPO-1, respectively (Fig 3.2.19 B ,C).

- Combined effect of BK channel blocker and Kv2.1 channel blocker on the myogenic response

The interaction of BK channels and Kv2.1 channels in the myogenic response was studied by application of the selective Kv2.1 channel blocker stromatoxin-1 (STTX) (Escoubas et al., 2002) and IBTX. The myogenic response was not strengthened in the presence of STTX at 10^{-7}M. Nevertheless, the myogenic response in the combined presence of IBTX and STTX was stronger than that in the presence of STTX alone (Fig 3.2.20 A). Meanwhile, the functional availability of Kv2.1 channels,

expressed as the contractile effect of STTX, was increased in the presence of IBTX (Fig 3.2.20 B), and the functional availability of BK channels was increased in the presence of STTX (Fig 3.2.20C).

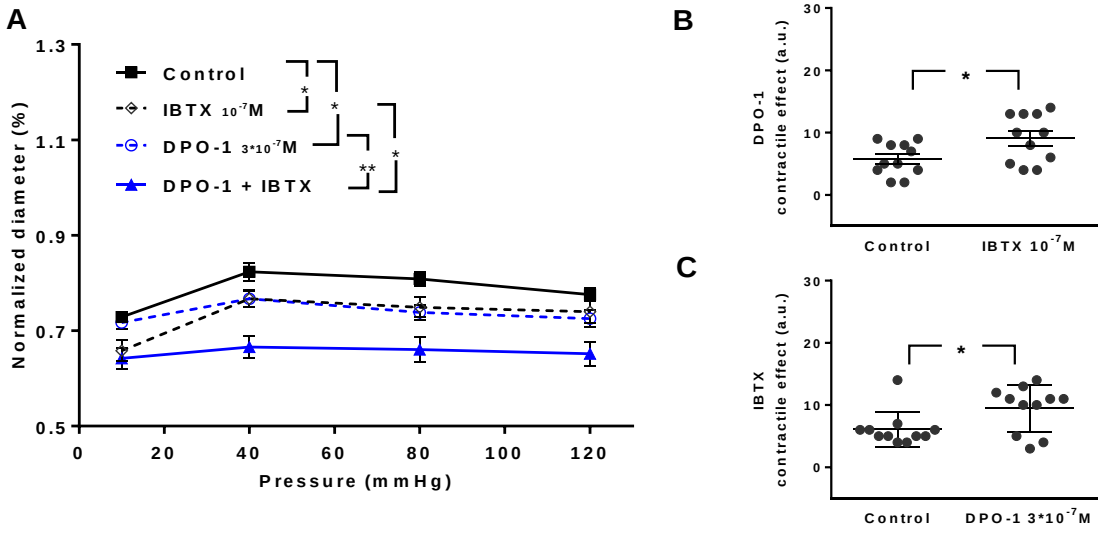

Figure3.2.19 Effect of DPO-1 and IBTX on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of DPO-1(DPO-1 $3*10^{-7}$M) and in the combined presence of DPO-1 and IBTX (DPO-1 + IBTX). **B)** DPO-1 contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of DPO-1 (DPO-1 $3*10^{-7}$M). n=11; * - p<0.05, **- p<0.01; *** - p<0.001.

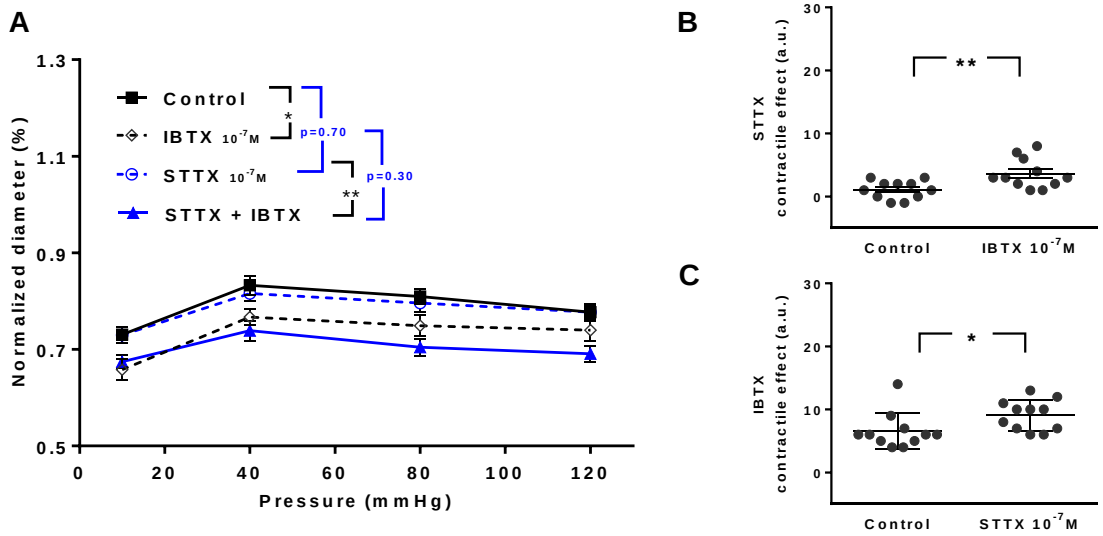

Figure 3.2.20 Effect of STTX and IBTX on the myogenic response

A) Normalized diameter of A.Gracilis at different intra-luminal pressures in the absence of potassium channel active agents (Control), in the presence of IBTX (IBTX 10^{-7}M), in the presence of STTX (STTX 10^{-7}M) and in the combined presence of STTX and IBTX (STTX + IBTX). **B)** STTX contractile effect in the absence (Control) and presence of IBTX (IBTX 10^{-7}M). **C)** IBTX contractile effect (BK channel functional availability) in the absence (Control) and presence of STTX (STTX 10^{-7}M). n=11; * - p<0.05, **- p<0.01; *** - p<0.001.

3.2.3 Summary of respective function of Kv7 channels and BK

When combining the results of all series in this study, for methoxamine-induced contractions it is shown that arterial contractions are enhanced by XE991 at intermediate concentrations of methoxamine ($3*10^{-7}$M and 10^{-6}M), whereas they are weakened by retigabine at higher concentration of methoxamine (10^{-6}M to 10^{-5}M). On the other hand, IBTX enhanced arterial tension in a relatively wide range of methoxamine concentrations (10^{-7}M to $3*10^{-6}$M), whereas NS19504 is similar to retigabine in that it also decreased contractions at higher concentrations of methoxamine (Table 3.2.1).

Table 3.2.1 Effect of blockers and activators of Kv7 and BK channels on arterial contractions at different methoxamine concentrations in the A. Saphena

Methoxamine	10^{-8}	$3*10^{-8}$	10^{-7}	$3*10^{-7}$	10^{-6}	$3*10^{-6}$	10^{-5}	n
XE991 $3*10^{-6}$	p=0.65	p=0.65	p=0.15	***	***	p=0.30	p=0.65	41
Retigabine 10^{-5}	p> 0.99	p> 0.99	p> 0.99	p=0.14	***	***	***	29
IBTX 10^{-7}	p=0.34	p=0.34	***	***	***	***	p=0.25	48
NS19504 $3*10^{-6}$	p> 0.99	p> 0.99	p> 0.99	p=0.20	***	***	***	29
$6*10^{-6}$	p> 0.99	p> 0.99	p= 0.98	p=0.08	***	***	***	33
10^{-5}	p> 0.99	p> 0.99	p=0.99	p=0.84	***	***	***	14

* - p<0.05, **- p<0.01; *** - p<0.001

Different from methoxamine-induced contractions, for pressure induced myogenic responses the data demonstrate that XE991 is similar to IBTX in that both enhanced myogenic responses at every pressure level. Retigabine as well as NS19504 respectively reduced myogenic responses at almost every pressure levels except 10mmHg (Table 3.2.2).

Table 3.2.2 Effect of blockers and activators of Kv7 and BK channels on the myogenic response at different pressure levels in the A. Gracilis

Pressure	10 mmHg	40 mmHg	80 mmHg	120 mmHg	n
XE991 $3*10^{-6}$	p=0.05	***	***	***	13
Retigabine $3*10^{-6}$	(-)	**	**	***	14
IBTX 10^{-7}	*	**	***	**	14
NS19504 $3*10^{-6}$	(-)	**	**	***	11

(-) before application of retigabine or NS19504, the diameter of arteries at 10mmHg was already similar to the maximum diameter in calcium-free solution

* - p<0.05, **- p<0.01; *** - p<0.001

3.2.4 Summary of the functional interaction of Kv7 and BK channels

Table 3.2.3 and Table 3.2.4 show the summary of the interaction effects of different active agents selectively targeting Kv7 channels and BK channels. As demonstrated, there were several obvious interactions between these active agents, and the effects of interaction were similar in methoxamine-induced contractions and in myogenic

responses. Specifically, XE991 abolished the effect of retigabine and increased the effects of NS19504 and IBTX. In turn, retigabine increased the effect of XE991, and it impaired the effects of IBTX and NS19504. On the other hand, IBTX abolished the effect of NS19504, whereas it improved the effects of XE991 and retigabine. NS19504 increased the effect of IBTX, and reduced the effects of retigabine and XE991.

Table 3.2.3 Interaction of active agents of Kv7 and BK channels in methoxamine-induced contractions

Effect / Agent	XE991 $3*10^{-6}$	Retigabine $3*10^{-5}$	IBTX 10^{-7}	NS19504 $3*10^{-6}$	$6*10^{-6}$	10^{-5}	IBTX10^{-7} +XE $3*10^{-6}$
XE991 $3*10^{-6}$		↓***	↑ ***	p=0.84	↑ ***		
Retigabine $3*10^{-5}$	↑ ***		↓**	↓**	↓***		
IBTX 10^{-7}	↑ ***	↑ **		↓***	↓***	↓***	
NS19504 $3*10^{-6}$	p=0.85	↓*	↑ **				
$6*10^{-6}$	↓***	↓***	↑ **				
10^{-5}			↑ **				↑ ***
IBTX 10^{-7} +XE $3*10^{-6}$						↓***	

* - p<0.05, **- p<0.01; *** - p<0.001

Table 3.2.4 Interaction of active agents of Kv and BK channels in the myogenic response

Effect / Agent	XE991 $3*10^{-6}$	Retigabine $3*10^{-6}$	IBTX 10^{-7}	NS19504 $3*10^{-6}$	DPO-1 $3*10^{-7}$	STTX 10^{-7}	HMR1556 10^{-5}
XE991 $3*10^{-6}$		↓***	↑ ***	p=0.92			
Retigabine $3*10^{-6}$	↑ ***		↓**	↓**			
IBTX 10^{-7}	↑ ***	↑ **		↓***	↑ *	↑ **	p=0.23
NS19504 $3*10^{-6}$	p=0.92	↓**	↑ ***				
DPO-1 $3*10^{-7}$			↑ *				
STTX 10^{-7}			↑ *				
HMR1556 10^{-5}			p=0.24				

* - p<0.05, **- p<0.01; *** - p<0.001

4 DISCUSSION

4.1 Expression of Kv7 and BK channels in rat skeletal muscle arteries

In this study, the gene expression of Kv7 (KCNQ) and BK channels in skeletal muscle arteries was detected using the qPCR technique.

According to the results, different subunits of Kv7 and BK channels are expressed in the A. Saphena and the A. Gracilis. In particular, BK channels (BK α and BK β1 subunits) are dominant as in other systemic blood vessels (Nelson and Quayle 1995; Tykocki et al. 2017). Regarding KCNQ genes, KCNQ4 is the most highly expressed, followed by KCNQ1 and KCNQ5, whose expression profile is similar to rat arteries studied previously (e.g. Gracilis arteries, cerebral arteries, coronary arteries, pulmonary arteries and mesenteric arteries) and human arteries(e.g. visceral arteries and mesenteric arteries) (Joshi et al., 2009; Ng et al., 2011; Zhong et al. 2010; Khanamiri et al. 2013; Zavaritskaya et al., 2013). Meanwhile, by comparison of the results obtained from vessel with and without endothelium, it was observed that the expression of Kv7 and BK channels is not dependent on the endothelium.

Therefore, both A. Saphena and A. Gracilis can be employed to examine the function of Kv7 and BK channels involved in arterial contractility. To enable the study of Kv7 and BK channels located in smooth muscle cells, vessels without endothelium can be used in the functional studies.

4.2 Function of Kv7 and BK channels in rat skeletal muscle arteries

4.2.1 Selection of appropriate methods

As known, in vivo there are numerous physiological factors, such as neurotransmitters, metabolites, hormones, mechanical factors (e.g. shear stress and transmural pressure), and interactions between VSMCs and endothelial cells or between proximal and distal vessel segments, etc., participating in the regulation of arterial contractility. In this complex situation, it is hard to interpret experimental data and clarify the exact role of any ion channel involved in the modulation of arterial contractility (Schubert, 2005). Meanwhile, the gene expression of the potassium channel in A. Saphena and A. Gracilis is independent of the endothelium as demonstrated above. In addition, as established previously, myogenic responses and agonist-induced contractions are functional independent of an intact endothelium (Kuo et al., 1990; Falcone et al., 1991; Meininger and Davis, 1992; Gaynullina et al., 2013). Thus, isolated arteries denuded of the endothelium were employed in this study. This enables us to better control the experimental conditions and to facilitate the interpretation of the data on the function of potassium channels involved in arterial contractility.

Moreover, the intrinsic myogenic response as well as vasoactive substance modulation are two main mechanisms governing vascular contractility (Brozovich et al., 2016). Therefore, in this study, the interaction of Kv7 and BK channels in the regulation of the contractility of isolated arteries was examined during pressure-induced myogenic responses (pressure myography) and agonist-induced contractions (wire myography) where methoxamine was used as a vasoconstrictor.

4.2.2 Respective function of Kv7 and BK channels

Before the discussion of the Kv7 and BK channel interaction, their respective role in the modulation of artery contractility will be clarified.

In general, the data of the presented study show that Kv7 and BK channels both participate in the maintenance of basic vascular tone of A.Saphena and A. Gracilis and appear to serve as negative feedback mechanisms in pressure-induced myogenic responses and vasoconstrictor-induced contractions, as XE991/IBTX or retigabine/NS19504 enhanced or reduced arterial contractility in pressure-induced myogenic responses and the methoxamine-induced contractions.

A detailed discussion of the roles of Kv7 and BK channels in the pressure-induced myogenic response and methoxamine-induced contraction will be presented in the following sections.

4.2.2.1 Roles of Kv7 and BK channels in the myogenic response

1) Mechanism of the myogenic response

In the vascular system, the myogenic response is an intrinsic reaction of small arteries to changes in vascular intraluminal pressure, in which the artery can constrict further or dilate from an original partially contracted state (myogenic tone). It is independent of influences from neurotransmitters, metabolites, hormones, etc. and plays a critical role in the establishment of basic vascular tone and the autoregulation of blood flow in the normal blood pressure range (Bayliss 1902; Davis & Hill 1999).

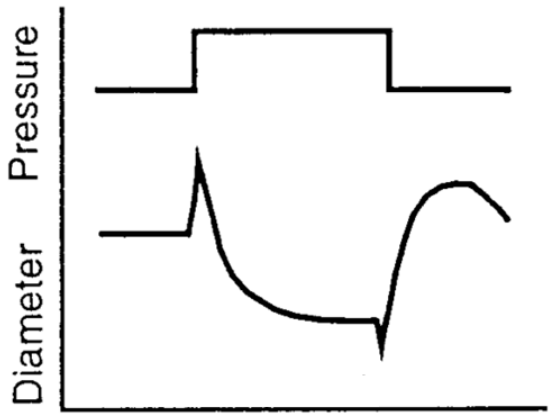

Figure 4.1 Example of a pressure-induced myogenic response in small arteries
(Davis & Hill 1999), modified

When the intraluminal pressure was increased immediately, the small artery displays a transient passive distension followed by vasocontriction, whereas when the intraluminal pressure reduced, the small artery shows vasodilatation following a transient collapse. Quantitative scales for time, pressure, and diameter depend on vessel type, size, species, and the method of study.

Although the mechanism of the myogenic response is yet to be thoroughly clarified, the basic mechanisms mediating this response have been demonstrated. The increased intraluminal pressure can cause VSMCs depolarization and an initial Ca^{2+} influx through VGCCs to activate the contractile proteins and to induce myogenic vasoconstriction. The pressure-induced VSMCs depolarization probably arises mainly from activation of mechanosensitive (MS) ion channels (e.g. nonselective cation channels) that allow Na^{+} or Ca^{2+} influx, stimulation of chloride channels that mediate Cl^{-} efflux, and/or a possible inhibition of potassium channels.

Besides that, pressure-induced mechanical deformation of the vascular wall can activate phospholipase C (PLC) and generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) to induce Ca^{2+} release from the

sarcoplasmic reticulum (SR) and activate protein kinase C (PKC) thereby modulating the activities of potassium channels, as it occurs in the receptor-mediated activation process (Meininger and Davis, 1992). Meanwhile, Ca^{2+} release from SR and increased Ca^{2+} sensitivity (Schubert et al., 2008) also have been supposed to be involved in the myogenic response.

However, pressure-induced myogenic vasoconstriction would finally arrive at a stable level rather than lead to progressive constriction. This is mainly ascribed to the activation of potassium channels by membrane depolarization, increased local Ca^{2+} or stretch itself, especially BK channels or Kv channels including Kv7 channels, serving as a brake on pressure-induced depolarization to limit the magnitude of the myogenic response(Brayden and Nelson 1992; Knot and Nelson 1995; Cole et al. 2005; Hill et al. 2010; Zhong et al. 2010). On the other hand, increased pressure is also known to produce some endogenous factors that can limit the activation of potassium channels, such as the BK channel inhibitor 20-hydroxyeicosatrienoic acid (20-HETE) produced from arachidonic acid (AA) metabolism in VSMCs(Zou et al. 1996; Davis and Hill 1999; Miyata and Roman 2005).

2) Overview of myogenic response in present study

In the present study, the characteristics of A.Gracilis myogenic responses to graded elevations of intraluminal pressure (10-120mmHg) are in accord with the characteristics of typical myogenic responses, in which with an acute increase in intraluminal pressure, vasoconstriction was induced following a short term passive distension, whereas with an acute reduction in intraluminal pressure, vasodilatation happened following a transient collapse (Fig 4.1) (Bayliss 1902; Davis and Hill 1999).

When the pressure increased stepwise from 40 mmHg to 80mmHg to 120 mmHg, the diameter of A.Gracilis at each pressure step is smaller than that at the previous, lower pressure step, which is consistent with the facts observed in previous electrophysiological experiments that with the stepwise increased pressure, a graded increased depolarization is recorded (Wesselman et al. 1997; Yang et al. 2009).

3) Roles of Kv7 and BK channels in the myogenic response

- Effect of blocker and activator of Kv7 channels on myogenic responses

It was observed that XE991 significantly increased the A.Gracilis myogenic constriction at pressures greater than 10 mmHg. Correspondingly, retigabine reduced myogenic constriction in the similar pressure range.

It seems that at lower pressure, Kv7 channels only weakly contribute to myogenic tone, which might due to the lower tension of an artery with relatively weak pressure-induced depolarization. With the pressure increased, at 40mmHg and higher, Kv7 channels are involved in the modulation of arterial myogenic tone. Under these conditions, inhibition of Kv7 channels leads to further constriction, while activation of Kv7 channels reduces myogenic tone.

These data are supported by findings from other experiments showing that inhibition of Kv channels caused VSMCs depolarization in pressurized arterioles and augmented myogenic tone. For example, in rat middle cerebral arteries, linopirdine

(another Kv7 channel blocker) suppressed Kv current amplitude and enhanced myogenic constriction at pressures ≥ 20mmHg, whereas S-1(another Kv7 channel activator) stimulated Kv currents and weakened myogenic responses at pressures greater than 20mmHg (Zhong et al., 2010). In rabbit cerebral arteries, 4-AP (a general Kv1-4 channel blocker) depolarized VSMCs and augmented myogenic tone at pressures greater than 40 mmHg (Knot and Nelson, 1995). In rat middle cerebral arteries, with expression of Kv1.5 channels suppressed, the myogenic response was strengthened with enhanced myogenic depolarization (Chen et al., 2006).

Therefore, the data of the present study suggest that Kv7 channels play a negative feedback role in the myogenic response to prevent excessive vasoconstriction by limiting pressure-induced depolarization.

- Effect of blocker and activator of BK channels on myogenic responses

Regarding BK channels, the present study showed that IBTX increased myogenic constriction in the pressure range from 10mmHg to 120mmHg; while NS19504 reduced myogenic constriction in the similar pressure range, which means inhibition of BK channels enhances the myogenic response, while activation of BK channels reduces myogenic constriction.

These findings are different from the observation in mesenteric arteries that demonstrated that closure of BK channels plays a major role in pressure-induced vasoconstriction rather than activation of BK channels limiting the myogenic response(Wesselman et al., 1997; Chlopicki et al., 2001). However, our results are in accordance with most other findings that BK channels can be significantly activated in the normal pressure range, and inhibition of BK channels caused VSMCs depolarization and enhanced myogenic constriction (Brayden and Nelson 1992; Knot and Nelson 1995; Wesselman et al. 1997; Davis and Hill 1999; Tykocki et al. 2017). The difference to the former experiments might mainly be ascribed to the special experimental condition where mesenteric arteries with very weak myogenic tone were treated with norepinephrine producing preconstriction to enable the myogenic responses.

Therefore, as with Kv7 channels, the data of the present study suggest that BK channels also participate in the negative feedback regulation of myogenic tone in the A. Gracilis

In summary, during myogenic responses, Kv7 and BK channels can be activated together and function to counteract myogenic constriction by precise control of myogenic depolarization.

4.2.2.2 Roles of Kv7 and BK channels in methoxamine-induced contraction

1) Mechanism of vasoconstrictor-induced arterial contraction

A number of vasoconstrictors, such as endothelin, angiotensin and thromboxane A2, can, through Gq-coupled receptors in the membrane of VSMCs, induce vascular contraction. Specifically, when the vasoconstrictor bind to the Gq-coupled receptors, PLC is activated, which generates the second messengers IP_3 and DAG. IP_3 can promote Ca^{2+} release from SR and the activation of MLCK to induce vasoconstriction

(Somlyo and Somlyo 1994; Davis and Hill, 1999; Somlyo and Somlyo 2003; Brozovich et al. 2016). DAG, on the other hand, can activate PKC altering the calcium sensitivity of the contractile apparatus and inhibiting the activities of potassium channels including Kv and BK channels by phosphorylation, which might contribute to the agonist-induced membrane depolarization and vasoconstriction (Fig.4.2) (Standen & Quayle 1998; Ko et al. 2008).

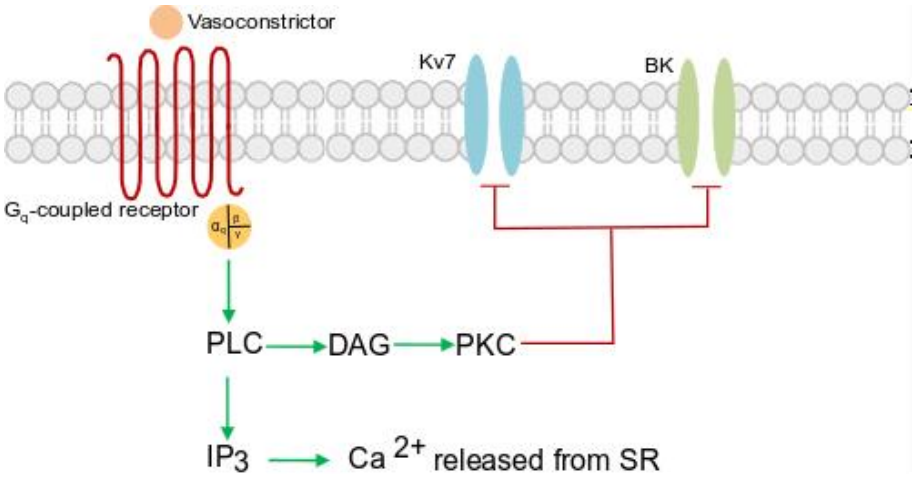

Figure 4.2 Schematic diagram of VSMC contraction induced by vasoconstrictors

Vasoconstrictors can, through Gq-coupled receptors in the membrane of VSMCs, activate phospholipase C (PLC), and generate the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can promote Ca^{2+} release from the sarcoplasmic reticulum (SR), while DAG can activate protein kinase C (PKC) to modulate the activities of ion channels including inhibition of Kv7 and BK channels.

2) Roles of Kv7 and BK channels in methoxamine-induced arterial contraction

In this study, methoxamine as a vasoconstrictor induced different intensity of A. Saphena contraction dependent on concentration.

- Effect of blocker and activator of Kv7 channels on methoxamine-induced arterial contraction

XE991 enhanced arterial contractions only at intermediate concentrations of methoxamine ($3*10^{-7}$M and 10^{-6}M), whereas contractions were weakened by retigabine at higher concentrations of methoxamine (10^{-6}M to 10^{-5}M). This suggests that at weaker contraction levels, Kv7 channels seem not to affect arterial contractility which might be due to the lower tension of the artery.

With the intensity of arterial contraction increased, inhibition of Kv7 channels can increase arterial contractility further, while activation of Kv7 channels can reduce arterial contractility. Meanwhile, the data show that the concentration-response relationship curve for methoxamine was shifted less by XE991 than by retigabine, which indicates that most Kv7 channels are closed during methoxamine -induced contraction, and at intermediate contractions, some more Kv7 channels begin to open as negative feedback mechanism to arterial contraction.

When the contraction achieved higher intensity, activation of Kv7 channels still can weaken arterial contractility, but inhibition of Kv7 channels cannot produce any effect

on arterial tension. This might be due to either almost all of the Kv7 channels have already been closed in order to contribute to the higher intensity of contraction caused by the inhibition effect of IP3 or the higher Ca^{2+} concentration on Kv7 channels, or there are still some Kv7 channels open, but the further contractile effect induced by inhibition of them is too small compared with the already very strong contraction.

Therefore, besides acting as a negative feedback, Kv7 channels might also contribute to promote further contraction at higher intensity level.

- Effect of blocker and activator of BK channels on methoxamine-induced arterial contraction

IBTX enhanced arterial tension in a relatively wide range of methoxamine concentrations (10^{-7}M to $3*10^{-6}$M), whereas NS19504, similar to retigabine, decreased contractions at higher concentrations of methoxamine.

As with Kv7 channels, BK channels do not contribute to the regulation of arterial tension at lower contraction levels. With enhanced contractile intensity, some BK channels are activated which might be due to depolarization and an increased intracellular Ca^{2+}, while some channels still keep closed. Under these conditions, inhibition of BK channels increases arterial contraction, while activation of BK channels reduces arterial tension. It is difficult to clarify which one, activated or closed, is the major state for BK channels during the methoxamine-induced contraction process. This is caused by the unspecific effect of NS19504 at higher concentration that hindered us to determine to what extend the methoxamine concentration-response curve can be really shifted when all of the BK channels are activated.

At the highest intensity of contraction, activation of BK channels still can weaken arterial contractility, whereas inhibition of BK channels cannot produce any effect on arterial tension. This might due to the same possible reasons as discussed for Kv7 channels.

Therefore, BK channels not only play a negative feedback role in the arterial contraction process, but might also serve as a positive feedback to achieve highest intensity of contraction.

In summary, as with pressure-induced myogenic responses, in methoxamine-induced contraction the activation of Kv7 and BK channels serves as a negative feedback modulating arterial contractility, and it seems that BK channel function is predominant in this process as BK channels perform negative control in a wider contractile intensity range. Meanwhile, the closure of Kv7 and BK channels might contribute to further contraction.

4.2.3 Functional interaction of Kv7 and BK channels

In order to investigate the interaction of Kv7 and BK channels, several combinations of activators and/or blockers of Kv7 and BK channels were tested as shown in the following table (table 4.1) in pressure-induced myogenic responses (pressure myography) and methoxmine-induced contractions (wire myography).

Table 4.1 combinations of activators and/or blockers of Kv7 and BK channels

	Kv7 channel blocker	Kv7 channel activator
BK channel blocker	XE991+IBTX	Retigabine+IBTX
BK channel activator	XE991+NS19504	Retigabine+NS19504

- Overview of interaction between Kv7 and BK channels

According to the results from experiments on both pressure-induced myogenic responses and methoxamine-induced contractions, XE991 increased the contractile effect of IBTX as well as the anticontractile effect of NS19504, whereas retigabine reduced the effect of both IBTX and NS19504. This suggested that inhibition of Kv7 channels could increase the functional availability of BK channels, while activation of Kv7 channels would decrease the functional availability of BK channels.

In turn, inhibition of BK channels can improve the functional availability of Kv7 channels, while activation of BK channels might decrease the functional availability of Kv7 channels, as IBTX increased both the contractile effect of XE991 and the anticontractile effect of retigabine, whereas NS19504 reduced the effect of retigabine and to some extent impaired the effect of XE991.

In other words, changes in the activity of Kv7 channels affect the activity of BK channels, and vice versa, which supports the idea that there is a dynamic equilibrium between the activities of Kv7 and BK channels when they perform similar function in regulation of arterial contractility.

- What are the possible mechanisms underlying this dynamic equilibrium?

This question will be discussed by focusing on Kv7 and BK channels themselves and essential factors of vasoconstriction (e.g. membrane potential and intracellular Ca^{2+}).

As mentioned in the introduction, Kv7 channels are voltage-dependent potassium (Kv) channels, which are directly activated by membrane depolarization. BK channels are large-conductance voltage-dependent and calcium-sensitive potassium channels, which are also activated by depolarization. Thus, membrane potential (depolarization) is the crucial trigger for the activation of both of these channels. In turn, their activities can affect the membrane potential. Especially, BK channels with large-conductance; activation of only a few of them would result in large changes of the membrane potential of VSMCs (Davis and Hill, 1999).

However, without Ca^{2+}, BK channels display low voltage sensitivity and are difficult to activate in the physiological membrane potential range. Meanwhile, there are some reports demonstrating that Kv channel activity can be inhibited by agonist-induced intracellular Ca^{2+} increases (Gelband et al., 1993; Ishikawa et al., 1993; Cox and Petrou, 1999). Therefore, intracellular Ca^{2+} is another key factor modulating the equilibrium between Kv7 and BK channels.

Moreover, these two types of K channels belong to the potassium channel family that is responsible for the transport of potassium ions to the extracellular space driven by electrochemical potential energy. The relationship between potassium ions, the activities of potassium channels and the membrane potential can be described as a plasma membrane equivalent circuit where each type of potassium channels is in

parallel contributing to the total resistance of potassium channels (R) (Fig. 4.3). A change in the activity of any potassium channel would lead to a change in potassium permeability (membrane conductance, 1/R). If the concentration of potassium ions is almost stable, potassium permeability will become an important determinant in the regulation of the membrane potential.

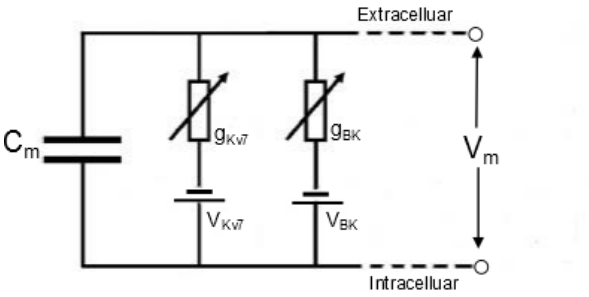

Figure 4.3 Equivalent circuit of the plasma membrane

In the equivalent circuit of the plasma membrane, every type of ion channel including Kv7 and BK channels is in parallel and contribute to the total membrane conductance (g).

Therefore, membrane potential, intracellular calcium, and membrane permeability might be the three key modulators of the dynamic equilibrium between the activities of Kv7 and BK channels.

In addition to the factors just mentioned, the exceeding diversity of these channels, arising from heteromultimerization, accessory proteins, alternate mRNA splicing or post-translational modification (Gutman et al., 2005), also should be taken into consideration. Because of channel subunit diversity, individual Kv7 or BK channels could possess different properties: Kv7 channels could display different voltage sensitivity; BK channels, besides having different voltage sensitivity, may have different Ca^{2+} sensitivity or different Ca^{2+} thresholds for voltage-dependent activation(Jackson and Blair 1998; Tykocki et al. 2017).

In other words, Kv7 and BK channels expressed in the same artery could be endowed with diverse activation thresholds, which probably account for the different extent of activation of Kv7 and BK channels with different intensity of stimulus. Specifically, at a certain stimulus concentration, a certain portion of Kv7/ BK channels has been activated, while the other Kv7/ BK channels with higher activation thresholds still stay at the resting state. At this point, if the activated Kv7 channels are inhibited, there would be a plasma membrane depolarization and an increase of calcium influx through VDCCs, which can activate some resting BK channels with relatively low activation threshold (to increase the IBTX-induced contractile effect) and bring more resting BK channels to approach their activation threshold (to increase the NS19504-induced anticontractile effect). Likewise, inhibition of BK channels also causes membrane potential depolarization. This depolarization can directly activate a portion of resting Kv7 channels (to increase the XE991-induced contractile effect) and bring more resting channels to be ready for activation (to increase the retigabine-induced anticontractile effect). On the other hand, activation of Kv7/BK channels would produce membrane potential hyperpolarization and hinder Ca2+ influx through VDCCs, and suppress BK/Kv7 channels activities.

In the view of the plasma membrane equivalent circuit, application of any one of the potassium channel blockers would reduce potassium permeability (increase membrane resistance). Based on this increased membrane resistance, application of another potassium channel blocker would produce a larger change in membrane potential according to Ohm’s law (U=IR).

Similarly, application of any potassium channel activator increases potassium permeability, i.e., reduces membrane resistance. Then, when another potassium channel activator is applied under conditions of a reduced resistance, a smaller change in membrane potential will be produced.

Therefore, it is reasonable to suggest that a dynamic equilibrium exists between Kv7 and BK channels and contributes to the modulation of arterial contractility. However, it is difficult to further investigate and clarify the specific interaction of each Kv7 subfamily with BK channels, as most of the Kv7 channel subfamilies, except Kv7.1, lack selective blockers/activators. The results obtained with the selective Kv7.1 channel blocker HMR1556 in pressure-induced myogenic responses showed that inhibition of Kv7.1 channels did not affect vascular tone and the activity of BK channels, while inhibition of BK channels also did not change the activity of Kv7.1 channels. Therefore, Kv7.1 channels appear not to participate in the maintenance of resting vascular tone and the regulation of myogenic responses, and these channels seem not to have an interaction with BK channels as well. This finding on Kv7.1 channels is consistent with other reports showing that coronary arteries were contracted by pan-Kv7 blockers, whereas HMR1556 had no effect on resting vascular tone (Chadha et al., 2012). Furthermore, preconstricted vessels were relaxed by selective Kv7.2-7.5 channel activators, whereas a Kv7.1 channel activator had no effect (Khanamiri et al. 2013; Tykocki et al. 2017).

Besides Kv7 channels, other Kv channel family members also can interact with BK channels. In this study, it was demonstrated that inhibition of Kv1.5 channels with DPO-1 and inhibition of Kv2.1/4.2 channels with STTX, respectively enhanced the functional availability of BK channels, while inhibition of BK channels also amplified the functional availability of Kv1.5 and Kv2.1/4.2 channels in pressure-induced myogenic responses. These findings are supported by other experiments (Tsvetkov et al., 2016). Moreover, these findings also support the conclusion obtained on the Kv7/BK channel interaction. However, the specific features of the Kv1.5 and Kv2.1 channel interaction with Kv7 channels were not further examined because they were out of the focus of the presented study.

- Comprehensive interpretation of the role of Kv7 and BK channels taking into account their interaction

Considering the interaction between Kv7 and BK channels, the data provided in the present study facilitate a more comprehensive analysis of the role of Kv7 and BK channels in arterial contractility.

For example, at the lowest pressure in the myogenic responses, and on weaker arterial contraction in the methoxamine-induced contraction process, XE991 or IBTX cannot induce any contractile effect. Thus, it seems that most of Kv7 and BK channels keep closed and do not contribute to the lower arterial tension., However, based on the interaction mechanism, there might be another explanation in that some Kv7 or BK channels are in an open state and XE991/IBTX can inhibit them which should lead to a contractile effect, but this effect is completely counteracted by the subsequent dilatory effect resulting from the activation of BK/Kv7 channels.

Specifically, for arteries with lower tension, application of XE991 to inhibit Kv7 channels, on the one hand, can induce membrane depolarization and Ca^{2+} influx via

VDCCs, and hence tend to produce arterial contraction. On the other hand, it would simultaneously activate some resting BK channels with relatively low activation threshold providing hyperpolarization to counteract vasoconstriction. Hence, this suggests that arterial tension should be extremely increased by XE991when the subsequent activation of BK channels was inhibited by IBTX, even at lower arterial tension. And the results in this study are corresponding to this supposition. Application of IBTX to inhibit BK channels would produce a similar effect. In this process, Kv7 channels also can be simultaneously activated. Although increased Ca^{2+} might to some extent suppress the activities of Kv7 channels, the depolarization induced activation of Kv7 channels can overwhelmingly override this Ca^{2+}inhibition effect, because the extent of the increased functional availability of Kv7 channels by IBTX is similar to that of BK channels enhanced by XE991. The Ca^{2+}inhibition effect on Kv7 channels can be neglected.

Corresponding to the plasma membrane equivalent circuit application of XE991would reduce potassium permeability (increase membrane resistance). Based on this increased membrane resistance, application of IBTX would produce a larger increase in membrane potential, and vice versa.

Therefore, in this study, for the lower arterial tension, there is no dilatory effect produced by activation of Kv7 and BK channels since the artery is at a relaxed state, while there is no contractile effect that could either be due to the almost closed Kv7 and BK channels at lower tension or to the effect of Kv7 and BK channel interaction in which the effect of subsequent BK/Kv7activation completely counteracts the original Kv7/BK inhibition.

4.2.4 Effect of the novel BK channel opener NS19504

The results presented in this study not only demonstrated the interaction of Kv7 and BK channels, but also provided new information on the novel BK channel opener NS19504 (Nausch et al., 2014). According to the results, it is important to note that the selectivity of NS19504 on BK channels is concentration dependent.

Firstly, the effect of NS19504 at higher concentrations (10^{-5}M) cannot be completely blocked by IBTX alone during methoxamine-induced contractions, but it can be completely blocked by IBTX combined with XE991, which indicated that NS19504 at higher concentrations not only potently activates BK channels but simultaneously stimulates Kv7 channels as well.

For the lower concentration of NS19504 ($3*10^{-6}$M), during pressure-induced myogenic responses and methoxamine-induced contractions, its effect seemed to be completely blocked by IBTX, but the effect is relative too weak to clearly clarify its interaction with Kv7 channel active agents.

Fortunately, NS19504 at $6*10^{-6}$M performed as a highly selective activator of BK channels, because the effect of NS19504 at this concentration can be completely blocked by IBTX. In addition, it decreased the respective effect of retigabine and XE991; in turn, XE991 enhanced its effect. Unfortunately, this concentration of NS19504 could not be tested on the myogenic response, because of the danger to loose myogenic reactivity.

Therefore, in this study, NS19504 at $6*10^{-6}$M can selectively activate BK channels, and definitely reduces the anticontractile effect of retigabine as well as the contractile effect of XE991, although NS19504 at higher concentrations seems to activate BK channels as well as some Kv7 channels. These results provide valuable information to direct us how to choose a selective concentration of NS19504 for the BK channel and how to analyze related results in a more objective and comprehensive way.

4.3 Conclusion

Kv7 channels and BK channels are expressed in rat skeletal muscle arteries, the A. Saphena and the A.Gracilis. They mainly function as a negative feedback in the regulation of the contractility of these arteries. Moreover, there is a dynamic equilibrium between Kv7 and BK channels, in which, membrane potential, concentration of Ca^{2+}, and membrane permeability might be the three major factors to build a bridge between Kv7 and BK channels.

Regarding NS19504, the selectivity of NS19504 for BK channels is concentration dependent. At $6*10^{-6}$ M NS19504 predominantly activates BK channels, whereas at higher concentrations, NS19504 is able to activate Kv7 channels as well.

4.4 Outlook

In this study, we have demonstrated for the first time that there is functional interaction between Kv7 and BK channels. However, we were unable to further clarify the respective roles of Kv7 subfamily members in this interaction process, because of a lack of corresponding selective blockers/activators, except for Kv7.1 channels that did not interact with BK channels. Meanwhile, it is a little bit unfortunate that NS19504 in this study was not able to activate BK channels completely with very satisfied high selectivity. Thus, the highest selective concentration used probably did not activate all BK channels so that we could not determine how vessel contractility alters under a full activation of BK channels. Therefore, exploitation and development of highly effective and selective blockers/activators of specific potassium channels is still very important for the thorough investigation of potassium channel function and their interaction. Moreover, with genetic techniques development, such as gene knockout or knockdown, siRNA interference etc., intervention with potassium channel expression has become another promising way to investigate the roles of specific potassium channels and their interaction. These genetic methods also can completely avoid the influence of interaction of active channel agents, although there is no report demonstrated there is obvious direct interaction between the activators and blockers employed in this study. The only limitation of this approach at the moment is that vessels have to be cultured for this purpose, but culture itself might induce changes in potassium channel expression.

Besides functional experiments, electrophysiological studies would directly provide more specific information on how membrane potential and potassium permeability changes during the interaction of Kv7 and BK channels. This electrophysiological information would provide helpful and forceful clues to further confirm and clarify the mechanism underlying this interaction. However, due to the large amount of experiments required for this approach, these were beyond the focus of the presented study.

Meanwhile, it is a promising direction to further investigate and clarify the possible factors involved in the modulation of the equilibrium between Kv7 and BK channels, especially the role of other ion channels, intracellular signaling molecules, such as second messengers (PKA, PKG and PKC) etc. However, a successful investigation in this direction depends on the effective intervention on the candidate factors.

In addition to physiological conditions, it is also worth to investigate how the equilibrium of Kv7 and BK channels changes in vascular diseases, which might provide some valuable indications to improve and innovate relevant intervention and treatment. It has been demonstrated that Kv7 and BK channels, respectively participate in several vascular pathophysiological process. But how they are exactly changed is still controversial. For example, in hypertension, expression and function of Kv7.4 channels are decreased in mesenteric arteries and coronary arteries(Jepps et al. 2011; Khanamiri et al. 2013), whereas it seems not to be altered in the gracilis arteries(Zavaritskaya et al., 2013); BK channel expression and function also have been reported to be enhanced as well as reduced in resistance arteries and arterioles(Tykocki et al., 2017). These controversial findings make the effect of a targeted therapy on single specific ion channels uncertain. It might be a promising breakthrough point for therapy to regulate the equilibrium between Kv7 and BK channels. However, till now, there are no studies to investigate this area. In the future, electrophysiological studies and functional studies combined with genetic techniques might facilitate a clear description and intervention of the changes in Kv7 and BK channel interaction in vascular diseases.

5 SUMMARY

The cardiovascular system is an organ system comprising three basic parts: heart, blood and vessels. Among them, the contractility of vessels plays an essential role in the determination of blood pressure and the regulation of blood distribution to different organs and tissues in order to meet the demand of the body. Membrane potential is one major determinant of vascular contractility, as the increased Ca^{2+} influx to trigger vascular contractile activity is mainly from the extracellular space through voltage-dependent Ca^{2+} channels.

Voltage-gated potassium (Kv) channels and large-conductance calcium-sensitive potassium (BK) channels are two major potassium channels identified in vascular smooth muscle cells, which provide hyperpolarizing conductance that contributes to the determination of membrane potential and Ca^{2+} influx, and hence play an important role in regulation of vascular contractility. Although they have become the spotlight of investigation in recent years there are only a few reports describing their expression and function in skeletal muscle arteries. Moreover, there is no report to clarify whether there is functional interaction of Kv7 and BK channels in intact arteries. Therefore, this study addressed the hypothesis that there is functional interaction between Kv7 and BK channels in skeletal muscle arteries under normal physiological conditions, which contributes to the maintenance of arterial contractility.

The Saphenous artery (A. Saphena, a conduit artery supplying blood to a large part of skeletal muscles on the hind limb) and the Gracilis artery (A. Gracilis, a resistance artery providing blood to the gracilis muscle on the hind limb) from Wistar rats (male, 8-12 weeks old) were isolated for this study. The expression of Kv7 and BK channels was detected by use of the qPCR technique; the function of Kv7 and BK channels and their interaction were examined on the A. Saphena during methoxamine-induced contractions using wire myography and on the A. Gracilis during pressure-induced myogenic responses using pressure myography.

The results show that several subfamily members of Kv7 channels (KCNQ) and BK channels were expressed in A. Saphena and A. Gracilis: the highest expression was observed for BKα, BKβ1 and KCNQ4, followed by KCNQ1, the other subfamily members were found at lower expression levels. Moreover, their expression was independent of the endothelium. In methoxamine-induced contractions and pressure-induced myogenic responses, inhibition of Kv7 channels or BK channels by XE991 (a selective blocker of Kv7 channels) or IBTX (a selective blocker of BK channels) enhanced arterial contractility, whereas activation of Kv7 channels or BK channels by retigabine (a selective activator of Kv7 channels) or NS19504 (a novel activator of BK channels) reduced arterial contractility. Furthermore, XE991 increased the contractile effect of IBTX as well as the anticontractile effect of NS19504, whereas retigabine reduced the effect of both IBTX and NS19504, which suggests that inhibition of Kv7 channels increases the functional availability of BK channels, while activation of Kv7 channels decreases the functional availability of BK channels. In turn, IBTX increased both the contractile effect of XE991 and the anticontractile effect of retigabine, whereas NS19504 reduced the effect of retigabine and impaired the effect of XE991, which indicates that inhibition of BK channels improves the functional availability of Kv7 channels, while activation of BK channels decreases the functional availability of Kv7 channels.

In conclusion, Kv7 and BK channels are expressed in rat skeletal muscle arteries and function as negative feedback modulators in the regulation of contractility of these arteries. Moreover, there is a dynamic equilibrium between Kv7 and BK channels, in which, membrane potential, concentration of Ca^{2+}, and membrane permeability might be the three major factors to build a bridge between Kv7 and BK channels.

In the future, functional studies and electrophysiological studies combined with genetic techniques might facilitate further clarification of the mechanism underlying Kv7 and BK channel interaction and might provide a promising direction in the treatment of relevant vascular diseases.

6 REFERENCES

Arya, M., Shergill, labal S., Williamson, M., Gommersall, L., Aryan，N, and Patel, H.R. (2005). Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn *Mar;5*, 209–219.

Bähring, R., Milligan, C.J., Vardanyan, V., Engeland, B., Young, B.A., Dannenberg, J., Waldschütz, R., Edwards, J.P., Wray, D., and Pongs, O. (2001). Coupling of Voltage-dependent Potassium Channel Inactivation and Oxidoreductase Active Site of Kvβ Subunits. J. Biol. Chem. *276*, 22923–22929.

Bayliss, W.M. (1902). On the local reactions of the arterial wall to changes of internal pressure. J. Physiol. *28*, 220–231.

Bevan, J.A., and Osher, J. V. (1972). A direct method for recording tension changes in the wall of small blood vessels in vitro. Agents Actions *2*, 257–260.

Blausen.com staff (2014). Medical gallery of Blausen Medical 2014.

Bradley, K.K., Jaggar, J.H., Bonev, a D., Heppner, T.J., Flynn, E.R., Nelson, M.T., and Horowitz, B. (1999). Kir2.1 encodes the inward rectifier potassium channel in rat arterial smooth muscle cells. J. Physiol. *515 Pt 3*, 639–651.

Brayden, J.E., and Nelson, M.T. (1992a). Regulation of arterial tone by activation of calcium-dependent potassium channels. Science *256*, 532–535.

Brayden, J.E., and Nelson, M.T. (1992b). Regulation of Arterial Tone by Activation of Calcium-Dependent Potassium Channels. *256*, 532–535.

Brenner, R., Peréz, G.J., Bonev, a D., Eckman, D.M., Kosek, J.C., Wiler, S.W., Patterson, a J., Nelson, M.T., and Aldrich, R.W. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. Nature *407*, 870–876.

Brozovich, F. V, Nicholson, C.J., Degen, C. V, Gao, Y.Z., Aggarwal, M., and Morgan, K.G. (2016). Mechanisms of Vascular Smooth Muscle Contraction and the Basis for Pharmacologic Treatment of Smooth Muscle Disorders. Pharmacol. Rev. *68*, 476–532.

Chadha, P.S., Zunke, F., Davis, A.J., Jepps, T.A., Linders, J.T.M., Schwake, M., Towart, R., and Greenwood, I.A. (2012). Pharmacological dissection of K v7.1 channels in systemic and pulmonary arteries. Br. J. Pharmacol. *166*, 1377–1387.

Chen, T.T., Luykenaar, K.D., Walsh, E.J., Walsh, M.P., and Cole, W.C. (2006). Key Role of Kv1 Channels in Vasoregulation.

Chlopicki, S., Nilsson, H., and Mulvany, M.J. (2001). Initial and sustained phases of myogenic response of rat mesenteric small arteries. Am. J. Physiol. Heart Circ. Physiol. *281*, H2176–H2183.

Cole, W.C., Chen, T.T., and Clément-chomienne, O. (2005). Myogenic regulation of arterial diameter : role of potassium channels with a focus on delayed rectifier potassium current 1. *765*, 755–765.

Cox, D.H., and Aldrich, R.W. (2000). Role of the β1 Subunit in Large-Conductance Ca2+-Activated K+ Channel Gating Energetics. J. Gen. Physiol. *116*, 411–432.

Cox, R.H., and Petrou, S. (1999). Ca2+ influx inhibits voltage-dependent and augments Ca2+-dependent K+ currents in arterial myocytes. Am. J. Physiol. Cell Physiol. *277*, C51-63.

Davis, M.J., and Hill, M. a (1999). Signaling mechanisms underlying the vascular myogenic response. Physiol. Rev. *79*, 387–423.

Escoubas, P., Diochot, S., Célérier, M.-L., Nakajima, T., and Lazdunski, M. (2002). Novel tarantula toxins for subtypes of voltage-dependent potassium channels in the Kv2 and Kv4 subfamilies. Mol. Pharmacol. *62*, 48–57.

Falcone, J.C., Davis, M.J., and Meininger, G.A. (1991). Endothelial independence of myogenic response in isolated skeletal muscle arterioles. Am. J. Physiol. *260*, H130-5.

Galvez, A., Gimenez-Gallego, G., Reuben, J.P., Roy-Contancin, L., Feigenbaum, P., Kaczorowski, G.J., and Garcia, M.L. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion Buthus tamulus. J. Biol. Chem. *265*, 11083–11090.

Gaynullina, D., Lubomirov, L.T., Sofronova, S.I., Kalenchuk, V.U., Gloe, T., Pfitzer, G., Tarasova, O.S., and Schubert, R. (2013). Functional remodelling of arterial endothelium during early postnatal development in rats. Cardiovasc. Res. *99*, 612–621.

Gelband, C.H., Ishikawa, T., Post, J.M., Keef, K.D., and Hume, J. (1993). Intracellular divalent cations block smooth muscle K+ channels. Circ. Res. *73*, 24–34.

Gogelein, H., Bruggemann, A., Gerlach, U., Brendel, J., and Busch, A.E. (2000). Inhibition of I(Ks) channels by HMR 1556. Naunyn. Schmiedebergs. Arch. Pharmacol. *362*, 480–488.

González, C., Baez-Nieto, D., Valencia, I., Oyarzún, I., Rojas, P., Naranjo, D., and Latorre, R. (2012). K+ channels: Function-structural overview. Compr. Physiol. *2*, 2087–2149.

Gordon, a. M., Huxley, a. F., and Julian, F.J. (1966). The variation in isometric tension with sarcomere length in vertebrate muscle fibres. J. Physiol. *184*, 170–192.

Greenwood, I.A., and Ohya, S. (2009). New tricks for old dogs: KCNQ expression and role in smooth muscle. Br. J. Pharmacol. *156*, 1196–1203.

Guia, A., Wan, X., Courtemanche, M., and Leblanc, N. (1999). Local Ca2+ entry through L-type Ca2+ channels activates Ca2+-dependent K+ channels in rabbit coronary myocytes. Circ. Res. *84*, 1032–1042.

Gutman, G.A., Chandy, K.G., Grissmer, S., Lazdunski, M., McKinnon, D., Pardo, L.A., Robertson, G.A., Rudy, B., Sanguinetti, M.C., Stühmer, W., et al. (2005). International Union of Pharmacology. LIII. Nomenclature and molecular relationships of voltage-gated potassium channels. Pharmacol. Rev. *57*, 473–508.

Heppner, T.J., Bonev, A.D., and Nelson, M.T. (1997). Ca(2+)-activated K+ channels regulate action potential repolarization in urinary bladder smooth muscle. Am J Physiol *273*, 110–117.

Herrera, G.M., and Nelson, M.T. (2002). Differential regulation of SK and BK channels by Ca(2+) signals from Ca(2+) channels and ryanodine receptors in guinea-pig urinary bladder myocytes. J. Physiol. *541*, 483–492.

Herrera, G.M., Heppner, T.J., and Nelson, M.T. (2000). Regulation of urinary bladder smooth muscle contractions by ryanodine receptors and BK and SK channels. Am. J. Physiol. Regul. Integr. Comp. Physiol. *279*, R60-8.

Hill, M.A., Yang, Y., Ella, S.R., Davis, M.J., and Braun, A.P. (2010). Large conductance, Ca2+-activated K+ channels (BKCa) and arteriolar myogenic signaling. FEBS Lett.

Hu, X.-Q., and Zhang, L. (2012). Function and regulation of large conductance Ca(2+)-activated K+ channel in vascular smooth muscle cells. Drug Discov. Today *17*, 974–987.

Ishikawa, T., Hume, J.R., and Keef, K.D. (1993). Modulation of K+ and Ca2+ channels by histamine H1-receptor stimulation in rabbit coronary artery cells. J. Physiol. *468*, 379–400.

Jackson, W.F. (2000). Ion channels and vascular tone. Hypertension *35*, 173–178.

Jackson, W.F. (2005). Potassium channels in the peripheral microcirculation. Microcirculation *12*, 113–127.

Jackson, W.F., and Blair, K.L. (1998). Characterization and function of Ca(2+)-activated K+ channels in arteriolar muscle cells. Am. J. Physiol. *274*, H27-34.

Jaggar, J.H., Porter, V.A., Lederer, W.J., and Nelson, M.T. (2000). Calcium sparks in smooth muscle. Am. J. Physiol. Cell Physiol. *278*, C235–C256.

Jepps, T.A., Chadha, P.S., Davis, A.J., Harhun, M.I., Cockerill, G.W., Olesen, S.P., Hansen, R.S., and Greenwood, I.A. (2011). Downregulation of Kv7.4 Channel Activity in Primary and Secondary Hypertension. Circulation *124*, 602–611.

Jiang, Z., Wallner, M., Meera, P., and Toro, L. (1999). Human and rodent MaxiK channel beta-subunit genes: cloning and characterization. Genomics *55*, 57–67.

Joshi, S., Sedivy, V., Hodyc, D., Herget, J., and Gurney, A.M. (2009). KCNQ modulators reveal a key role for KCNQ potassium channels in regulating the tone of rat pulmonary artery smooth muscle. J. Pharmacol. Exp. Ther. *329*, 368–376.

Khanamiri, S., Soltysinska, E., Jepps, T.A., Bentzen, B.H., Chadha, P.S., Schmitt, N., Greenwood, I.A., and Olesen, S.P. (2013). Contribution of Kv7 channels to basal coronary flow and active response to ischemia. Hypertension *62*, 1090–1097.

Klockner, U., and Isenberg, G. (1985). Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). Pflugers Arch *405*, 329–339.

Knot, H.J., and Nelson, M.T. (1995). Regulation of membrane potential and diameter by voltage-dependent K+ channels in rabbit myogenic cerebral arteries. Am. J. Physiol. *269*, H348–H355.

Knot, H.J., and Nelson, M.T. (1998). Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure. J. Physiol. 199–209.

Ko, E. a, Han, J., Jung, I.D., and Park, W.S. (2008). Physiological roles of K+ channels in vascular smooth muscle cells. J. Smooth Muscle Res. *44*, 65–81.

Korovkina, V.P., and England, S.K. (2002). Molecular diversity of vascular potassium channel isoforms. Clin. Exp. Pharmacol. Physiol. *29*, 317–323.

Kuo, L., Chilian, W.M., and Davis, M.J. (1990). Coronary arteriolar myogenic response is independent of endothelium. Circ. Res. *66*, 860–866.

Lagrutta, A., Wang, J., Fermini, B., and Salata, J.J. (2006). Novel , Potent Inhibitors of Human Kv1 . 5 K 2 Channels and Ultrarapidly Activating Delayed Rectifier Potassium Current. J. Pharmacol. Exp. Ther. *317*, 1054–1063.

Lang, D.G., and Ritchie, A.K. (1990). Tetraethylammonium blockade of apamin-sensitive and insensitive Ca2(+)-activated K+ channels in a pituitary cell line. J. Physiol. *425*, 117–132.

Meininger, G.A., and Davis, M.J. (1992). Cellular mechanisms involved in the vascular myogenic response. Am J Physiol *263*, H647-59.

Miyata, N., and Roman, R.J. (2005). Role of 20-hydroxyeicosatetraenoic acid (20-HETE) in vascular system. J. Smooth Muscle Res. *41*, 175–193.

Morales-Cano, D., Moreno, L., Barreira, B., Pandolfi, R., Chamorro, V., Jimenez, R., Villamor, E., Duarte, J., Perez-Vizcaino, F., and Cogolludo, A. (2015). Kv7 channels critically determine coronary artery reactivity: Left-right differences and down-regulation by hyperglycaemia. Cardiovasc. Res. *106*, 98–108.

Mulvany, M.J., and Halpern, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res *41*, 19–26.

Nausch, B., Rode, F., Jørgensen, S., Nardi, A., Korsgaard, M.P.G., Hougaard, C., Bonev, A.D., Brown, W.D., Dyhring, T., Strøbæk, D., et al. (2014). NS19504: a novel BK channel activator with relaxing effect on bladder smooth muscle spontaneous phasic contractions. J. Pharmacol. Exp. Ther. *350*, 520–530.

Nelson, M.T., and Quayle, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. Am. J. Physiol. *268*, C799–C822.

Ng, F.L., Davis, A.J., Jepps, T.A., Harhun, M.I., Yeung, S.Y., Wan, A., Reddy, M., Melville, D., Nardi, A., Khong, T.K., et al. (2011). Expression and function of the K + channel KCNQ genes in human arteries. Br. J. Pharmacol. *162*, 42–53.

Ohya, S., Sergeant, G.P., Greenwood, I.A., and Horowitz, B. (2003). Molecular variants of KCNQ channels expressed in murine portal vein myocytes: A role in delayed rectifier current. Circ. Res. *92*, 1016–1023.

Osol, G., Brekke, J.F., McElroy-Yaggy, K., and Gokina, N.I. (2002). Myogenic tone, reactivity, and forced dilatation: a three-phase model of in vitro arterial myogenic behavior. Am. J. Physiol. Heart Circ. Physiol. *283*, H2260-7.

Pallotta, B.S., Magleby, K.L., and Barrett, J.N. (1981). Single channel recordings of Ca2+-activated K+ currents in rat muscle cell culture. Nature *Oct 8 293*, 471–474.

Pongs, O., and Schwarz, J.R. (2010). Ancillary Subunits Associated With Voltage-Dependent K+ Channels. Physiol Rev *90*, 755–796.

Pongs, O., Leicher, T., Berger, M., Roeper, J., Bähring, R., Wray, D., Giese, K.P., Silva, a J., and Storm, J.F. (1999). Functional and molecular aspects of voltage-gated K+ channel beta subunits. Ann. N. Y. Acad. Sci. *868*, 344–355.

Quayle, J.M., Nelson, M.T., and Standen, N.B. (1997). ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. Physiol. Rev. *77*, 1165–1232.

Robbins, J. (2001). KCNQ potassium channels: Physiology, pathophysiology, and pharmacology. Pharmacol. Ther. *90*, 1–19.

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X.B., Sausbier, U., Feil, S., Kamm, S., Essin, K., Sailer, C.A., et al. (2005). Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. Circulation *112*, 60–68.

Schreiber, M., and Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. Biophys. J. *73*, 1355–1363.

Schubert, R. (2005). Isolated vessels. Pract. Methods Cardiovasc. Res. 198–211.

Schubert, R., Lidington, D., and Bolz, S.S. (2008). The emerging role of Ca2+ sensitivity regulation in promoting myogenic vasoconstriction. Cardiovasc. Res. *77*, 8–18.

Soldovieri, M. V., Miceli, F., and Taglialatela, M. (2011). Driving With No Brakes: Molecular Pathophysiology of Kv7 Potassium Channels. Physiology *26*, 365–376.

Somlyo, A.P., and Somlyo, A. V. (1994). Signal transduction and regulation in smooth muscle. Nature *372*, 231–236.

Somlyo, A.P., and Somlyo, A. V. (2003). Ca2+ Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase. Physiol. Rev. *83*, 1325–1358.

Sperelakis, N., and Ohya, Y. (1989). Electrophysiology of Vascular Smooth Muscle. In Physiology and Pathophysiology of the Heart, N. Sperelakis, ed. (Boston, MA: Springer US), pp. 773–811.

Standen, N.B., and Quayle, J.M. (1998). K+ channel modulation in arterial smooth muscle. Acta Physiol Scand *164*, 549–557.

Stott, J.B., Jepps, T.A., and Greenwood, I.A. (2014). Kv7 potassium channels: A new therapeutic target in smooth muscle disorders. Drug Discov. Today *19*, 413–424.

Stott, J.B., Povstyan, O. V, Carr, G., Barrese, V., and Greenwood, I.A. (2015). G-protein betagamma subunits are positive regulators of Kv7.4 and native vascular Kv7 channel activity. Proc Natl Acad Sci U S A *112*, 6497–6502.

Tatulian, L., Delmas, P., Abogadie, F.C., and Brown, D.A. (2001). Activation of expressed KCNQ potassium currents and native neuronal M-type potassium currents by the anti-convulsant drug retigabine. J Neurosci *21*, 5535–5545.

Thorneloe, K.S., and Nelson, M.T. (2005). Ion channels in smooth muscle: regulators of intracellular calcium and contractility. Can. J. Physiol. Pharmacol. *83*, 215–242.

Thorneloe, K., Chen, T., Kerr, P., Grier, E., Horowitz, B., Cole, W., and Walsh, M. (2001). Molecular Composition of 4-Aminopyridine-Sensitive Voltage-Gated K+ Channels of Vascular Smooth Muscle. Circ. Res. *89*, 1030–1037.

Tsvetkov, D., Tano, J., Kassmann, M., Wang, N., and Schubert, R. (2016). The Role of DPO-1 and XE991-Sensitive Potassium Channels in Perivascular Adipose Tissue-Mediated Regulation of Vascular Tone. Front. Physiol. *7*, 335.

Tykocki, N.R., Boerman, E.M., and Jackson, W.F. (2017). Smooth Muscle Ion Channels and Regulation of Vascular Tone in Resistance Arteries and Arterioles. Compr. Physiol. *7*, 485–581.

Uebele, V.N., Lagrutta, A., Wade, T., Figueroa, D.J., Liu, Y., McKenna, E., Austin, C.P., Bennett, P.B., and Swanson, R. (2000). Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K+ channel. J. Biol. Chem. *275*, 23211–23218.

Wallner, M., Meera, P., and Toro, L. (1996). Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca(2+)-sensitive K+ channels: an additional transmembrane region at the N terminus. Proc. Natl. Acad. Sci. U. S. A. *93*, 14922–14927.

Wesselman, J.P., Schubert, R., VanBavel, E.D., Nilsson, H., and Mulvany, M.J. (1997). KCa-channel blockade prevents sustained pressure-induced depolarization in rat mesenteric small arteries. Am. J. Physiol. *272*, H2241–H2249.

Wickenden, a D., Yu, W., Zou, a, Jegla, T., and Wagoner, P.K. (2000). Retigabine, a novel anti-convulsant, enhances activation of KCNQ2/Q3 potassium channels. Mol. Pharmacol. *58*, 591–600.

Wulff, H., Castle, N.A., and Pardo, L.A. (2009). Voltage-gated potassium channels as therapeutic targets. Nat. Rev. Drug Discov. *8*, 982–1001.

Yang, Y., Murphy, T. V, Ella, S.R., Grayson, T.H., Haddock, R., Hwang, Y.T., Braun, A.P., Peichun, G., Korthuis, R.J., Davis, M.J., et al. (2009). Heterogeneity in function of small artery smooth muscle BK Ca : involvement of the β 1-subunit. J. Physiol. *12*, 3025–3044.

Yeung, S.Y.M., Pucovský, V., Moffatt, J.D., Saldanha, L., Schwake, M., Ohya, S., and Greenwood, I. a (2007). Molecular expression and pharmacological identification of a role for K(v)7 channels in murine vascular reactivity. Br. J. Pharmacol. *151*, 758–770.

Zavaritskaya, O., Zhuravleva, N., Schleifenbaum, J., Gloe, T., Devermann, L., Kluge, R., Mladenov, M., Frey, M., Gagov, H., Fésüs, G., et al. (2013). Role of KCNQ Channels in Skeletal Muscle Arteries and Periadventitial Vascular Dysfunction. Hypertension *61*, 151–159.

Zhang, J., and Yan, J. (2014). Regulation of BK channels by auxiliary ?? subunits. Front. Physiol. *5*, 1–7.

Zhong, X.Z., Harhun, M.I., Olesen, S.P., Ohya, S., Moffatt, J.D., Cole, W.C., and Greenwood, I.A. (2010). Participation of KCNQ (Kv7) potassium channels in myogenic control of cerebral arterial diameter. J Physiol *588*, 3277–3293.

Zou, A.-P., Fleming, J.T., Falck, J.R., Jacobs, E.R., Gebremedhin, D., Harder, D.R., Roman, R.J., and Department (1996). 20-HETE is an endogenous inhibitor of the large- conductance Ca(2+)-activated K+ channel in renal arterioles. Am. J. Physiol. *270*, R228-37.

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Ma D., Wang B.*, Cheng Y., Song B., Hu Z. (2013). The Significance of Heart Rate Variability in Patients with Idiopathic Ventricular Premature Contractions Originating from the Right Outflow Tract. Journal of Clinical Electrocardiology. *22*(1),17-20.

Ma D., Wang B.*, Hu Z , Cheng Y , Chen D , Liu M., Cao Z., Song B., Fang N. (2013). The Combination of Hepatocyte Growth Factor and Valsartan Attenuate Myocardial Fibrosis in Spontaneously Hypertensive Rats. Acta Universitatis Medicinalis Anhui. *48*(10),1159-1163

Fang N., Hu Z.,Wang B.*, **Ma D**., Zhou Q., Wang y. (2013). Effect of Melatonin on Blood Lipids, Toll-like Receptor 4 Protein, Inflammatory Cytokines and the Development of Experimental Atherosclerosis in Rabbits, Acta Universitatis Medicinalis Anhui. *48*(9), 1010-1013.

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