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KCNQ channel opening as a powerful mechanism for vasodilation

DISSERTATION

to obtain the academic degree
Doctor of Medical Sciences (doctor rerum humanarum)
from the Medical Faculty at University of Rostock, Germany

presented by Nadezda Zhuravleva
born in 12.09.1984 in Moscow (USSR)

Rostock, Mai 2011

urn:nbn:de:gbv:28-diss2012-0095-9

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Date of submission: 24.06.2011
Date of defence: 28.02.2012

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1. INTRODUCTION

This research is devoted to the study of KCNQ channels in vascular smooth muscle cells (VSMCs) of small arteries of the rat. In particular, we investigated the effects of new substances which are expected to work as KCNQ channels openers.

1.1. Basis for this investigation

KCNQ channels were widely studied in the nervous system because regulation of their activity is important for many neurological disorders. Based on these investigations new medications were discovered representing pharmacological modulators of KCNQ channels.

In the last years it was revealed that KCNQ channels are also present in some smooth muscle cells where they take part in contraction. The presence of KCNQ channels in vascular smooth muscle cells (VSMCs) is especially important. Theoretically, regulation of the activity of KCNQ channels gives the possibility to regulate the contractility of VSMCs that in turn is connected with vascular tone. The connection between KCNQ channels and vascular tone was confirmed by a few newer studies. In 2007 it was discovered that the hormone vasopressin leads to vasoconstriction by inhibition of KCNQ5 channels in VSMCs (Brueggemann et al., 2007). Another example showed that baroreceptors of the aortic arch express KCNQ channels which regulate the sensitivity of the mechanosensitive neurons to changes in arterial blood pressure (Wladyka et al., 2008).

Thus, investigations of vascular reactions with participation of KCNQ channels are necessary for a more exact understanding of the processes of vascular tone regulation. In addition, such studies may help to develop new medications for cardiovascular diseases.

1.2. Basic features of vessel physiology

All arteries have in principle the same structure. The arterial vascular wall consists of three layers: the outer tunica adventitia, a central tunica media, and the inner tunica intima. Depending on the amount of muscular and elastic components in the tunica media all arteries are divided into 3 types: elastic, muscular and mixed arteries. *Small arteries* belong to the muscular type (fig. 1.1).

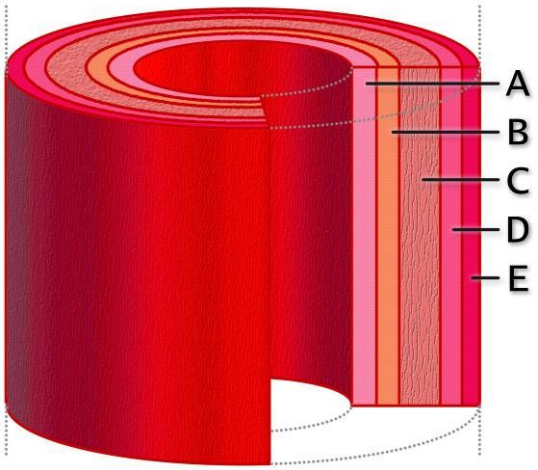


Figure 1.1 Structure of a small artery: A - tunica intima, B - internal elastic lamina, C - tunica media, D - external elastic lamina, E - tunica adventitia.

In small arteries, the force of the pulse-wave essentially decreases because of remoteness from the heart. However, these vessels possess a considerable muscular component in the tunica media in order to facilitate blood flow regulation. The diameter of these arteries can decrease during constriction and increase during relaxation of VSMCs. The thickness of the wall of these arteries essentially exceeds the diameter of the aperture. Such vessels create a resistance for the streaming blood. Therefore they are often called “resistive”.

Intima

The tunica intima is a comparatively thin layer which consists of one layer of endothelial cells and the internal elastic lamina. The internal elastic lamina represents one layer of elastic fibers. This lamina supports the endothelial cells which are in direct contact with the blood flow. Endothelial cells, along with other factors, play important roles in vessel relaxation and contraction (Furchgott et al., 1989).

Endothelium-dependent relaxation occurs because of three different endothelium-derived relaxing factors (EDRFs): nitric oxide – NO (Ignarro et al., 1987), prostacyclin – PGI_2 (Bunting et al., 1976), and an unidentified endothelium-derived hyperpolarizing factor – EDHF (Feletou et al., 1988; Chen et al., 1988). EDRFs are released by a large number of agents (e.g., acetylcholine, ATP and ADP, substance P, bradykinin, histamine, thrombin, serotonin) and by shear stress. Endothelium-dependent relaxation occurs in resistance vessels as well as in larger arteries.

There is also endothelium-dependent vasoconstriction produced by a few endothelium-derived contracting factors (EDCFs) such as Endothelin-1 – ET-1 (Yanagisawa et al., 1988), Angiotensin II – Ang II (Hernandez Schulman et al., 2007), Thromboxane A_2 – TXA_2 (Oates et al., 1988) and prostaglandin H_2 – PGH_2 (Davidge, 2001).

Media

The tunica media of small arteries consists of smooth muscle cells and elastic fibers located spirally. The spiral arrangement of the VSMCs promotes greater reduction of the vessel aperture. The tunica media borders on the internal elastic lamina at the luminal side, but there is no elastic lamina within the media (Carlson et al., 1982; Lee et al., 1983a). The volume fraction occupied by VSMCs in small arteries is greater than in larger vessels (Todd et al., 1983). The number of VSMCs in small arteries depends on vessel diameter: from approximately six layers in 300-µm vessels (Lee et al., 1983b) to one cell layer in 30- to 50-µm arterioles (Gattone et al., 1986, Miller et al., 1987, Walmsley et al., 1982).

Adventitia

The tunica adventitia of small arteries consists of connective tissue (elastin and collagen), fibroblasts, mast cells, macrophages and Schwann cells with associated nerve axons (Laher et al., 1986; Rhodin, 1980). In small arteries, nerves do not penetrate the media (Griffith et al., 1982; Luff et al.1987; Owen et al., 1983, Rhodin, 1980). The volume fraction of nerves in the adventitia depends on vessel location: from 1% in proximal small arteries to 3% in the more distal small arteries (Lee et al., 1983a; Nilsson et al., 1986; Smeda et al., 1988). Nerve endings have a great influence on vascular function.

Vessel contraction

Contraction of small arteries, as in large arteries (Deth et al., 1974), is dependent on the presence (Mulvany et al., 1980) and influx (Cauvin et al., 1984) of extracellular calcium. Calcium gets into the VSMC trough potential-dependent calcium channels (electromechanical coupling), receptor- or store-activated calcium permeating channels (pharmacomechanical coupling) and from the sarcoplasmic reticulum.

Pharmacomechanical coupling is a major mechanism of VSMC contraction. This kind of contraction or dilatation could be caused by such substances as catecholamines (phenylephrine), histamine, acetylcholine, serotonin, angiotensin, nitric oxide (NO), carbon dioxide (CO_2), K^+ and H^+ ions, and prostaglandins (tromboxane, U46619). Some of these substances were used in the present study as vessel preconstrictors in the myograph experiments.

The diameter of small vessels depends on different factors released from nerve endings or surrounding tissues and transported by the blood flow (hormones). In addition, there are mechanical factors like shear stress and transmural pressure as well as influences from proximal and distal vessel segments.

That's why the investigation of vessel contractility mechanisms is not easy. For example, at studying VSMC-based mechanisms the endothelium should be removed, the action of sympathetic nerve endings be blocked, and resting tension selected according to the vessel location, e.g. 100 mmHg for *Gracilis* and 15 mmHg for *Intrapulmonary* arteries (Steeds et al., 1997; Chou et al., 2002).

1.3. KCNQ channels

KCNQ channels (also known as Kv7 channels) are a small family of voltage-activated potassium channels.

1.3.1. Potassium channels

All cells are surrounded by a lipid membrane which works as a barrier to the diffusion of many substances, including ions. Transporting necessary ions through the membrane is possible due to proteinaceous channels integrated into the membranes. Ions cross the membrane when these channels are open. Potassium channels (K^+ channels) are a subset of membrane channels selective for potassium ions (K^+).

The concentration of K^+ ions is usually approximately 25-times higher inside then outside the cell because of the activity of Na^+/K^+-ATPases. When potassium channels open (activate), K^+ tends to leak out of the cell, producing a measurable electrical current that establishes a charge difference across the membrane (membrane potential). This polarization of the membrane is the basis of cellular electrical signaling.

1.3.2. Voltage-activated potassium channels

Voltage-activated potassium channels (Kv channels) are a subset of the K^+ channels which are sensitive to changes of the cell's membrane potential. They play a crucial role during action potentials in returning the depolarized cell to a resting condition. Kv channels are activated at relatively negative potentials (~ -60 mV, close to the resting potential for many excitable cells). Thus, Kv outward currents determine at least partly the membrane resting potential.

Structure

The typical Kv channel represents an assembly of four identical (or similar) transmembrane subunits surrounding a central pore (fig. 1.2 A, B).

Both NH_2- and COOH- termini of the subunits are situated on the intracellular side of the membrane. Each subunit has six transmembrane crossing segments (S1–S6). Different segments form physiologically important structures. The segments S1–S4 constitute a voltage sensor (the S4 region has multiple positive charges), S5 and S6 form a pore, the central structure of Kv channels. The narrowest part of the pore, the selectivity filter, is formed by the loop between S5 and S6. The parts of the subunits responsible for ion selectivity were discovered during channel mutation studies. The selectivity filter of Kv channels includes the amino acid sequence Thr-Val-Gly-Tyr-Gly (Doyle et al., 1998).

Opening and closing of the pore regulates the ion transport through the membrane.

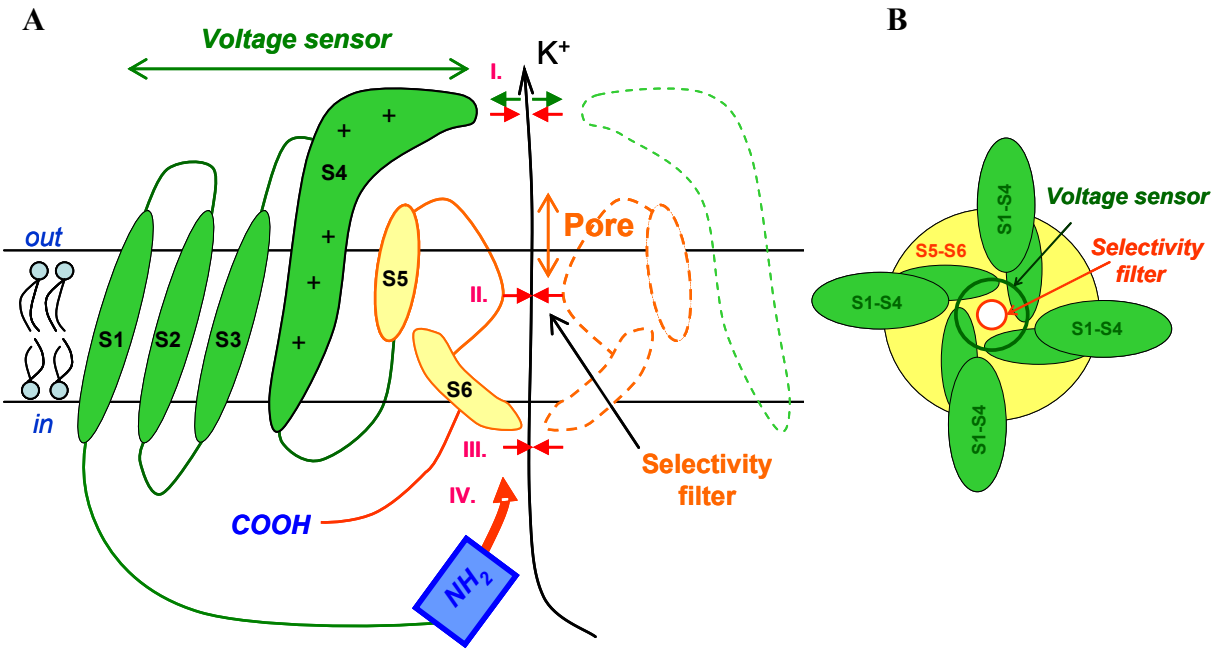


Figure 1.2 Scheme of Kv channel architecture.

A. Longitudinal section of the Kv channel (presented is one subunit from four). Conformations: I – "paddle" -open/close conformation; II – selectivity filter-inactivation; III – S6-inactivation; IV – "ball-and-chain" inactivation. Green arrows indicate activation of the Kv channel, red – inactivation. B. Cross section of the Kv channel.

Gating

Gating is a set of conformational changes resulting in opening and closing of the Kv channel pore (Yellen, 2002). The voltage sensor is responsible for the gating, it contains a region "paddle" composed of the S3 and S4 segments (fig. 1.2 A). When the membrane potential becomes more positive inside the cell (depolarisation), the voltage sensor initiates channel opening (activation) which leads to K^+ efflux. The opposite change of the membrane potential (hyperpolarisation) leads to the movement of the "paddle" into the closed conformation.

Besides gating there is also an inactivated conformation; in this case the channel cannot open, even if the transmembrane voltage is suitable. Inactivation is governed by several mechanisms. The three most known mechanisms are S6-, pore- ("selectivity filter") and NH_2-("ball-and-chain") inactivation (fig. 1.2A). In the case of the "ball-and-chain" inactivation, the NH_2-terminus of the channel's subunit forms a ball which is connected to the rest of the protein through a loop (the chain). During inactivation, the ball moves into the inner porehole, and doesn't allow ions to move through the channel (Armstrong et al., 1973; Murrell-Lagnado et al., 1993).

Depolarization of the cell membrane activates Kv channels. It works also as the inactivating factor for many Kv channels. Thus, the amount of current flowing through the Kv channel represents the result of the balance between channel activation and inactivation processes (Nelson et al., 1995).

Blockers of Kv channels

Kv channels are blocked by Ba^{2+} ions: half-block occurs at concentrations > 1 mM (Pfrunder et al., 1992).

The canonical pore-blocking substance tetraethylammonium (TEA) inhibits most Kv channels at higher concentrations then K_{Ca} channels: half-block is observed at 10 mM (Overturf KE et al., 1994).

4-aminopyridine (4-AP) is widely used as an inhibitor of vascular smooth muscle Kv channels because of its selectivity: half-block is seen at 0,2-1,1 mM (Hara et al., 1980).

Some other Kv channel inhibitors have been described: phencyclidine (Robertson et al., 1994), tedisamil, a class 3 antiarrhythmic (Pfrunder et al., 1992), and quinidine (Beech et al., 1989).

Glibenclamide, charybdotoxin and iberiotoxin, inhibtors of non-Kv channels expressed in vascular smooth muscle cells, have no blocking effect on Kv channels.

1.2.3. The KCNQ subfamily

Voltage-activated potassium channels include the KCNQ (Kv7) subfamily. Architecturally, KCNQ channels are similar to other Kv channels, but they possess a long and variable C-terminus (Schmitt et al., 2000). Other distinctions of the KCNQ subfamily such as pharmacologic, biophysical and physiologic features will be reviewed later.

Variety of KCNQ channels

The KCNQ family includes five subtypes encoded by five genes (KCNQ1-5). KCNQ channels are present in different excitable tissues.

KCNQ1 channels are found predominantly in the heart. In contrast to other KCNQ members, KCNQ1 (KvLQT1) is not expressed in neurons (Jespersen et al., 2005) and can form only homomeric channels of four identical (KCNQ1) transmembrane subunits (Schwake et al., 2003). KCNQ1 is insensitive to activators of other KCNQ channels (Gamper et al., 2005; Munro et al., 2007).

KCNQ1 channels produce a slow delayed-rectifier K^+ current (I_{Ks}) which is important for the repolarization of the cardiac action potential. These channels play a crucial role in the forming of the QT interval of the electrocardiogram. Mutations affecting the function of KCNQ1 channels could lead to the long QT syndrome dangerous because of the induction of arrhythmias, ventricular fibrillation, and cardiac arrest (Chiang et al, 2000; Herbert et al., 2002; Jespersen et al., 2005).

Members of the KCNE family of Kv channels can interact with KCNQ1 with varying effects on channel function (McCrossan et al., 2004; Jespersen et al., 2005).

KCNQ2 and KCNQ3 subtypes are mostly present in the nervous system. They create a heteromeric complex. These channels produce ACh-regulated M-currents important for neuronal excitability. Mutations in KCNQ2 and KCNQ3 channels lead to Benign Familial Neonatal Convulsions (BFNC), a subtype of generalized epilepsy (Charlier et al., 1998).

KCNQ4 channels locate mostly in neurons, sensory cells of the cochlea and vestibular organs. These subtypes can form homomeric and also heteromeric channels (in association with the KCNQ3 subtype). Defects in KCNQ4 lead to the nonsyndromic sensorineural deafness type 2 (DFNA2), an autosomal dominant form of progressive hearing loss (Kubisch et al., 1999).

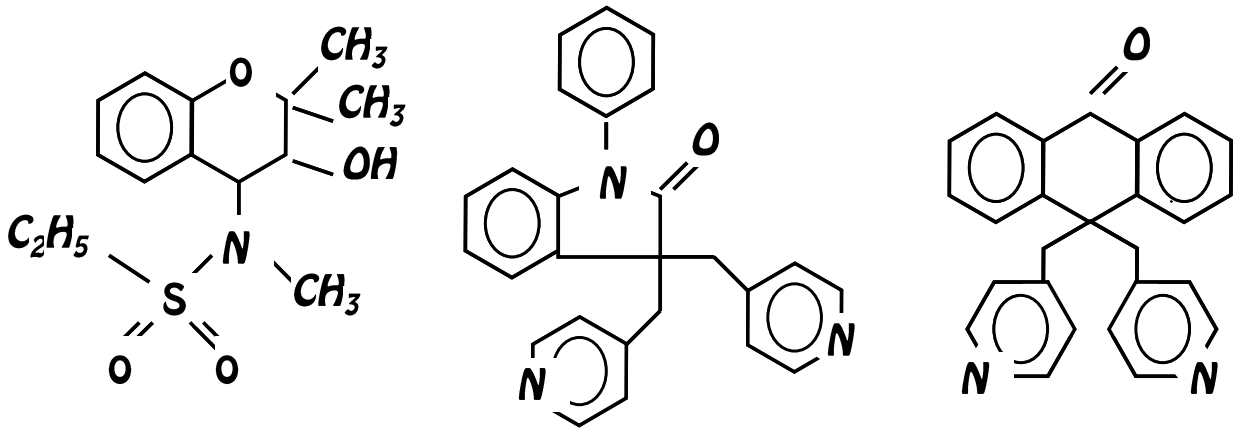
KCNQ5 channels are expressed in some neurons and in other excitable tissues, including visceral smooth muscle (Jensen et al., 2005) and skeletal muscle (Lerche et al., 2000; Schroeder et al., 2000). KCNQ5 also have been found to interact with KCNQ3 channels.

Biophysics and functions

KCNQ channels represent a slowly inactivating or non-inactivating (delayed rectifier) channel. These channels are very important for excitable tissues because of their role in the regulation of the membrane potential (Robbins, 2001; Delmas et al., 2005). Based on investigations of mutations in the KCNQ channel genes leading to several human diseases it was possible to analyze their functions (Brown, 2008).

Pharmacology

KCNQ channels have a unique pharmacology. Due to the existence of highly selective blockers it was possible to get more information concerning their properties in many tissues, including vascular smooth muscle (fig. 1.3). The classical K^+ channel blockers tetraethylammonion (TEA) and 4-aminopyridine (4-AP) inhibit a large majority of Kv channels, but from the KCNQ family only KCNQ2 is blocked by TEA (KCNQ2 alone: 0.16 mM; KCNQ2/KCNQ3: 0.5 mM) (Wang et al., 1998; Robbins, 2001). KCNQ channels are largely resistant to 4-AP. Chromanol 293B (C293B), linopirdine (DuP 996) and its more potent analogue 10,10-bis(4-pyridinyl-methyl)-9(10H)-anthracenone or XE991 (Earl et al., 1998) are potent and selective blockers of KCNQ channels (Jentsch, 2000; Robbins, 2001). The activity of these channels is enhanced (through an activation voltage-shift) by an anticonvulsant, retigabine.

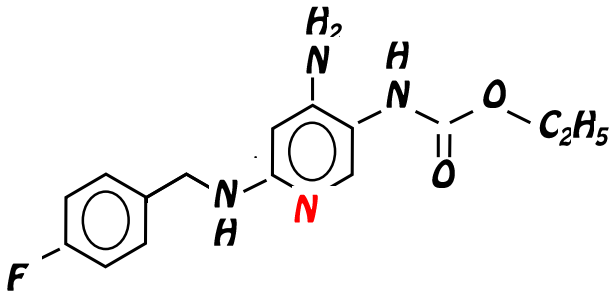


Chromanol 293B Linopirdine XE991

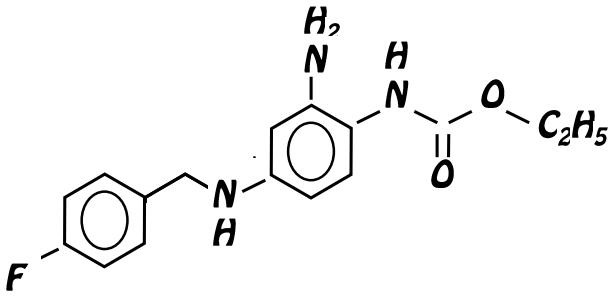
Figure 1.3 KCNQ channel blockers.

At concentrations of 10 μM or less linopirdine and XE991 are effective and relatively selective blockers of all five subtypes of KCNQ channels, other types of K^+ channels these drugs influence at higher concentrations (Schnee et al., 1998; Wladyka et al., 2006).

Flupirtine [ethyl-*N*-[2-amino-6-(4-fluorophenylmethyl-amino)pyridine-3-yl]carbamate] and retigabine [ethyl-*N*-[2-amino-4-(4-fluoro-phenylmethyl-amino)phenyl]carbamate] are commonly used activators of KCNQ channels (fig. 1.4). Chemically they differ only by one nitrogen atom. They activate all of the subtypes except KCNQ1.



Flupirtine



Retigabine

Figure 1.4 KCNQ channel activators.

The structurally dissimilar agent, Bristol-Myers Squibb compound (Acrylamide S-1) blocks KCNQ1 and activates KCNQ2-5 currents (Bentzen et al., 2006).

Regulation

M-currents (currents produced by neuronal KCNQ channels) are inhibited by activation of M1 muscarinic acetylcholine receptors.

KCNQ channels are also regulated by membrane phosphatidyl-inositol-4,5-bisphosphate (PIP_2) and variably by intracellular Ca^{2+} (via channel-associated calmodulin) and by protein kinase C (PKC), and are inhibited by transmitters acting on receptors coupled to the G proteins Gq and G_{11} (Selyanko et al., 2000), providing a mechanism whereby such transmitters can increase neuronal excitability (Brown, 1983). They are involved in the regulation of nociceptive transmission (Passmore et al., 2003).

1.4. KCNQ channels in blood vessels

In contrast to the extensive research of KCNQ channels in the CNS and heart, there are only a few studies on these channels in smooth muscle cells (SMCs). To date, KCNQ1 transcripts have been identified in the rat stomach (Ohya et al., 2002) and the murine portal vein (Ohya et al., 2003). More recently KCNQ4 and KCNQ5 were also found to be expressed in the murine portal vein (Yeung et al., 2008). Examination of KCNQ channel gene expression in arterial smooth muscle cells from different vessels such as the thoracic aorta, carotid artery and femoral artery of mice showed transcripts for KCNQ1, KCNQ4 and KCNQ5 (Yeung et al., 2007). The expression of KCNQ1, KCNQ4 and KCNQ5 was shown in adult rat aorta; A7r5 cells derived from this vessel expressed only KCNQ5 (Brueggemann et al., 2007). KCNQ1, 4 and 5 transcripts were also found in rat mesenteric artery smooth muscle cells (Mackie et al., 2008a) that confirms the findings in murine mesenteric artery. KCNQ1, 3, 4 and 5 transcripts were found in rat pulmonary artery, myocytes from this vessel did not contain KCNQ3 message (Joshi et al., 2009). The newest data show the presence of KCNQ1, 4 and 5 messages in smooth muscle cells from rat middle cerebral arteries (Zhong et al., 2010). All KCNQ genes except the KCNQ2 member were expressed in 2 kinds of human vessels: arteries from visceral adipose tissue and proximal mesenteric arteries (Ng et al., 2011).

Physiological roles

The vasoconstrictor hormone Arg^8-vasopressin (AVP) inhibits KCNQ5 channels in A7r5 smooth muscle cells (Brueggemann et al., 2007). Vasoconstriction occurs as the result of AVP binding to G_q-coupled V_{1a} vasopressin receptors (fig. 1.5). The effects of AVP are protein kinase C (PKC)-dependent: they disappear after PKC inhibition by calphostin. PKC-dependent inhibition of KCNQ currents by AVP causes membrane depolarization and activation of L-type Ca^{2+}channels, resulting in Ca^{2+} entry and cellular contraction.

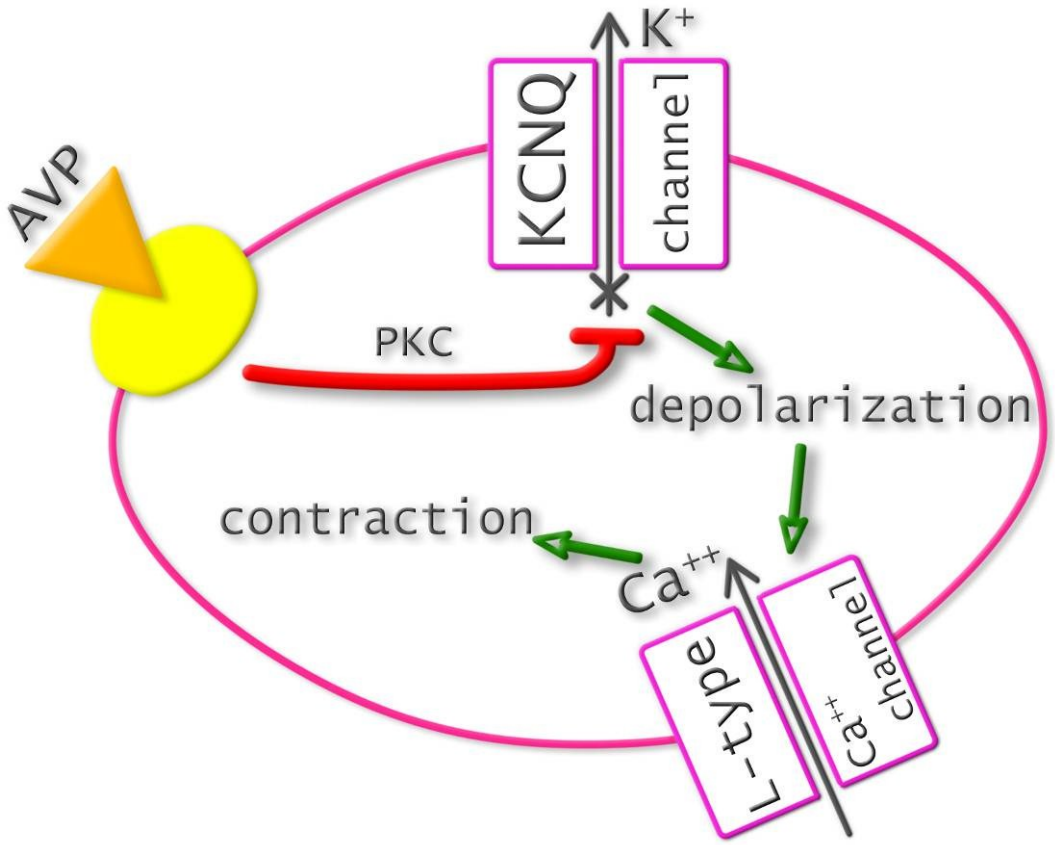


Figure 1.5 Effects of AVP: red line indicates inhibitory action, green lines - stimulatory action.

This was the first evidence that vascular KCNQ channels may play a role as targets of vasoconstrictor hormones. Later, an AVP-induced inhibition of KCNQ5 channels was also found in the rat mesenteric artery (Mackie et al., 2008a). In summary, information about the contribution of KCNQ channels to vascular contractility are very limited.

1.5. Hypothesis

The investigation of KCNQ channels in the circulatory system is a relatively new area in physiology. However, the participation of KCNQ channels in vessel contractility regulation, especially in relaxation mechanisms, is still not well known. Some data were obtained by studying the effects of the KCNQ channel activators retigabine and flupertine. But the involvement of KCNQ channels in vessel responses to endogenous vasodilator substances has not been reported yet. Therefore, we have tested the hypothesis that KCNQ channels are powerful targets for relaxation of small systemic and pulmonary arteries.

2. MATERIAL AND METHODS

2.1. Material

Experimental subjects:

The experiments presented in this work were performed on male normotensive Wistar and spontaneously hypertensive SHR rats. The animals were kept under standard laboratory conditions with free access to food and water. Rats 10-15 weeks old had body weights between 250-300 g. All experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23 revised 1996).

Materials:

The following chemicals and kits were used (table 2.1):

Procedure	Name	Firma	Order number
RNA-isolation	RNeasy Fibrouse Tissue Mini Kit (50)	Qiagen	Cat. No.74704
	RNase-Free DNase Set (50)	Qiagen	Cat. No. 9254
RT-PCR	Transcriptor One-Step RT-PCR Kit (50)	Roche	Cat. No. 04655877001
	PCR-Master	Roche	Cat.No. 11636103001
	Primers (table 2.2)	Qiaqen	on demand
Visualisation of PCR results	Agarose		
	Tris-base		
	Boric acid		
	EDTA		
	Ethidium bromide		
	DNA marker		

Myography	Acetylcholine		
	Phentolamine		
	XE991	Tocris	
	EGTA		
Preconstrictors	Serotonin	Sigma-Aldrich	
	U46619	Sigma-Aldrich	
	Phenylephrine	Sigma-Aldrich	
KCNQ openers	Retigabine	Valeant Research North America	
	VRX0530727		
	VRX0621238 (ICA-27243)		
	VRX0621688		

Table 2.1 Chemicals and kits.

2.2. Methods

The expression of KCNQ channels in *Gracilis* arteries, a representative of a small artery of the systemic circulation, was studied using the PCR-method. The functional effects of KCNQ channel openers were investigated on isometric preparations of intact *Gracilis* and *Intrapulmonary* arteries using wire myography.

2.2.1. PCR

Each kind of KCNQ channel basically represents a unique complex of transmembrane proteins. The amino acid sequence of these proteins is known and coded by a sequence of nucleotide triplets represented by mRNA. The presence of these mRNA molecules in the cell is a prerequisite for the presence of KCNQ channels. Thus, the existence of a certain mRNA in the vessel suggests the expression of the corresponding channel in this tissue. For the isolation of mRNA from *Gracilis* arteries the RNeasy Fibrouse Tissue Mini Kit (Qiagen) was used.

Practically it is not possible to read nucleotide sequences from mRNA. So it is necessary to get the complimentary DNA fragment (cDNA) from the mRNA molecule by reverse transcription.

For the different KCNQ channels we used the following specific primers (table 2.2).

Gene	Primer	Primer Pair Sequences (5'-3')	Predicted Size
KCNQ1	forward	ggc ata ctt ggc tct ggg ttt g	486 base pairs
	reverse	gca gct gtg aca cat ggg tga tg	
KCNQ2	forward	acg cct tct acc gca agc tgc	363 base pairs
	reverse	aag aca ttg ccc tgg gag cc	
KCNQ3	forward	aag acc aaa gca tga tgg gga agt t	764 base pairs
	reverse	tgg aag ggg tcc ata tgg aat ctg	
KCNQ4	forward	cgc ttc cgg gcc tct cta aga c	560 base pairs
	reverse	gtc ctc gtg gtc tac agg gct gtg	
KCNQ5	forward	gat gcc agt gtg acg tgt ccg tgg	392 base pairs
	reverse	cct ttc cga gga cct gct ggt ag	

Table 2.2 Primer pair sequences used for KCNQ channel identification.

After reverse transcription (T=45°C, 30 minutes) cDNA enters into the polymerase chain reaction (PCR) which consists of 3 principle stages (repeat: 35 cycles):

1. *Denaturation* (T=94°C, 2 minutes) – dissociation of the RNA/cDNA hybrid, melting of the cDNA template by disrupting the hydrogen bonds between complementary bases yielding single strands of DNA.

2. *Annealing* (T=56°C, 30 seconds) – annealing of the primers to the single-stranded DNA template.

3. *Elongation* (T=68°C, 1 minute 10 seconds) – the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs (Deoxynucleotide Triphosphates) that are complementary to the template in the 5' to 3' direction.

All steps were performed using the Transcriptor One-Step RT-PCR Kit (Roche).

Procedure of working with ready kits

2.2.1.1. RNA isolation from Gracilis arteries

1) Rats were sacrificed by decapitation, *Gracilis* arteries from both legs were isolated quickly under the microscope and placed into a small (0,5ml) glass tube for homogenization.

2) 200µl RNase-free water, 50µl of ready to use RLT Buffer (with β-ME) and 20µl of Proteinase K from the RNeasy Fibrouse Tissue Mini Kit (Qiagen) were applied.

3) The tissue was thoroughly disrupted and homogenized using a glass pestle.

4) The mixture was transferred to a 1,5ml plastic tube and 200µl RNase-free water was applied.

5) The resulting solution was incubated at 55°C for 10 minutes and mixed with ultrasound.

6) 250µl of 96–100% ethanol was applied and mixed.

7) The sample was transferred to the RNeasy column (from the kit), centrifuged for 15 seconds at ≥8000 x g, and the flow-through was discarded.

8) 350µl RW1 Buffer was applied to the RNeasy column, centrifuged for 15 seconds at ≥8000 x g, and the flow-through was discarded.

9) A mix of 10µl DNase stock solution and 70µl RDD Buffer (from RNase-Free DNase Set, Qiagen) was applied to the RNeasy membrane and incubated for 15 min at 20–30°C.

10) 350µl RW1 Buffer was applied to the RNeasy column, centrifuged for 15 seconds at ≥8000 x g, and the flow-through was discarded.

11) 500µl ready to use RPE Buffer (with ethanol) was applied to the RNeasy column, centrifuged for 15 seconds at ≥8000 x g, and the flow-through was discarded.

12) 500µl ready to use RPE Buffer (with ethanol) was applied to the RNeasy column, centrifuged for 2 minutes at ≥8000 x g, and the flow-through was discarded.

13) The RNeasy column was placed in a new 1,5ml tube, 50µl RNase-free water was applied and centrifuged for 1 minute at ≥8000 x g. The eluate contained the RNA to be investigated.

For positive controls RNA was isolated from brain and heart of the animal. Here, 30 mg of tissue was used. The RNA-isolation procedure was the same as described above and in the RNeasy Fibrouse Tissue Mini Kit manual.

2.2.1.2. RT-PCR

RT-PCR was performed according to the instruction of the Transcriptor One-Step RT-PCR Kit (Roche). Initially, primers, 5xReaction buffer and Transcriptor enzyme mix from the kit as well as RNA template were put on ice. The following mix of the constituents was prepared (table 2.3):

	for 1 tube, µl	for 5 normal tubes, µl
template RNA	3,00	15,00
Sterile H_2O	10,15	50,75
5x Reaction buffer	5,00	25,00
Transcriptor Enzyme mix	0,25	1,25
Summary volume	18,40	92,00

Table 2.3 Working PCR mix.

Samples were put into the PCR-machine (Mastercycler gradient, Eppendorf) and the PCR program (see attachment I) was started. After the first pause, each primer pair mix: 3,3µl forward + 3,3µl reverse primer (concentration $3*10^{-6}$M) was applied to the corresponding tubes. The PCR program was continued. Then, the control mix (table 2.4) for the control tubes was prepared using the PCR-Master kit (Roche):

	for 1 tube, µl	for 5 normal tubes, µl
template RNA	3,00	15,00
Sterile H_2O	2,90	14,50
Special PCR-Master	12,50	62,50
Summary volume	18,40	92,00

Table 2.4 Control Mix with PCR-Master kit.

Control tubes were put into the PCR-machine at the second pause, the primer mix was applied as described above and the PCR program was continued. After completion of the program all tubes were allowed to cool down. In addition, an 1,6% agarose gel (table 2.5, table 2.6) containing 12 slots was prepared.

	for 100ml
Agarose	1,6g
TBE*10 solution	5ml
Ethidium bromide	40µl

Table 2.5 Agarose gel.

	for 1 l
Tris-base	108g
Boric acid	55g
EDTA	9,3g

Table 2.6 TBE*10 solution.

The gel was placed into the standard Gel Electrophoresis system filled with TBE Buffer. In each slot a mix of 8µl DNA, 3µl Blue ink and 4µl water was applied. The first and last slots were used to load DNA-markers (table 2.7).

	slot	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
DNA	name	DNA marker	KCNQ1	KCNQ1 control	KCNQ2	KCNQ2 control	KCNQ3	KCNQ3 control	KCNQ4	KCNQ4 control	KCNQ5	KCNQ5 control	DNA marker
	µl	1	8	8	8	8	8	8	8	8	8	8	1
ink	µl	3	3	3	3	3	3	3	3	3	3	3	3
water	µl	11	4	4	4	4	4	4	4	4	4	4	11

Table 2.7 Template disposition on the gel.

2.2.2. Wire myography

Rats were sacrificed by decapitation. Thereafter, arteries were isolated from the animal and mounted on the small vessel myograph. Vessel reactivity was evaluated by measuring isometric tension.

2.2.2.1. Vessel isolation

The experiments reported in this study were performed on systemic small arteries and on pulmonary arteries. In particular, fragments of *Gracilis* arteries (Fig. 2.1, A) and *Intrapulmonary* arteries (Fig. 2.1, B) with an internal diameter of about 300 μm were chosen. At the beginning, a leg or the lung was cut off and placed into cooled (4°C) preparation solution (solution II, see attachment II). The arteries were carefully cleaned from connective tissue under the microscope using small forceps and scissors. Thereafter, they were dissected into 2 mm rings, transferred to the myograph chamber and mounted on the wires. In some cases the endothelium was removed using a rat's whisker (Schubert, 2004).

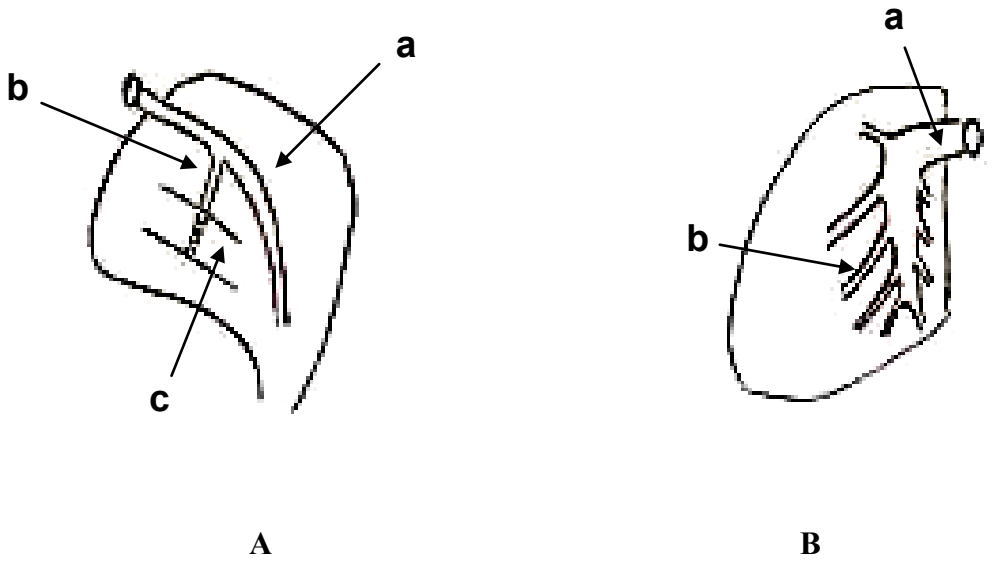


Figure 2.1 Scheme of vessels localisation: A – medial surface of the left hindlimb: a) a. saphena, b) a. gracilis, c) m. gracilis; B – lower lobe of the left lung (dorsal surface): a) a. pulmonalis sinistra, b) 3rd order branch of a. pulmonalis (*Intrapulmonary* artery)

2.2.2.2. Principle of myography

In this work, a 2-channel DMT myograph (model 410A) was used. Each channel contains two stainless steel jaws. Two mounting wires (diameter 40 µm) were threaded through the vessel lumen and each wire was secured to the jaws (see Fig. 2.2). One of the mounting jaws was attached to a micropositioner for the adjustment of vessel circumference and the application of tension. The other jaw was attached to a built-in sensitive force transducer.

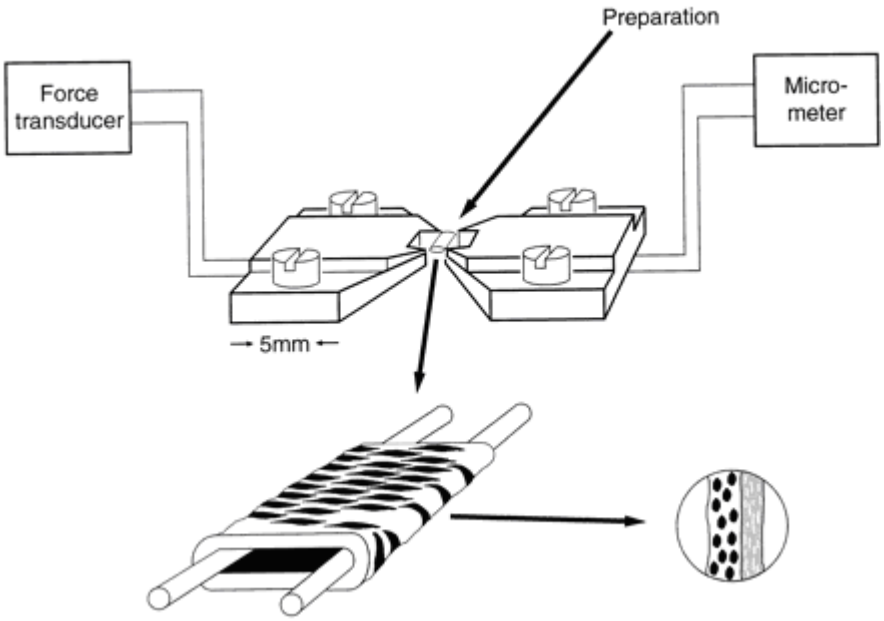


Figure 2.2 Scheme of vessel fixation on the mounting jaws. Vessel segments are threaded on 2 stainless steel wires that are fastened to a force transducer and a micrometer. (Mulvany et al., 1977).

After vessels mounting, the myograph was connected to the computer and the recording program Myodaq 2.01 (Myonic Software) was started. Thereafter, the myograph was heated up to 37°C during 30 minutes. To achieve optimal conditions for determining vessel contractility, the so called normalization procedure was performed (Mulvany et al., 1977).

2.2.2.3. Normalization procedure

In practice, the normalization is performed by distending the segment stepwise with use of the micropositioner and taking sets of micrometer and force readings (Fig. 2.3). These data are converted into values of internal circumference (in μm) and wall tension T (in mN/mm) respectively (DMT 410A, User Manual).

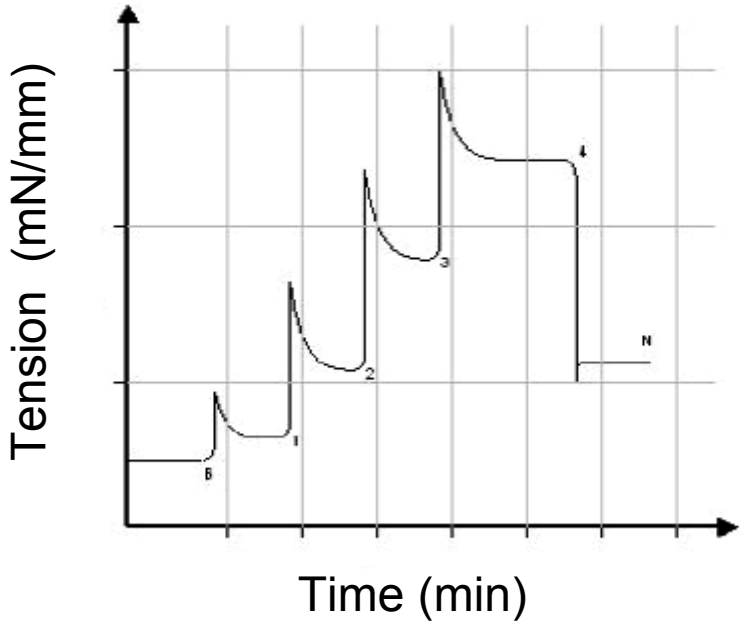


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Figure 2.3 Illustration of the stepwise normalization procedure (picture taken from the user manual 410A).

Plotting wall tension against internal circumference reveals an exponential curve and by applying the isobar curve corresponding to 100 mmHg for *Gracilis* arteries or 15 mmHg for *Intrapulmonary* arteries, respectively the IC100 (IC15 for *Intrapulmonary* artery) is calculated from the point of intersection using the Laplace relation (Fig. 2.4). IC100 (IC15) is the internal circumference of the vessel at 100 mmHg (15 mmHg).

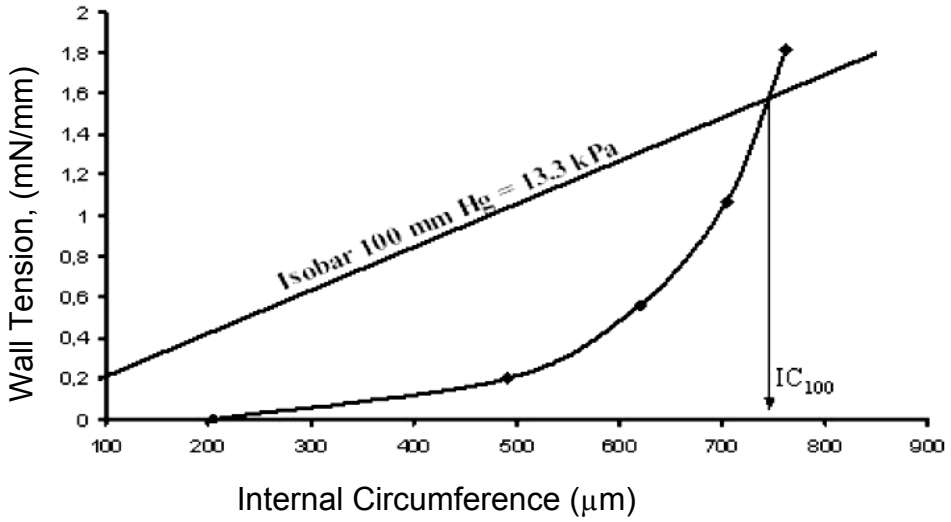

Figure 2.4 Illustration of the exponential curve fitting and determination of IC100 (picture taken from the user manual 410A).

Thereafter, the internal circumference at which the active force production as well as the sensitivity to agonists of the segment is maximal (IC1) was calculated from IC100 (IC15) by multiplying the latter by a certain factor. The factor used in this work was 0.9, thus IC1=0,9xIC100 (IC1=0,9xIC15), as established by Mulvany et al. (Mulvany et al., 1977).

After the normalisation procedure, the preparation solution in the myograph chambers was changed to the experimental solution (solution I, see attachment II) that was oxygenated constantly with a gas mixture of 95% O_2 and 5% CO_2 in the experiments with *Gracilis* arteries. The experimental solution for *Intrapulmonary* arteries contained no $NaHCO_3$ (Joshi et al., 2009) and was called solution I without $NaHCO_3$ (see attachment II), which was aerated with air. Ten - fifteen minutes after normalization the main experiment was started.

2.2.2.4. Experimental protocols

For the investigation of the effect of KCNQ channel openers, the arteries were preconstricted to a level of about 30-50% of maximal possible constriction. After stabilization of preconstriction, a substance to be studied was applied by adding increasing doses in a stepwise manner yielding a **dose-response relationship** (Fig. 2.5). The **maximum contraction** of each vessel was determined using solution I, where 120 mM KCl was added and a corresponding amount of NaCl was removed and 10^{-5} M serotonin was applied. The removal of all tested substances was done by a **wash out procedure**: the solution in the experimental chamber was changed to the normal experimental solution 3 times, one more time after 5 minutes and once more after 10 minutes. After solution changes, vessels were allowed to equilibrate for 5 minutes. For vessel **preconstriction** one of the following substances was used: serotonin, U46619, or phenylephrine. In some cases also solution I, where 60 mM KCl was added and a corresponding amount of NaCl was removed was used for preconstriction. To determine the **fully relaxed state** of the vessel, $2x10^{-3}$M EGTA was added into the experimental solution at the end of an experiment. In some cases, when the endothelium was removed, acetylcholine (10^{-5}M final concentration) was used to test for successful functional removal of the endothelium.

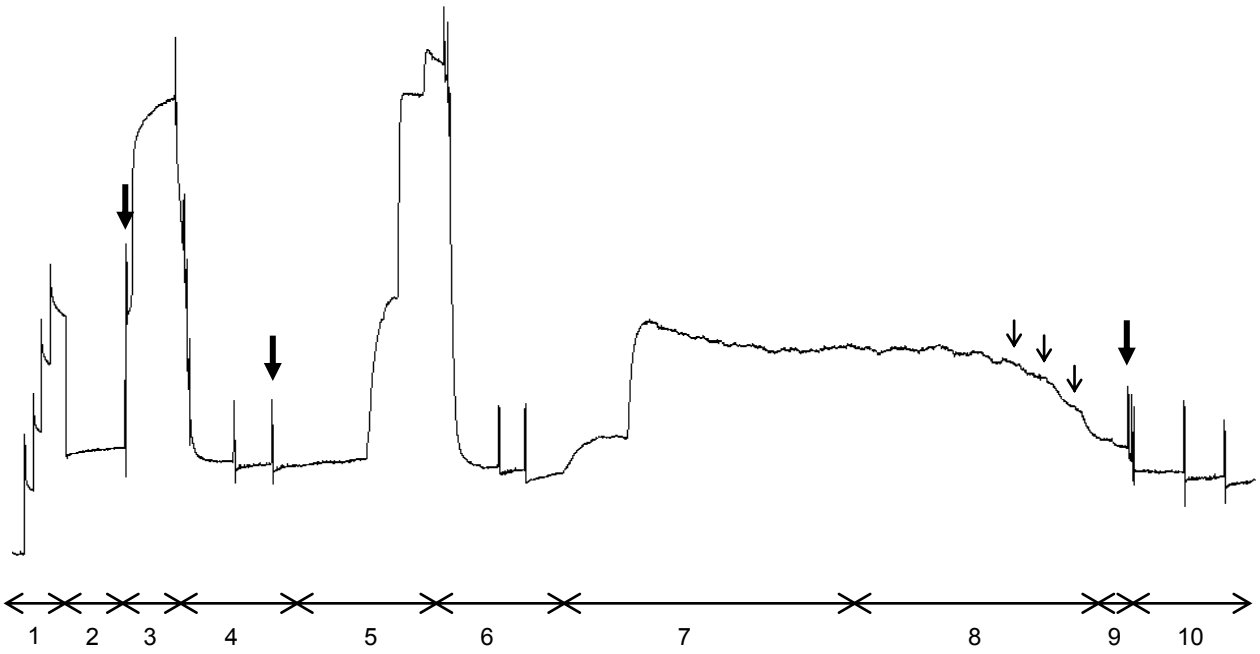

Figure 2.5 Original trace, illustrating the general protocol of the experiments testing KCNQ channel openers: 1-normalization, 2-stabilization after normalization, 3-initial contraction in 120mM KCl + 10^{-5}M serotonin, 4-wash out, 5-dose-response curve for preconstrictor, 6-wash out, 7-preconstriction, 8-step-wise addition of KCNQ channel opener (addition shown by small arrows), 9-full relaxation, 10-wash out. Big arrow shows the usual artifact caused by changing the solution in the chamber.

All values are given as mean ± SEM. Paired and unpaired Student‘s *t* tests or ANOVA were used as appropriate. A value of $p < 0.05$ was considered statistically significant; n represents the number of animals tested.

3. RESULTS

In order to test the hypothesis of the present study, KCNQ channel expression in *Gracilis* arteries was investigated using the PCR technique and KCNQ channel function in these vessels was evaluated using wire myography.

3.1. Expression of KCNQ channels in rat *Gracilis* arteries

The PCR-based experiments were performed to clarify the presence of KCNQ channels in *Gracilis* arteries and to determine their subtypes.

In *Gracilis* arteries we found mRNA expression for KCNQ1, KCNQ3, KCNQ4 and KCNQ5 channels (Fig. 3.1). Similar findings were obtained in 4 other experiments.

For positive controls, brain and heart was used. Expression of all KCNQ channels tested was found in control tissues. To show that isolated mRNA and other reagents had no DNA-contamination, a negative control was done. To prepare negative controls, the RT-step (reverse transcription) was omitted.

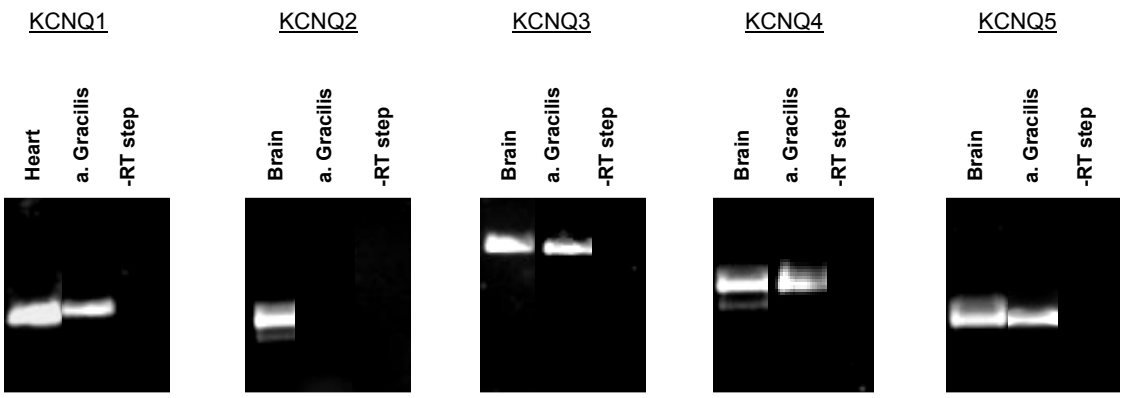

Figure 3.1 Detection of KCNQ1, KCNQ3, KCNQ4 and KCNQ5 by PCR in isolated rat *Gracilis* arteries. Positive control: heart or brain tissues; negative control: *Gracilis* arteries without RT-step.

The presence of KCNQ channel mRNAs in *Gracilis* arteries initiated the following series of experiments aimed at determining the functional role of these channels.

3.2. Functional effect of KCNQ channel opener on rat *Gracilis* arteries

These experiments were done to determine the influence of KCNQ channel opening on the contractile properties of *Gracilis* arteries.

Functional effects of KCNQ channel opener were studied on arteries preconstricted with different contractile agents (serotonin, phenylephrine or U-46619). Experimental KCNQ channel opener were applied stepwise with the following final concentrations: 10^{-8}, $3*10^{-8}$, 10^{-7}, $3*10^{-7}$, 10^{-6}, $3*10^{-6}$, 10^{-5} and $3*10^{-5}$M. The interval between subsequent concentrations was 3-5 minutes.

3.2.1. Effects on serotonin-preconstricted Gracilis arteries

All three substances, VRX0530727, VRX0621238 and VRX0621688, relaxed *Gracilis* arteries preconstricted with serotonin (n=8; $p<0.01$; n=9; $p<0.01$ and n=7; $p<0.01$, respectively).

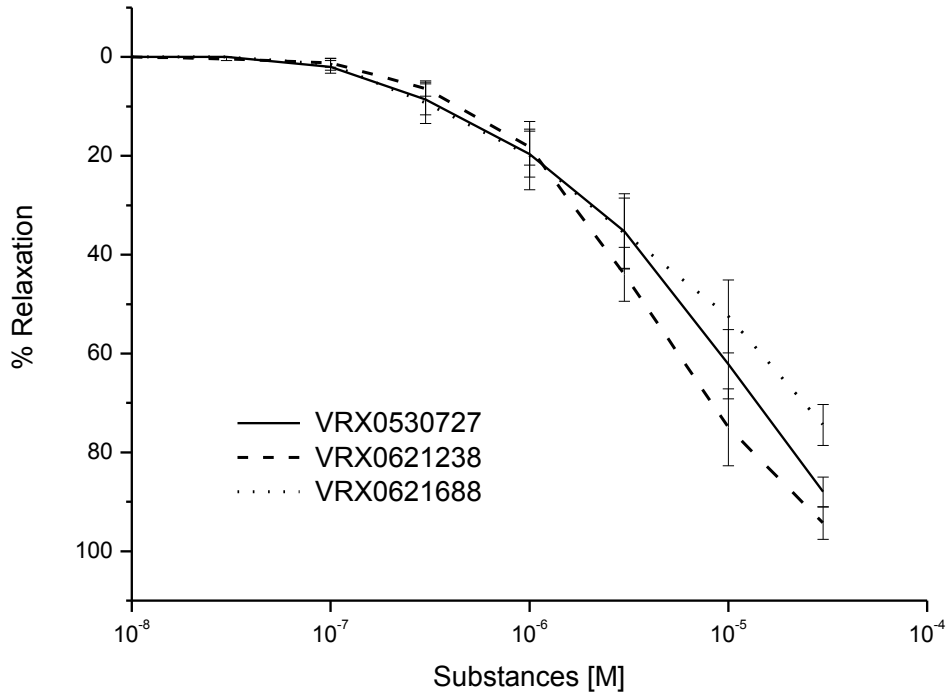

Figure 3.2 KCNQ channel opener relax serotonin-preconstricted *Gracilis* arteries.

The relaxation was similar for all substances (n=8 for VRX0530727, n=9 for VRX0621238, n=7 for VRX0621688; p=0.39; Fig. 3.2).

3.2.2. Effects on phenylephrine-preconstricted Gracilis arteries

In order to determine whether the effect of the KCNQ channel opener depends on the mode of preconstriction, vessels were also preconstricted with phenylephrine.

Under these conditions, only VRX0621238 and VRX0621688 produced considerable relaxation (n=7; $p<0.01$ and n=9; $p<0.01$, respectively; Fig. 3.3), whereas VRX0530727 did not alter vessel tension (n=6; p=0.07).

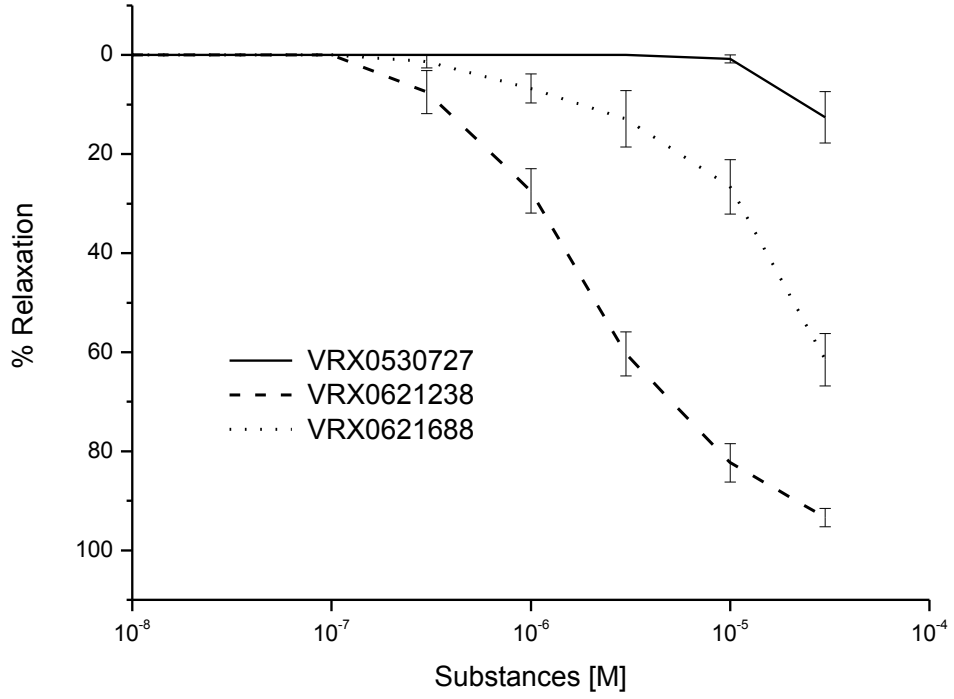

Figure 3.3 KCNQ channel opener relax phenylephrine-preconstricted *Gracilis* arteries.

In addition, the relaxing effect of VRX0621238 and VRX0621688 was different (n=7 for VRX0621238, n=9 for VRX0621688; $p<0.01$; Fig. 3.3).

3.2.3. Effects on U-46619-preconstricted Gracilis arteries

To confirm that the effect of the KCNQ channel opener depends on the mode of preconstriction, another substance, the thromboxane-analogue U-46619, was used to preconstrict the vessels.

Under these conditions, VRX0621238 and VRX0621688 produced considerable relaxation (n=11; $p<0.01$ and n=9; $p<0.01$, respectively; Fig. 3.4). VRX0530727 produced vessels constriction (n=6; $p<0.05$).

The relaxing effect of VRX0621238 and VRX0621688 was different (n=11 for VRX0621238, n=9 for VRX0621688; $p<0.01$; Fig. 3.4).

These data show that the KCNQ channel opener VRX0621238 and VRX0621688 are potent vasodilators acting largely independent of the mode of preconstriction. In order to be able to study the effect of these substances in more detail without consideration of the mode of preconstriction, serotonin was used to preconstrict the vessels in the following experiments.

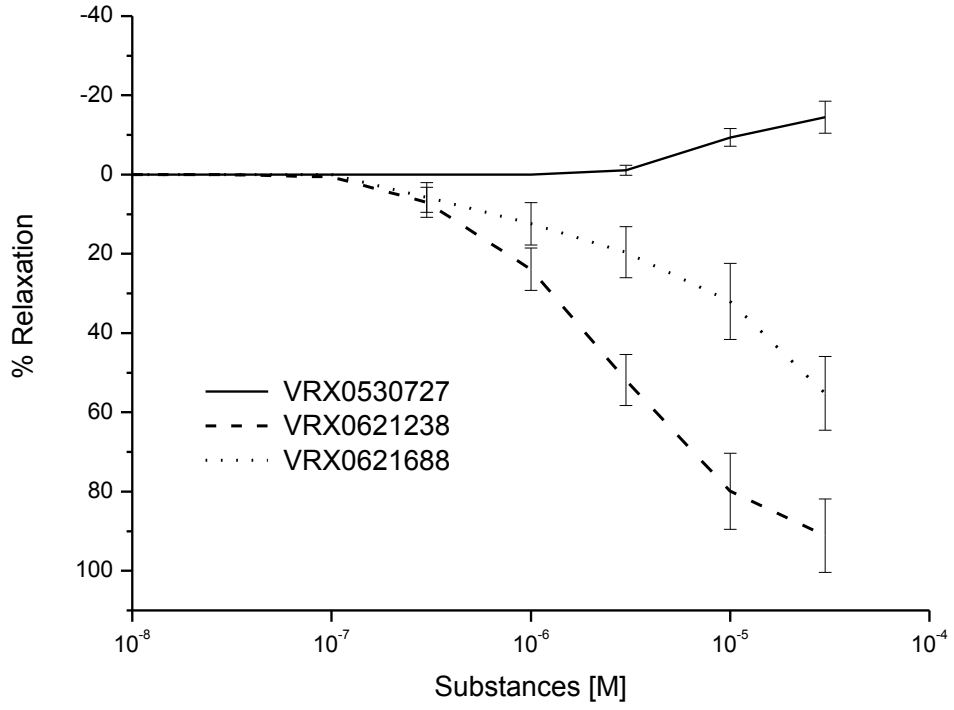

Figure 3.4 KCNQ channel opener relax U46619-preconstricted *Gracilis* arteries.

3.3. Mechanism of action of KCNQ channel opener

The mechanism of action of the KCNQ channel opener was studied to better understand the relationship between KCNQ channel opening and vessel relaxation. As a representative of the opener tested, VRX0530727 was used.

3.3.1. Role of the endothelium

To identify the contribution of the endothelium to the relaxing effect of the KCNQ channel opener, the endothelium was functionally removed using a rat's whisker. Removal of the endothelium did not affect the relaxation of *Gracilis* arteries preconstricted with serotonin by VRX0530727 (n=7; p=0.67; Fig. 3.5). Hence, the endothelium plays no role in the relaxation of the vessels induced by VRX0530727.

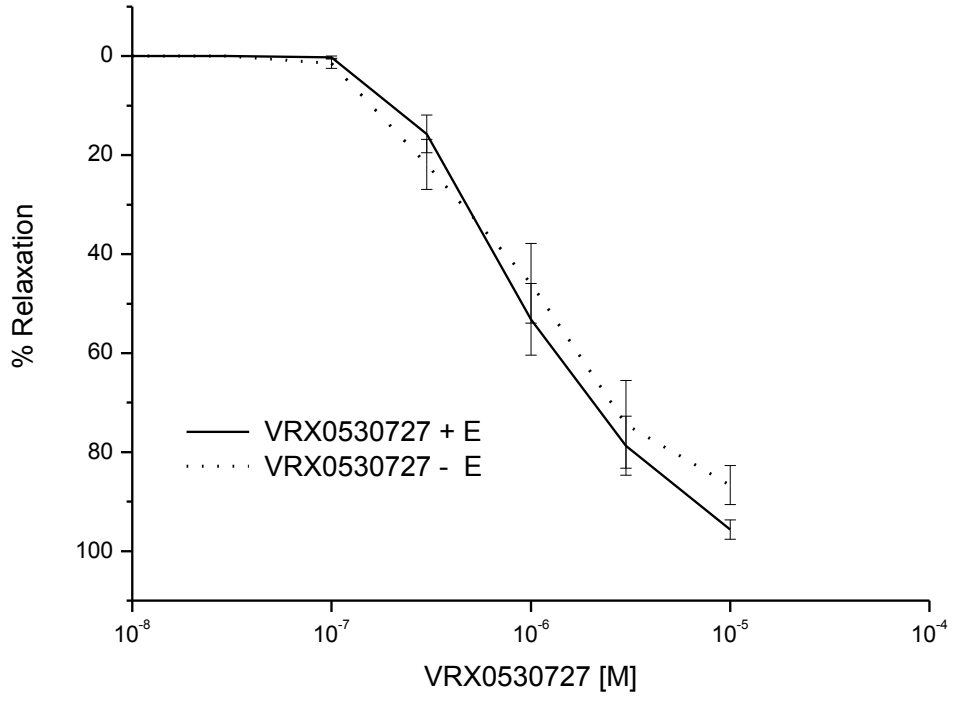

Figure 3.5 VRX0530727 relaxes serotonin-preconstricted *Gracilis* arteries with (+E) and without (-E) endothelium.

3.3.2. Role of sympathetic nerve endings

To determine whether sympathetic nerve endings contribute to the vessel relaxation induced by VRX0530727, another set of experiments was performed. The influence of sympathetic nerve endings was functionally eliminated by blocking postsynaptic receptors with phentolamine at a concentration of 10^{-6} M.

The relaxation of *Gracilis* arteries preconstricted with serotonin by VRX0530727 in the presence of intact and blocked sympathetic nerve endings was not different (n=4; p=0.94; Fig. 3.6). We determined that sympathetic nerve endings were not involved in the relaxation of the vessels induced by VRX0530727.

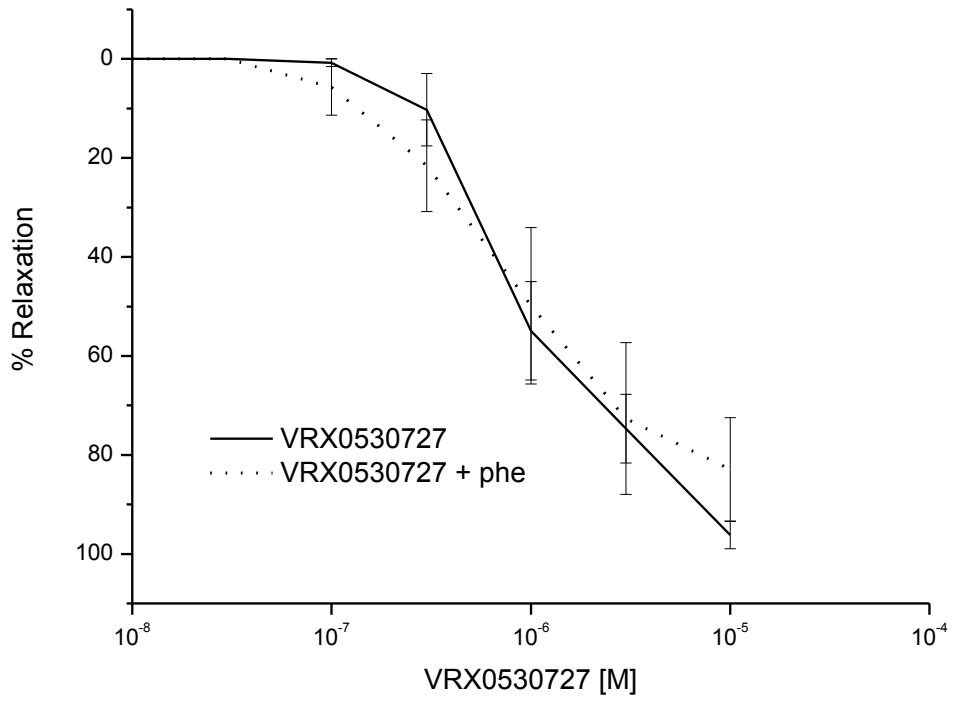

Figure 3.6 VRX0530727 relaxes serotonin preconstricted *Gracilis* arteries in the presence of intact and phentolamine-blocked (+phe) sympathetic nerve endings.

3.3.3. Contribution of K^+ channels

The substances tested in this study have been reported to show a potent opening effect on KCNQ channels in expression systems. Therefore, we tested the contribution of K^+ channels to the relaxation of an intact vessel induced by VRX0530727.

In a first series of experiments, K^+ channels were functionally eliminated by rising the extracellular potassium concentration to 60mM. This brings the actual membrane potential close to the potassium equilibrium potential and, therefore, suppresses potassium fluxes through open potassium channels. In order to prevent transmitter release from nerve endings evoked by KCl-induced depolarization, vessels were additionally treated with phentolamine.

The relaxation of *Gracilis* arteries preconstricted with serotonin and with 60mM KCl by VRX0530727 was different, KCl-induced preconstriction reduced the relaxation induced by VRX0530727 (n=5; $p<0.01$; Fig. 3.7).

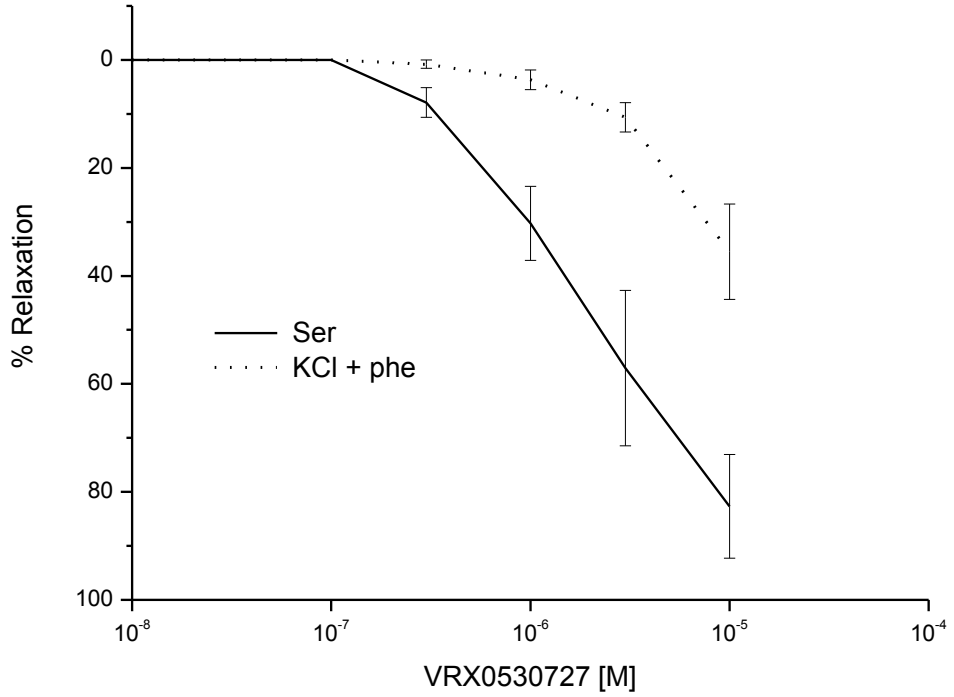

Figure 3.7 VRX0530727 relaxes *Gracilis* arteries preconstricted with KCl in the presence of phentolamine (KCl + phe) less than vessels preconstricted with serotonin (Ser).

These data suggest that K^+ channels contribute to the relaxation of the vessels by VRX0530727.

3.3.4. Involvement of KCNQ channels

In a second series of experiments, the contribution of KCNQ channels to the relaxation induced by VRX0530727 was tested. For this purpose, the specific KCNQ channel inhibitor XE991 at 10^{-5}M was used.

In the presence of XE991, the relaxation of *Gracilis* arteries by VRX0530727 was considerably reduced (n=5; $p<0.01$; Fig. 3.8). Thus, channels from the KCNQ family mediate the VRX0530727-caused relaxation of *Gracilis* arteries.

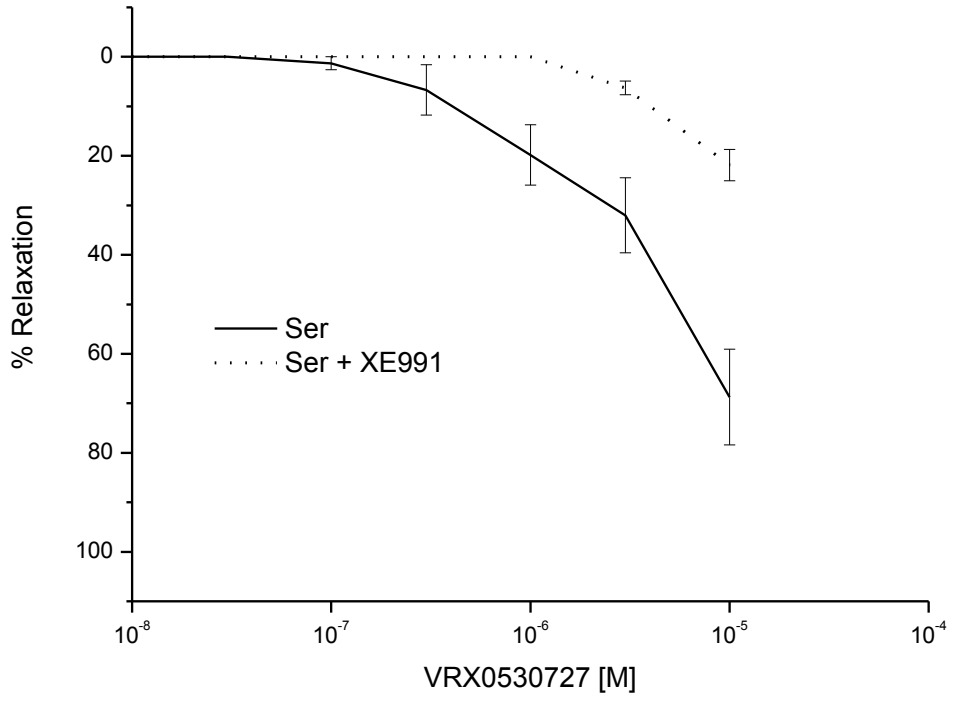

Figure 3.8 VRX0530727 relaxes serotonin-preconstricted *Gracilis* arteries in the presence of the KCNQ channel inhibitor XE991 (Ser + XE991) less than serotonin-preconstricted vessels in its absence (Ser).

3.4. Effect of KCNQ channel opener on *Gracilis* arteries of SHR rats

Since opening of potassium channels, especially KCNQ channels, might be an interesting therapeutic strategy in disease accompanied by increased peripheral vascular resistance, the effect of the KCNQ channel opener was tested also on *Gracilis* arteries from spontaneously hypertensive rats (SHR).

All three substances, VRX0530727, VRX0621238 and VRX0621688, relaxed *Gracilis* arteries from SHR preconstricted with serotonin (n=4; $p<0.01$; n=4; $p<0.01$ and n=4; $p<0.05$, respectively; Fig. 3.9).

The relaxation was similar for all substances (n=4 for VRX0530727, n=4 for VRX0621238, n=4 for VRX0621688; p=0.49) and was not different from their effects in normotensive animals (VRX0530727: n=8 for normotensive rats, n=4 for SHR; p=0.28; VRX0621238: n=9 for normotensive rats, n=4 for SHR; p=0.51; VRX0621238: n=7 for normotensive rats, n=4 for SHR; p=0.63).

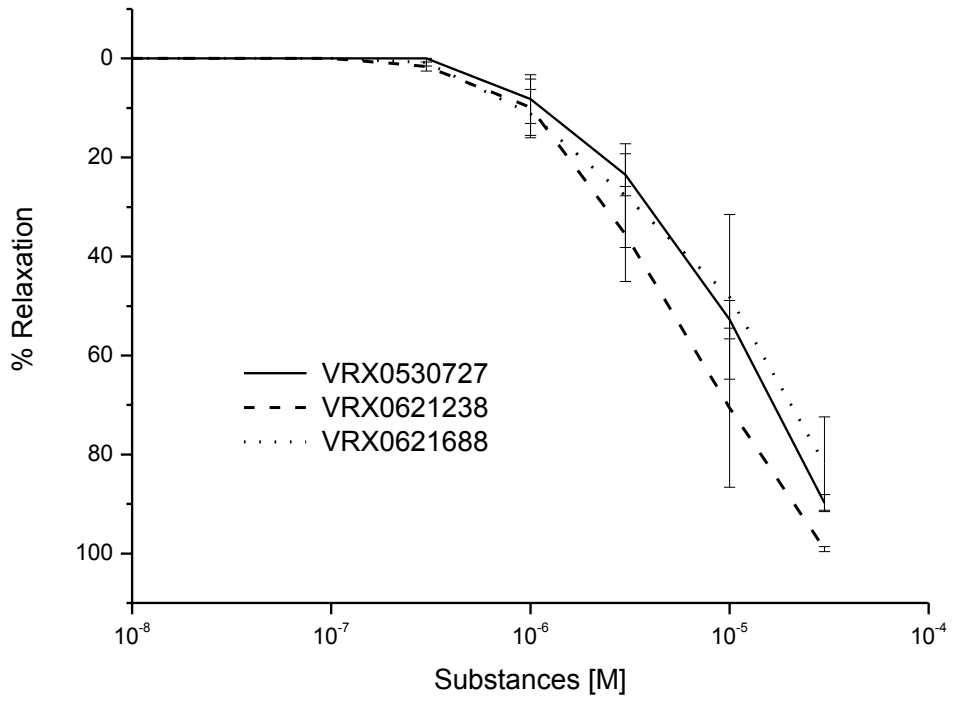

Figure 3.9 KCNQ channel opener relax serotonin-preconstricted *Gracilis* arteries from SHR.

In addition, we studied the effect of retigabine, a known KCNQ channel opener, on *Gracilis* arteries from spontaneously hypertensive rats.

Retigabine relaxed *Gracilis* arteries from SHR preconstricted with serotonin (n=4; $p<0.01$; Fig. 3.10).

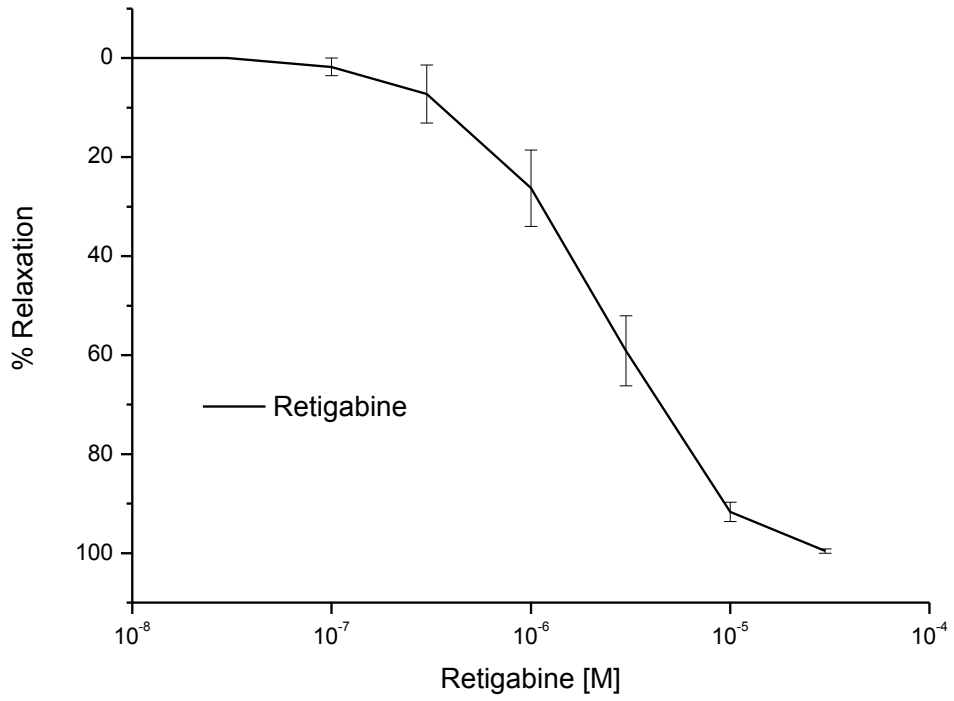

Figure 3.10 Retigabine relaxes serotonin-preconstricted *Gracilis* arteries from hypertensive rats.

Thus, all KCNQ channel opener dilate also arteries from hypertensive rats in the same way like in normotensive rats. The KCNQ channel opener tested in this study have the same action on *Gracilis* arteries from hypertensive rats as the potent KCNQ opener Retigabine.

3.5. Functional effect of KCNQ channel opener on rat *Intrapulmonary* arteries

All previously described experiments were conducted on a vessel representing the systemic circulation. In order to understand whether the KCNQ channel opener act also on the pulmonary circulation, additional experiments were performed on isolated *Intrapulmonary* arteries.

In the experiments with *Intrapulmonary* vessels, some parameters were changed to account for the specific features of the pulmonary circulation, for example the different transmural pressure (for more details see methods section). Further, the thromboxane-analogue U-46619 was used to preconstrict *Intrapulmonary* arteries, because preliminary experiments showed that this is the only stable preconstrictor for these vessels.

The vasorelaxing action of the KCNQ channel opener was of primary interest. Thus, the effect of VRX0621238 and VRX0621688, producing relaxation of U-46619-preconstricted *Gracilis* arteries, was studied on *Intrapulmonary* arteries.

3.5.1. Effects on U-46619-preconstricted Intrapulmonary arteries

VRX0621238 and VRX0621688 relaxed *Intrapulmonary* arteries ($n=9$; $p<0.01$ and $n=8$; $p<0.01$, respectively). The relaxation was similar for both substances ($n=9$ for VRX0621238 and $n=8$ for VRX0621688; $p=0.27$; Fig. 3.11).

These experiments show that the KCNQ channel opener VRX0621238 and VRX0621688 produce a relaxation in the pulmonary circulation resembling their effect on systemic arteries.

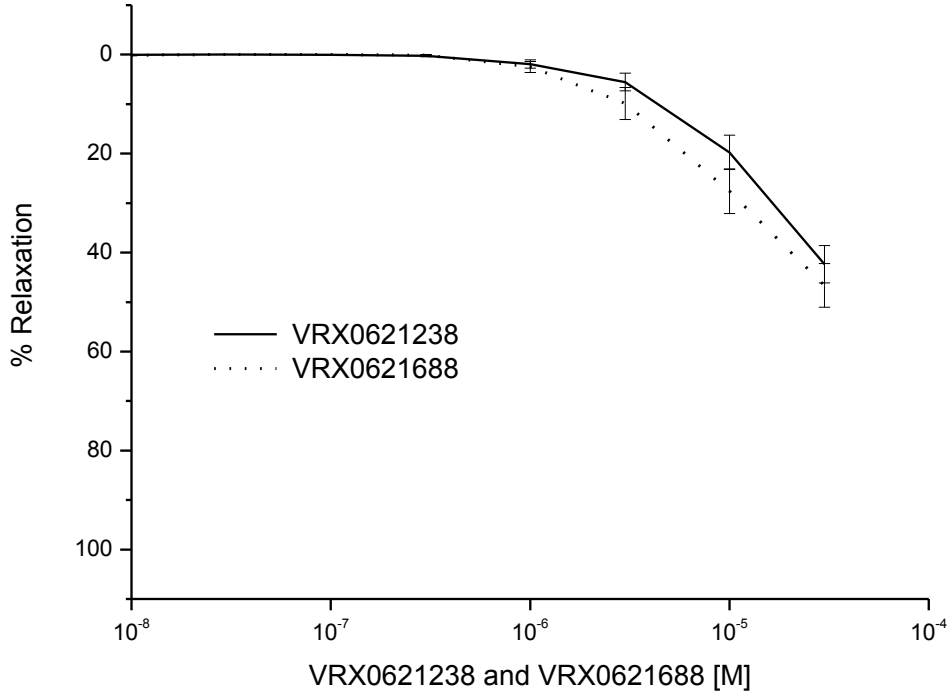

Figure 3.11 VRX0621238 and VRX0621688 relax U-46619-preconstricted *Intrapulmonary* arteries.

3.5.2. Comparison of the effects on arteries of the systemic and pulmonary circulation

The relaxation of *Gracilis* and *Intrapulmonary* arteries preconstricted with U46619 by VRX0621238 was different (n=9 for *Gracilis* arteries and n=7 for *Intrapulmonary* arteries; $p<0.01$; Fig. 3.12). The effect on *Gracilis* arteries was stronger.

In contrast, the relaxation by VRX0621688 was similar (n=9 for *Gracilis* arteries and n=5 for *Intrapulmonary* arteries; $p=0.39$; Fig. 3.13).

Thus, the effect of KCNQ channel opener in the systemic and the pulmonary circulation is different for VRX0621238 and similar for VRX0621688.

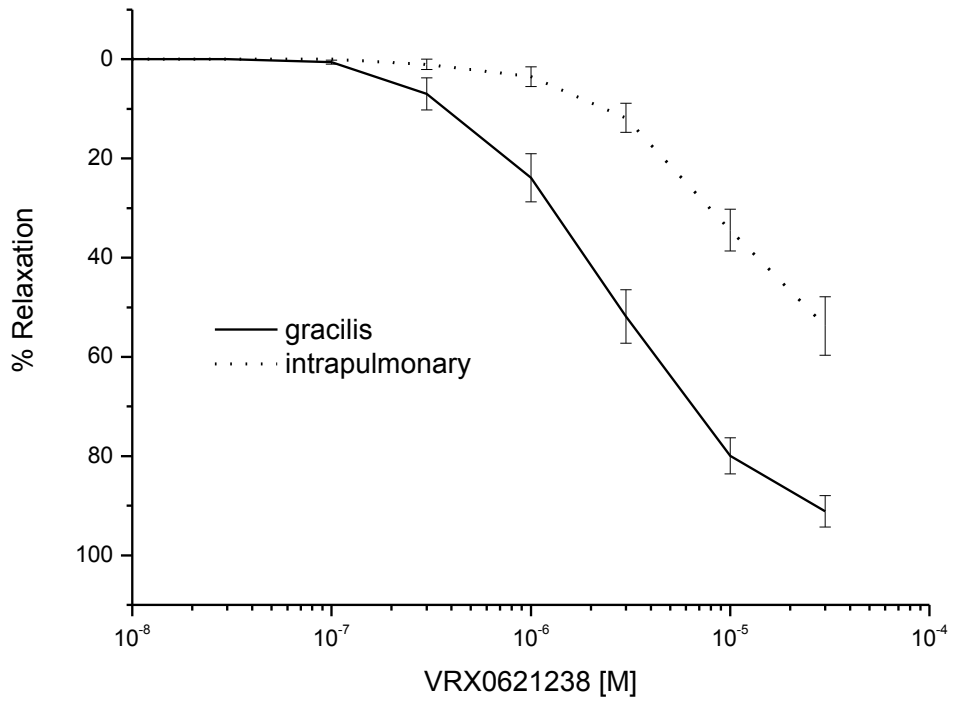

Figure 3.12 VRX0621238 relaxes U-46619-preconstricted vessels from the systemic and pulmonary circulation.

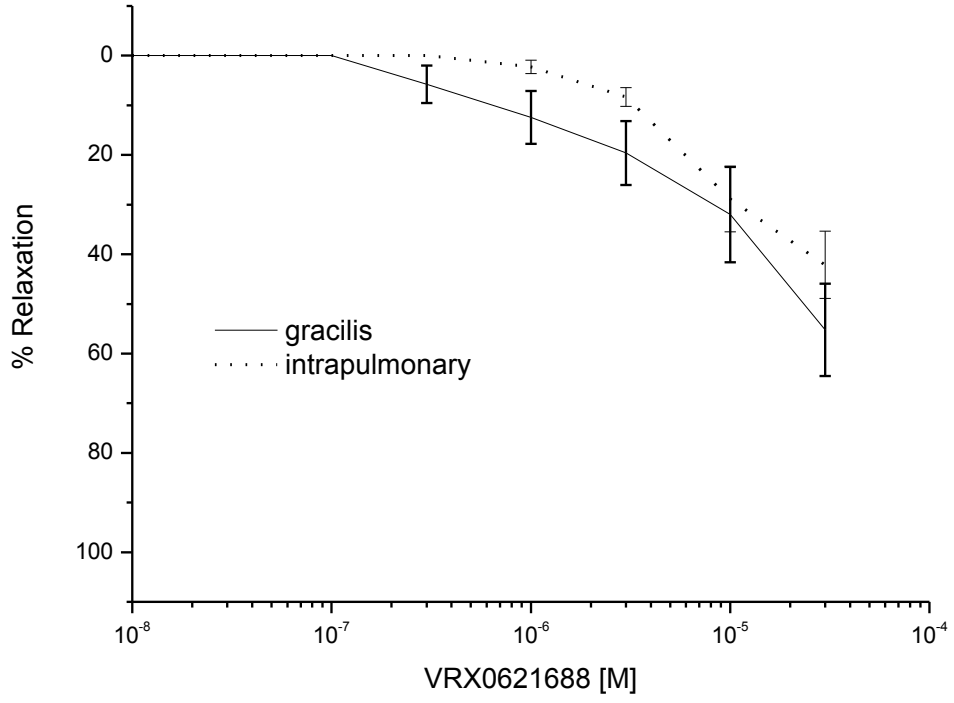

Figure 3.13 VRX0621688 relaxes U-46619-preconstricted vessels from the systemic and pulmonary circulation.

4. DISCUSSION

This study shows that KCNQ channel mRNA expression in a non-visceral artery, the rat *Gracilis* artery, is not different from other vessels studied previously. We showed for the first time that the putative KCNQ channel opener VRX0530727, VRX0621238 and VRX0621688 have a potent vasorelaxing effect in rat *Gracilis* arteries. We demonstrated that KCNQ channels are involved in the relaxation induced by the KCNQ channel opener. KCNQ channel opener also relax arteries from hypertensive rats. In addition, KCNQ channel opener produce a relaxation in the pulmonary circulation as well.

4.1. Expression of KCNQ channels in rat *Gracilis* arteries

In our study on rat *Gracilis* arteries we found mRNA expression for KCNQ1, KCNQ3, KCNQ4 and KCNQ5 channels.

From previous studies we know that KCNQ1, KCNQ4 and KCNQ5 transcripts are present in several, mainly visceral vessels isolated from mouse, rat and humans. In the mouse, KCNQ1, 4 and 5 channel messages were found in the portal vein, thoracic aorta, carotid and femoral artery (Ohya et al., 2003; Yeung et al., 2007-2008). The same transcripts were detected in the rat aorta, mesenteric, pulmonary and small cerebral arteries (Brueggemann et al., 2007; Mackie et al., 2008a; Joshi et al., 2009; Zhong et al., 2010). In a study on human arteries from visceral adipose tissue and the mesenteric bed the same findings were reported (Ng et al., 2011).

Transcripts for KCNQ2 channels were always absent in vascular tissue in all previous investigations.

Our findings concerning the presence of KCNQ1, 4 and 5 channels and the absence of KCNQ2 transcripts in rat *Gracilis* arteries confirm data obtained in other vessels studied so far.

Expression of mRNA for KCNQ3 channels is not typical for blood vessels. To this moment, KCNQ3 transcripts were found only in rat pulmonary arteries (Joshi et al., 2009) and some human arteries (Ng et al., 2011). A more detailed analysis of isolated smooth muscle cells derived from rat pulmonary arteries demonstrated the absence of KCNQ3 message (Joshi et al., 2009). Unfortunately, such analysis was not performed on arteries from visceral adipose tissue and proximal mesenteric arteries (Ng et al., 2011). Nevertheless, based on these data we suppose that KCNQ3 channels may not be present in the smooth muscle cells of *Gracilis* arteries, but in cells of the other layers of the vessel wall. To confirm or refute this supposition, additional studies are required using isolated cells, a method not employed in this study.

Taking all data together, we suppose that KCNQ2 and KCNQ3 channels play no role in vascular smooth muscle cells because of their rare existence in this tissue. In addition, we suppose that pharmacological interventions targeting these subunits in the nervous system do not show side effects in the cardiovascular system. In contrast, KCNQ4 and KCNQ5 channels are present in both types of tissues (KCNQ1 is not expressed in the brain) and this may be the reason for some unexpected manifestations of drug applications. For example, the anticonvulsant retigabine was used at a concentration of 10μM in the neurvous system and at the same concentration it produces significant relaxation of preconstricted vessel (Yeung et al., 2007).

4.2. Functional effect of KCNQ channel opener on rat *Gracilis* arteries

To this moment, several KCNQ channel opener such as retigabine, flupirtine, acrylamide S-1 and the novel KCNQ openers VRX0530727, VRX0621238, and VRX0621688 were reported to relax preconstricted isolated systemic arteries. The effects of these substances are reversed by KCNQ channel inhibition with XE991 (Yeung et al., 2007; Joshi, et al., 2009; Schleifenbaum et al., 2010; Ng et al., 2011).

In particular, it was shown that retigabine (2 - 20 μmol/l) relaxes segments of mouse aorta (preconstricted with phenylephrine) with maximal relaxation of about 40%. Application of flupirtine (20 μmol/l) in the same vessels produces also considerable relaxations (Yeung et al., 2007).

Retigabine also produces concentration-dependent relaxation of mouse mesenteric arteries preconstricted with serotonin or U-46619 with a half-maximal effect at ~ 3-5 µmol/l. Interestingly, the relaxation of serotonin preconstricted vessels was stronger then in case of U-46619 preconstricted vessels. The same arteries preconstricted with serotonin show significant relaxation also after VRX0530727, VRX0621238 and VRX0621688 application (Schleifenbaum et al., 2010).

Recently it was shown that retigabine and acrylamide S-1 (3 – 10 µmol/l) relax preconstricted human visceral adipose and mesenteric arteries dose-dependently (Ng et al., 2011).

Our experiments show that VRX0530727, VRX0621238 and VRX0621688 relax rat *Gracilis* arteries preconstricted with serotonin in a similar way with a half-maximal effect at ~ 5-10 µmol/l. These data confirm available information about effect of KCNQ openers on mouse mesenteric arteries.

In addition, we tested retigabine on rat *Gracilis* arteries preconstricted with serotonin from hypertensive rats. Retigabine application produced vessel relaxation with a half-maximal effect at ~ 3-5 µmol/l. Our findings confirm previous data on vessels from normotensive mice. The available data show that the relaxing effect of retigabine does not depend on the vessel type studied, but may depend on the way of preconstriction.

The latter conclusion is supported by our data on the effect of the novel KCNQ channel openers in vessels preconstricted with different agents. Thus, *Gracilis* arteries preconstricted with phenylephrine were not affected by VRX0530727. However, VRX0621238 and VRX0621688 relax those arteries, but with different half maximal effects (~ 3 and 10 µmol/l respectively). In addition, the use of the thromboxane-analogue U-46619 as a preconstrictor demonstrates a different result: VRX0621238 and VRX0621688 produce considerable relaxation (half maximal effect at ~ 5 and 7 µmol/l respectively) but VRX0530727 produce vessel constriction.

The mechanisms of vessel constriction of the used preconstrictor agents show some differences (Fig. 4.1). All substances used bind to their appropriate receptors at the smooth muscle cell membrane; serotonin binds to the 5-hydroxytryptamine receptors (5-HT_{2A}), phenylephrine – to the α1-adrenoreceptors (α1-ARs) and the thromboxane-analogue U-46619 to

the thromboxane A2 receptors (TBXA2R). All these receptors represent G protein-coupled receptors (GPCR). There are few different G-protein-mediated signaling pathways depending on the sub-classes of G-proteins (Wettschureck et al., 2005). We will discuss two of them which are important for smooth muscle cell contraction (calcium-sensitive and -insensitive pathways).

a) Ca^{++} dependent contraction (Fig. 4.1, a)

In this case GPCR represent the Gq/G_{11} coupled receptors (for example AT_1-angiotensin, α1, $5\text{-}HT_{2A}$ receptors etc.). When a signal substrate-molecule binds to a GPCR, a G-protein becomes activated on the intracellular side. In turn, the G-protein binds and activates phospholipase C (PLC), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdInsP2) and releases inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). In the sarcoplasmic reticulum IP3 binds to the appropriate receptors (IP3R) that releases calcium. In the cytosol, Ca^{++} first binds to calmodulin. The Ca^{++}/calmodulin-complex activates myosin light chain kinase (MLCK) and this induces increased myosin light chain (MLC) phosphorylation and cell contraction. Furthermore, Ca^{++} and released DAG (see above) activate protein kinase C (PKC), which leads to L-type calcium channel phosphorylation. This calcium also participates in myosin phosphorylation.

b) Ca^{++} independent contraction so called Ca^{++} sensitization (Fig. 4.1, b)

Most Gq/G_{11}-coupled vasoconstrictor receptors also activate the G_{12}/G_{13} family of G proteins (receptors for endothelin-1, vasopressin, angiotensin II, thrombin, thromboxane A2 etc.). G_{12}/G_{13} proteins activate the second signaling pathway for smooth muscle cell contraction through RhoA/Rho-kinase activation that leads to MLCP inhibition and an increased MLC phosphorylation.

In addition, PKC activates also CPI-17, the inhibitor of the myosin light chain phosphatase (MLCP) catalytic subunit that also increases MLC phosphorylation (Somlyo et al., 2000). This mechanism plays a minor, transient role in Ca^{++} independent cell contraction.

It is suggested that from these two mechanisms of vessel contraction Gq/G_{11} conveys a fast, transient response and G_{12}/G_{13} proteins mediate a sustained, tonic contraction (Hersch et al., 2004).

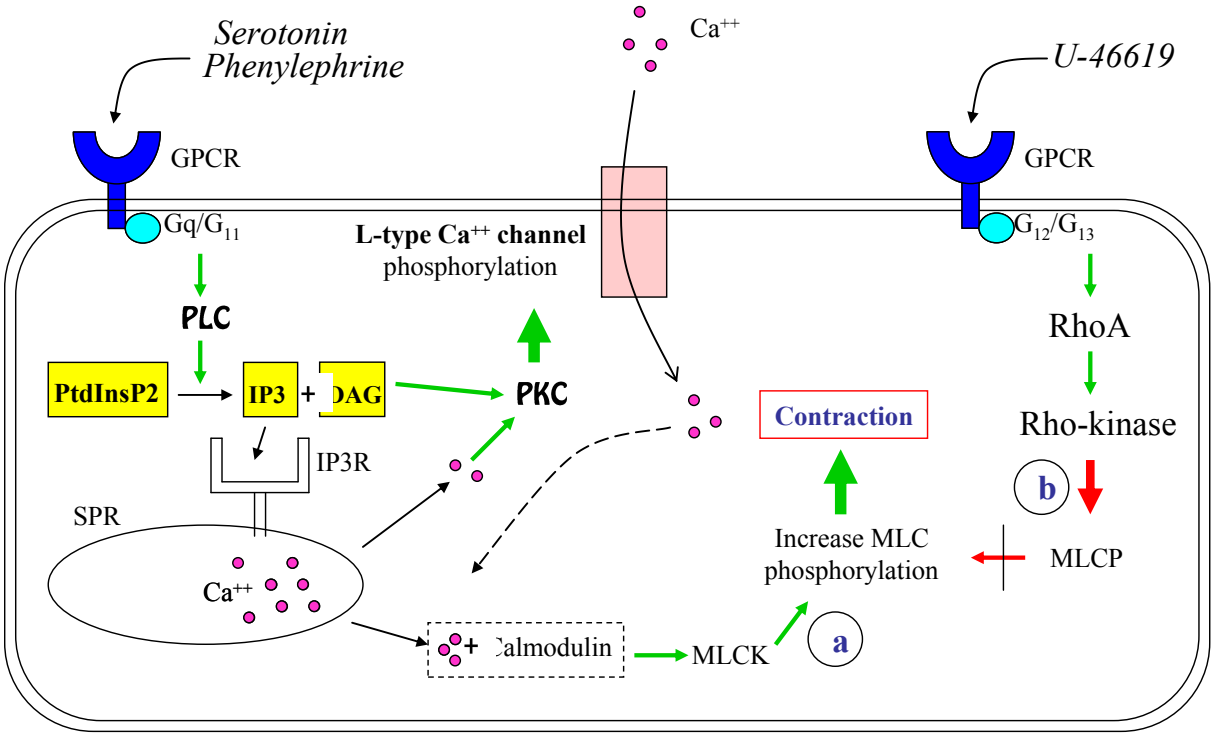

Figure 4.1 Vessel constriction caused by GPCR activation: GPCR - G protein-coupled receptor, PLC - phospholipase C, PtdInsP2 - phosphatidylinositol-4,5-bisphosphate, IP3 - inositol-1,4,5-trisphosphate, DAG – diacylglycerol, IP3R - inositol-1,4,5-triphosphate receptor, SPR - sarcoplasmic reticulum, PKC - protein kinase C, MLCK - myosin light chain kinase, MLCP - myosin light chain phosphatase, MLC - myosin light chains; a) calcium-sensitive pathway, b) calcium-insensitive pathway.

The membrane potential depolarization associated with the action of the contractile agents used leads, along with the two mechanisms producing contraction, also to a compensatory activation of Kv and Ca^{++} dependent potassium channels (K_{Ca}). Thus, these channels limit the agonist-induced contraction.

However, the contractile agents used in our study employ these mechanisms, the calcium-sensitive and the calcium-insensitive pathway, to different degrees. Thus, phenylephrine and serotonin are using mostly the calcium-sensitive pathway (the contribution of Ca^{++} sensitization is very small and realized by the CPI-17 component). In contrast, the U-46619-induced contraction is mediated in a large part by the calcium-insensitive contraction mechanism (Wettschureck et al., 2005).

We suggest that these differences in the intracellular mechanisms employed by the contractile agents used in this study explain the variability of the effects of the KCNQ channel openers tested.

All together, we can conclude that the novel KCNQ openers act differently depending on the way of vessel preconstriction, but VRX0621238 and VRX0621688 always show a relaxing effect, thus they are the most potent vasodilators.

4.3. Mechanism of action of KCNQ channel opener

It seems logic that KCNQ channel openers act on the KCNQ channels located in smooth muscle cells of the arterial tunica media. However, such channels may also exist in the endothelium or in nerve endings in the vessel wall. Thus, in our study we first investigate if tunica intima (endothelium) or tunica adventitia (sympathetic nerve endings) could mediate the action of KCNQ channel openers in arteries of the systemic circulation.

We found that the endothelium plays no role in the relaxation of the vessels induced by VRX0530727.

For these experiments, the endothelium of some vessels was mechanically removed by a rat whisker. The endothelial layer was scraped off by several motions of the whisker inserted in the vessel lumen. Complete functional destruction of the endothelial cells was confirmed by the absence of the response to the application of 10^{-5}M acetylcholine on preconstricted arteries. In contrast, control arteries demonstrated strong vasorelaxation to acetylcholine application.

The data about an endothelium independent relaxation induced by VRX0530727 confirm previous information. Analogous experiments addressing the role of the endothelium were performed on vessels from the pulmonary circulation. They demonstrated that the vasodilator action of retigabine occurred independently of a functional endothelium in rat pulmonary arteries (Joshi, et al., 2009).

In addition, we showed that sympathetic nerve endings are not involved in the relaxation of vessels induced by VRX0530727. For these experiments, a possible influence from

sympathetic nerve endings was blocked by the application of 10^{-5}M phentolamine in order to prevent the action of transmitter substances released from the nerve endings.

Taken together it is clear that the target for the new KCNQ openers is the tunica media, most likely the KCNQ channels in the smooth muscle cells.

Indeed, after elimination of the functional impact of K^+ channels on vessel contractility by rising the extracellular potassium concentration to 60 mM, relaxation of the vessels by VRX0530727 was significantly decreased.

The increase of the extracellular potassium concentration changes the equilibrium potential for potassium. It is known that the concentration of potassium ions inside the cell is normally ~ 25-times higher then in the extracellular surroundings as the result of the activity of Na^+/K^+-ATPases. Increasing the concentration of potassium ions in the physiological solution brings the K^+ amount inside the cell closer to its amount outside the cell. In this case K^+ outflow through potassium channels is reduced and the K^+ ions not leaving the cell any more produce a depolarization of the membrane potential that brings the membrane potential close to the equilibrium potential for potassium. Consequently, the difference between the membrane potential and the equilibrium potential for potassium, i.e. the driving force for potassium ions, is so small that even in the case of active potassium channels they are unable to affect the contractile state of the vessels. .

The same effect was observed after the selective blockade of KCNQ channels by 10 μM of XE991. XE991 produced significant changes: the relaxation of the vessels was blocked considerably, but not completely. These data suggest that K^+ channels and in particular KCNQ channels contribute to the relaxation of the vessels induced by VRX0530727.

In our experiments we used the following concentrations of K^+/ KCNQ channel blockers: 60 mM KCl solution and 10 μM of XE991. At this concentration XE991 is an effective and selective blocker for all KCNQ channels (Gutman et al., 2005). The relaxing effect of VRX0530727 in our study was blocked incompletely. We will discuss this observation below.

Our findings on the effect of the new KCNQ channel opener are consistent with previously published data. Thus, in mouse thoracic aorta it was shown that retigabine has no effect on contractions produced by 60 mM KCl or by 10 μM XE991 (Yeung et al., 2007). In addition, in human arteries the relaxation produced by retigabine or acrylamide S-1 was reversed

completely by the subsequent application of 10 μM XE991 or was prevented by application of XE991 before the preconstriction (Ng et al., 2011).

The same picture was observed in the pulmonary circulation. The effect of retigabine was greatly reduced when pulmonary arteries were preconstricted with 50 mM KCl. Retigabine also reduced the vasoconstrictor effects of linopirdine (10 μM) and XE991 (1 μM) almost abolishing vasoconstriction at a concentration of 100 μM (Joshi, et al., 2009).

Finally, relaxation of mouse mesenteric arteries induced by retigabine, VRX0530727, VRX0621238, and VRX0621688 was considerably reduced, albeit not completely, after inhibition of KCNQ channels with 30 μM XE991 (Schleifenbaum et al., 2010) that confirm our data.

In our study we observed that blockade of potassium channels and in particular KCNQ channels leads to a significant decrease of vessel relaxation induced by VRX0530727. However, this blockade did not abolish the relaxation completely.

Studies on mouse portal vein showed that the half-blocking concentration of XE991 is 5.8 μM (Ohya et al., 2003). We used 10 μM XE991. At this concentration, KCNQ channels will not be blocked completely. However, higher concentrations, that are able to produce a full block of these channels, may have side effects not related to KCNQ channels (Zhong et al., 2010). In order to be able to draw a clear conclusion from the data obtained, it was decided to use a concentration of XE991 that allows a selective action on KCNQ channels even it the block is incomplete.

4.4. Effect of KCNQ channel opener on *Gracilis* arteries of SHR rats

It is known that the expression of Kv channels in vascular smooth muscle cells from normo- and hypertensive rats may differ. For example the expression of Kv1.2, Kv1.5 and Kv2.1 channels is higher in mesenteric arteries of SHR rats compared with normotensive animals. In addition, Kv currents associated with these channels are larger in myocytes from SHR mesenteric arteries (Cox et al., 2008).

We showed here that all KCNQ channel opener and retigabine dilate *Gracilis* arteries from hypertensive rats. Moreover, VRX0530727, VRX0621238, and VRX0621688 act with the same potency as in normotensive rats. This demonstrated that the functional availability of KCNQ channels is not different between normotensive and hypertensive rats. Although we did not investigate the expression of KCNQ channels in the arteries from SHR, these data also indicate that KCNQ channel expression is not different in SHR and normotensive rats.

These findings suggest that the novel KCNQ channel openers may be used as a treatment for hypertensive conditions. If one supposes that the high blood pressure does not depend on KCNQ channel expression in the vessels, these channels could be used as targets for the treatment against hypertension. We suggest that in the future investigations should be performed to prove or refute the therapeutic potential of the KCNQ channel openers. In the case that KCNQ channel opener may be successfully applied for the treatment of hypertensive conditions, these opener may comprise a new class of antihypertensive drugs.

4.5. Functional effect of KCNQ channel opener on rat *Intrapulmonary* arteries

From previous studies we know that activation of KCNQ channels is possible also in the pulmonary circulation. Thus, in rat intrapulmonary arteries preconstricted with phenylephrine the KCNQ channel activator retigabine produced a reduction of the contractile response in a concentration-dependent manner with a half-maximal effect at ~ 10 µmol/l (Joshi et al., 2009).

We determined that the new KCNQ channel opener VRX0621238 and VRX0621688 also produce a relaxation in the pulmonary circulation resembling their effect on systemic arteries. For our study we used U-46619 for preconstriction because it was the only stable preconstrictor for intrapulmonary arteries. The concentration for the half-maximal effect was approximately the same as in a previously published study (Joshi et al., 2009).

We also found that the effect of KCNQ channel opener in the systemic and pulmonary circulation is different for VRX0621238 but similar for VRX0621688, namely VRX0621238 acts much weaker in the pulmonary circulation. These findings might be useful for the in vivo

situation. It is important to take into account that the effect of KCNQ channel opener appears in both, systemic and pulmonary, circulations and that the contribution of the KCNQ channel opener in those circulations is variable.

5. SUMMARY

In this study we have tested the hypothesis that KCNQ channels are powerful targets for relaxation of small systemic and pulmonary arteries. We employed the PCR-method and small vessel myography on small skeletal muscle and pulmonary arteries from normotensive Wistar and hypertensive SHR rats. The following observations were made:

1. Representatives of the KCNQ channel family are present in small systemic and pulmonary arteries. We have found mRNA expression for KCNQ1, KCNQ3, KCNQ4 and KCNQ5 channels now also in rat Gracilis arteries. Thus, they could be powerful targets for relaxation of these vessels.

2. Preconstricted systemic and pulmonary arteries relax after the application of KCNQ channel opener. In particular, we showed strong relaxations of rat *Gracilis* and *Intrapulmonary* arteries by the novel KCNQ channel opener VRX0530727, VRX0621238 and VRX0621688. This relaxation was shown to be mediated by KCNQ channels (especially in the rat *Gracilis* artery). This finding shows that KCNQ channels are targets for the induction of vessel relaxation.

3. The vasodilation induced by KCNQ channels opening does not depend on vessel type, but depends on the mechanism of vessel preconstriction. VRX0621238 and VRX0621688 relax vessels preconstricted by all studied agents, so they are the most potent vasodilators.

4. The KCNQ channel opener VRX0530727, VRX0621238 and VRX0621688 as well as retigabine relax also arteries from hypertensive animals (*Gracilis* arteries from SHR rats). This additional finding demonstrates the premise that KCNQ channels may serve as therapeutic targets in the case of pathological hypertensive conditions.

Attachment I

PCR program

	Command	Time	Process
	CNTRL	TUBE	
	LID105°		Warm up lid
	WAIT	AUTO	
1.	**T=45°**	00:00:10	
2.	Sound 5		
3.	PAUSE "PRIMER MIX"		Add primers
4.	**T=45°**	00:30:00	Reverse Transcription
5.	Sound 5		
6.	PAUSE “CONTROL”		Add control tubes, add primers
7.	T=94°	00:02:00	Denaturation of DNA
8.	T=94°	00:00:10	Denaturation of DNA
9.	**T=56°**	00:00:30	Annealing
10.	T=68°	**00:01:10**	Elongation
11.	GOTO 8	REP 9	(10 cycles)
12.	T=94°	00:00:10	Denaturation of DNA
13.	**T=56°**	00:00:30	Annealing
14.	T=68°	**00:01:10**	Elongation
		0:05 s	(ferment activity compensation)
15.	GOTO 12	REP **24**	(25 cycles)
16.	T=68°	00:07:00	Elongation
17.	SOUND 5		
18.	HOLD 4°		Cooling down
	end		

Attachment II

Solutions

Solution I

Compounds	Molecular weight, g/mol	Final concentration, mM	Amount for 1l
NaCl	58,40	120,00	7,008g
$NaHCO_3$	84,01	26,00	2,184g
KCl	74,60	4,50	0,336g
$NaH_2PO_4 \times H_2O$	137,99	1,20	0,166g
$MgSO_4$	120,40	1,00	0,120g
$CaCl_2$	147,00	1,60	0,235g
Glucose	180,16	5,50	0,991g
(Na)EDTA (25mM stock solution)	372,24	0,03	1ml
(Na)Hepes 37°C (0,5M)	238,30	5,00	10ml

Solution I modified with 120 mM KCl

Compounds	Molecular weight, g/mol	Final concentration, mM	Amount for 1l
NaCl	58,40	4,50	0,263g
$NaHCO_3$	84,01	26,00	2,184g
KCl	74,60	120,00	8,952g
$NaH_2PO_4 \times H_2O$	137,99	1,20	0,166g
$MgSO_4$	120,40	1,00	0,120g
$CaCl_2$	147,00	1,60	0,235g
Glucose	180,16	5,50	0,991g
(Na)EDTA (25mM stock solution)	372,24	0,03	1ml
(Na)Hepes 37°C (0,5M)	238,30	5,00	10ml

Solution I without $NaHCO_3$

Compounds	**Molecular weight, g/mol**	**Final concentration, mM**	**Amount for 1l**
NaCl	58,40	146,00	8,5274g
$NaHCO_3$	84,01	—	—
KCl	74,60	4,50	0,336g
$NaH_2PO_4 \times H_2O$	137,99	1,20	0,166g
$MgSO_4$	120,40	1,00	0,120g
$CaCl_2$	147,00	1,60	0,235g
Glucose	180,16	5,50	0,991g
(Na)EDTA (25mM stock solution)	372,24	0,03	1ml
(Na)Hepes 37°C (0,5M)	238,30	5,00	10ml

Solution II

Compounds	**Molecular weight, g/mol**	**Final concentration, mM**	**Amount for 1l**
NaCl	58,40	145,00	8,468g
KCl	74,60	4,50	0,336g
$NaH_2PO_4 \times H_2O$	137,99	1,20	0,166g
$MgSO_4$	120,40	1,00	0,120g
$CaCl_2$	147,00	0,10	0,2ml
(Na)EDTA (25mM stock solution)	372,24	0,025	1ml
(Na)Hepes 4°C (0,5M)	238,30	5,00	10ml

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ACKNOWLEGEMENT

I would like to thank Prof. Dr. med. Maik Gollasch for inviting me to work on this interesting project and his group in Berlin (especially Kirill Essin, Galyna Dubrovska and Johanna Schleifenbaum) for their help with the first steps on this way.

I am very thankful to my supervisor Prof. Dr. med. Rudolf Schubert for teaching me the myograph technique, data analysis, for detailed discussions and comprehensive help during the work at the dissertation.

I would like to thank also my colleges Torsten Gloe, Olga Zavaritskaya, Raissa Keppen and others for helping me with new ideas and in the case of technical problems during this study.

Many thanks go to my family and friends for the great support throughout my work at the project and during the process of writing the dissertation.

THESES

KCNQ channels are a family of membrane potassium channels which are widely expressed in many organs. These channels play an important role in a variety of widespread diseases; their pharmacological modulators are used as effective medications in the nervous system. Although it was already shown that they play a very important role in excitable tissues, there are not much data about KCNQ channels in the vascular system.

In our study we have tested the hypothesis that KCNQ channels are powerful targets for relaxation of small systemic and pulmonary arteries.

We investigated the distribution of these channels in blood vessels. Their expression was studied in *Gracilis* arteries using the PCR-method. We investigated also the functional role of KCNQ channels in vessel relaxations induced by the novel KCNQ channel opener VRX0530727, VRX0621238 and VRX0621688 on isometric preparations of intact *Gracilis* and *Intrapulmonary* arteries using wire myography. We have tested our hypothesis on male normotensive Wistar and spontaneously hypertensive SHR rats.

In rat *Gracilis* arteries we found mRNA expression for KCNQ1, KCNQ3, KCNQ4 and KCNQ5 channels. This artery, preconstricted with diverse agents (serotonin, phenylephrine or U-46619), showed different responses to KCNQ channel opener application, but VRX0621238 and VRX0621688 always produced vessel relaxation.

In serotonin-preconstricted *Gracilis* arteries relaxation by VRX0530727 was shown to be independent on the endothelium and sympathetic nerve endings. Vasodilation was mediated by KCNQ channels (proved in experiments with the specific KCNQ channel blocker XE-991).

The novel KCNQ channel opener and retigabine dilate also arteries from hypertensive rats in the same way like in normotensive rats.

KCNQ channel opener produce a relaxation in the pulmonary circulation resembling their effect on systemic arteries (we tested the effect of VRX0621238 and VRX0621688 on U-46619-preconstricted *Intrapulmonary* arteries).

Our results confirm and extend already existing data on the role of KCNQ channels in small arteries reported by previously by other groups.

Taking all our observations together we proved our hypothesis that KCNQ channels are powerful targets for relaxation of small systemic and pulmonary arteries. This conclusion is based on the following novel findings:

1. Representatives of the KCNQ channel family are present in small non-visceral arteries.

2. Preconstricted systemic and pulmonary arteries relax after the application of KCNQ channel opener. This relaxation is mediated by KCNQ channels.

3. Novel KCNQ channel opener relax also arteries from hypertensive animals. This additional finding demonstrates the premise that KCNQ channels may serve as therapeutic targets in the case of pathological hypertensive conditions.