Charles University in Prague

Faculty of Natural Sciences

Department of Physiology

The Role of Calcium Influx and Calcium Sensitization in Contraction of Isolated Arteries of Normotensive and Hypertensive Rat

Ph.D. Thesis

Mgr. Michal Bencze

The Czech Academy of Sciences

Institute of Physiology

Supervisor: MUDr. Josef Zicha, DrSc.

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Declaration

I hereby declare that this thesis has been written by me and comprises only my original work and that it has not been submitted elsewhere for the same or higher degree. Where other sources of information have been used, they have been acknowledged.

Date: …….

Signature: …….

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Abstrakt

Cévní odpor je dán především kontrakcí hladkého svalu cév, která je regulována fosforylací lehkého řetězce myosinu. Hladkosvalová cévní kontrakce je zahájena vstupem vápnatých iontů do buněk, který je zprostředkovaný kanály typu TRP (transient receptor potential) a napěťově závislými Ca^{2+} kanály typu L (L-type voltage-dependent Ca^{2+} channels, L-VDCC). Vápníková sensitizace je mechanismus zvyšující cévní kontraktilitu při dané úrovni intracelulárního vápníku pomocí RhoA/Rho kinázou zprostředkovanou inhibici fosfatázy lehkého řetězce myosinu. V této disertační práci prezentuji data 1) o úloze TRP kanálů v mechanismu kontrakce hladkého svalu cév, 2) o zvýšené kontraktilitě artérií spontánně hypertenzních potkanů (spontaneously hypertensive rats, SHR) a 3) o rozdílech v kontrakci artérií u normotenzních a hypertenzních potkanů, spojených s úlohou RhoA/Rho kinázové dráhy u třech modelů experimentální hypertenze (SHR, Ren-2 transgenní potkani a sůl-sensitivní Dahlovi potkani).

Ve studii věnující se TRP kanálům jsem porovnával vlivy třech běžně používaných neselektivních inhibitorů TRP kanálů (2-APB, SKF-96365 a FFA) na kontrakci izolované artérie. Z těchto inhibitorů byl nejzajímavější 2-APB, jehož inhibiční působení bylo závislé na typu kontrakčního stimulu a zároveň na přítomnosti sodíku v inkubačním roztoku. Ve studii týkající se zvýšené kontraktility artérií SHR potkana bylo prokázáno několik mechanismů, které za ni mohou být zodpovědné: vliv noradrenalinu uvolněného z vaskulárních varikosit, nedostatečné otevírání K^+ kanálů a především změna membránového potenciálu. Jak vstup vápnatých iontů, tak vápníková sensitizace přispívají k adrenergní kontrakci artérií. Vstup vápnatých iontů se zdá být důležitější u potkanů s genetickou hypertenzí (SHR a Ren-2 transgenní potkani), zatímco úloha vápníkové sensitizace je u nich snížená. Naopak úloha vápníkové sensitizace je zvýšená při kontrakci artérií u sůl-sensitivních Dahlových potkanů.

Lepší pochopení mechanismů hladkosvalové cévní kontrakce ve zdraví a nemoci je důležité pro vývoj budoucích léčiv.

Summary

Vascular resistance is mainly determined by the contraction of vascular smooth muscle (VSM), which is regulated by the phosphorylation of myosin light chain (MLC). VSM contraction is initiated by calcium influx into the VSM cells, which is mediated by transient receptor potential (TRP) channels and L-type voltage-dependent calcium channels (L-VDCC). On the other hand, calcium sensitization is a mechanism enhancing vascular contractile response at a given level of intracellular calcium by RhoA/Rho kinase pathway-mediated inhibition of myosin light chain phosphatase. In this thesis I present the data about i) the role of TRP channels in the mechanisms of vascular smooth muscle contraction, ii) enhanced contractility of arteries from spontaneously hypertensive rats (SHR), and iii) the differences in contraction of arteries from normotensive and hypertensive rats related to the role of RhoA/Rho kinase pathway in three types of experimental hypertension (SHR, Ren-2 transgenic rats and salt-sensitive Dahl rats).

In the study concerning TRP channels, I compared the effects of three commonly used non-selective TRP channels inhibitors (2-APB, SKF-96365, FFA) on isolated arteries. Among them 2-APB was the most interesting because the observed inhibitory effects of 2-APB were dependent on the type of contraction stimulus and also on Na^+ presence in bathing solution. In the study on enhanced contractility of SHR arteries the participation of several mechanisms was suggested to be responsible: the increased influence of norepinephrine vascular varicosities, insufficient opening of K^+ channels and especially altered membrane potential. Both calcium entry and calcium sensitization contribute to the adrenergic vasoconstriction of arteries. Calcium entry seems to be more important in rats with genetic hypertension (SHR and Ren-2 TGR), in which the role of calcium sensitization is attenuated. On the other hand, the role of calcium sensitization is enhanced in contraction of arteries in hypertensive salt-sensitive Dahl rats.

Better understanding of mechanisms of vascular smooth muscle contraction in health and disease might be important for future drug development.

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Publication A

Bencze M, Behuliak M, Vavřínová A, Zicha J. **Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat.** Eur J Pharmacol. 2015; 765: 533-540. IF=2.730

Publication B

Bencze M, Behuliak M, Vavřínová A, Zicha J. **Altered contractile responses of arteries from spontaneously hypertensive rat: The role of endogenous mediators and membrane depolarization.** Life Sci. 2016; 166: 46-53. IF=2.685

Publication C

Behuliak M, Pintérová M, Bencze M, Petrová M, Líšková S, Karen P, Kuneš J, Vaněčková I, Zicha J. **Ca^{2+} sensitization and Ca^{2+} entry in the control of blood pressure and adrenergic vasoconstriction in conscious Wistar-Kyoto and spontaneously hypertensive rats.** J Hypertens. 2013; 31: 2025-2035. IF=5.062

Publication D

Behuliak M, Vavřínová A, Bencze M, Polgárová K, Ergang P, Kuneš J, Vaněčková I, Zicha J. **Ontogenetic changes in contribution of calcium sensitization and calcium entry to blood pressure maintenance of Wistar-Kyoto and spontaneously hypertensive rats.** J Hypertens. 2015; 33: 2443-2454. IF=5.062

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Zicha J, Behuliak M, Pintérová M, Bencze M, Kuneš J, Vaněčková I. **The interaction of calcium entry and calcium sensitization in the control of vascular tone and blood pressure of normotensive and hypertensive rats.** Physiol Res. 2014; 63: 19-27. IF=1.643

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List of abbreviations

2-APB	2-aminoethoxydiphenyl borate
ATP	adenosine triphosphate
BK_{Ca}	big conductance Ca^{2+}-activated K^+ channel
BP	blood pressure
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
DAG	diacylglycerol
FFA	flufenamic acid
GEF	guanosine triphosphate exchange factor
GTP	guanosine triphosphate
IK_{Ca}	intermediate conductance calcium-activated potassium channel
IP_3	inositol trisphosphate
K_{IR}	inwardly rectifying potassium channel
KPSS	physiological saline solution with Na^+ substituted by K^+
L-VDCC	L-type voltage-dependent Ca^{2+} channels
NE	norepinephrine
NO	nitric oxide
MLC	myosin light chain
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKG	cGMP-dependent protein kinase
PLC	phospholipase C
RAS	renin-angiotensin system
Ren-2 TGR	Ren-2 transgenic rat
SHR	spontaneously hypertensive rat
SK_{Ca}	small conductance calcium-activated potassium channel
SKF	SKF-96365
SNS	sympathetic nervous system
SS/Jr	Dahl salt-sensitive rat
SR	saroplasmatic reticulum
SR/Jr	Dahl salt-resistant rat
TRP	transient receptor potential channel
TRPC	canonical transient receptor potential channel
VSM	vascular smooth muscle
WKY	Wistar-Kyoto rat

1. Introduction

1.1 Blood pressure regulation and hypertension

There are three main denominators of blood pressure (BP) - blood volume, cardiac output and vascular resistance. Blood volume is determined mainly by the action of kidneys. Cardiac output is the volume of blood pumped by the heart per unit time and it is mainly determined by end-diastolic volume, myocardial contractility and heart rate. Vascular resistance is mainly determined by arterial contraction and it is regulated by tissue metabolism, sympathetic activity and endothelium action. Taken together, brain, kidneys, heart and blood vessels all regulate blood pressure (Guyton, 1968; Henderson et al., 2010).

To maintain constant BP, all these BP denominators must be balanced by humoral and autonomic nervous systems. One of the most important humoral systems regulating blood pressure and fluid homeostasis is renin-angiotensin aldosterone system (Crowley and Coffman, 2012). Autonomic nervous system regulates blood pressure via sympathetic and parasympathetic nerves that innervate blood vessels, heart and kidneys. Adrenal medulla as a major source of catecholamines also participates in blood pressure control. The arterial baroreceptors control sympathetic nerve activity and have a dominant role in both short-term and long-term BP regulation. The sympathetic nerve activity at rest is presumed to be the most crucial parameter for long-term BP control. In the central nervous system (CNS) the important role for BP regulation is played by a core network of neurons that reside in the hypothalamus, the nucleus of the solitary tract and the rostral ventrolateral medulla. All these centres are responsible for the long-term BP control. Limbic, cortical and midbrain structures are responsible for the short-term changes in sympathetic tone, related to behaviour and stress (Guyenet 2006). Endothelium, consisting of a layer of cells lining the lumen of blood vessels, is an effective force balancing vasoconstrictor stimuli. It is an important source of nitric oxide (NO) and other relaxing factors (Deanfield et al., 2007). Attenuated function of endothelium or enhanced function of any vasoconstrictor system (contributing to BP

maintenance discussed above) lead to impaired BP balance and might result in hypertension.

Hypertension is, by definition, a chronic elevation of the 24-hour average BP (Mancia et al., 2013). Arterial hypertension represents a global problem in the developed societies. The overall prevalence of hypertension is around 30–45% and is increasing with ageing of the population. Hypertension is associated with the incidence of several cardiovascular complications such as stroke, myocardial infarction or renal failure (Mancia et al., 2013). These events are referred as end-organ damage and may be the actual cause of death. Cardiovascular diseases are now responsible for 30% of all death causes worldwide. The recent rapid rise in the mortality by cardiovascular disease is attributable mainly to the changes in environmental risk factors such as unhealthy diet and low physical activity (Kearney et al., 2005). Essential (primary) hypertension is defined as high BP in patients in which the causes of secondary hypertension (such as renovascular disease, renal failure, hyperaldosteronism, etc.) or monogenic forms were excluded. Essential hypertension accounts for 95% of all cases of hypertension and its causes are still elusive. The causal factors that lead to high BP may differ in this heterogeneous disorder (Carretero et al., 2000).

Essential hypertension is considered as multifactorial disorder, which might be closely dependent on the function of central nervous system (CNS) and its neuroendocrine influences. Alterations in CNS function might be triggered by psychosocial challenges characteristic for modern lifestyle, commonly referred to as stress (Folkow 2001). Stress might be viewed as a condition characterized by enhanced patterned response of its effectors. Various stressors elicit different patterns of activation of the sympathetic nervous, adrenomedullar, hypothalamic-pituitary-adrenocortical and other effector pathways, with negative feedback loops (Goldstein et al., 2007). Since high BP is usually without any symptoms, the early diagnosis is crucial in the prevention of above mentioned complications. A considerable number of effective antihypertensive drugs are available, but due to the multifactorial nature of primary hypertension, most of the patients need two or more drugs (and even that might not be sufficient for long-term control of hypertension). Appropriate lifestyle changes are also necessary for successful management of hypertension (Mancia et al., 2013).

1.2 Arterial contraction and relaxation

Contractile state of arteries, which is a result of vasoconstrictor and vasodilator processes in vascular smooth muscle, most importantly contributes to vascular resistance. The contraction of small arteries is highly regulated by the sympathetic nervous system. Several structures of the CNS are responsible for the maintenance of sympathetic tone and blood pressure (Guyenet 2006). Sympathetic neurons end in arterial wall as sympathetic varicosities – globular endings of neurons from which transmitters are released (Astrand and Stjärne, 1989). Norepinephrine, along with ATP, released from sympathetic varicosities initiates the contraction through the mechanisms associated with propagating Ca^{2+} waves (Lamont et al., 2003). Sympathetic neurons also activate transcellular negative feedback, elevating endothelial Ca^{2+} signals to oppose vasoconstriction (Nausch et al., 2012).

Adrenergic receptors (adrenoceptors) are the sites through which norepinephrine and epinephrine act both in the periphery and in the central nervous system. There are nine subtypes of adrenoceptors α_{1A}, α_{1B}, α_{1D}, $\alpha_{2A/D}$, α_{2B}, $\alpha_{2A/D}$, β_1, β_2, and β_3. α_1-adrenoceptors are located in the vicinity of sympathetic nerve terminals to be activated by norepinephrine coming out from the nerves, whereas α_2-adrenoceptors are situated extrajunctionally to be activated preferentially by circulating catecholamines. A similar differential location was also observed among β-adrenoceptors. β_1-adrenoceptors that are very responsive to norepinephrine mediate responses to sympathetic nerve activity, whereas β_2-adrenoceptors, that are insensitive to norepinephrine, function as receptors for epinephrine from the adrenal medulla (Guimarães and Moura 2001).

Along with norepinephrine and ATP, further vasoconstricting agents contributing to long-term BP regulation are angiotensin II, arginine-vasopressin, endothelin, neuropeptide Y and many others. All these substances together create important connection between arterial contraction and blood pressure. Neuropeptide Y, released by sympathetic activity, is responsible for a prolonged contraction of blood vessels (Zukowska et al., 2003). Arginine vasopressin (AVP) regulates blood pressure and fluid volumes by exerting diverse functions. AVP receptor (V_{1A})–deficient mice are hypotensive due to blunted AVP-induced vasopressor response, impaired arterial baroreceptor reflex, decreased sympathetic nerve activity and

decreased blood volume (Aoyagi et al., 2009). V_{1A} receptors are found in VSM and cause vasoconstriction. V_{1B} receptors act in pituitary gland where they activate several signaling pathways. V_2 receptors regulate water excretion from the kidney by increasing the osmotic water permeability of the renal collecting duct by inducing signalling pathway causing insertion of aquaporine-2 water channels into apical membrane of the collecting duct principal cells (Holmes et al., 2003). The renin-angiotensin system through its effectors exerts fundamental control over sodium and water handling in the kidney, its deregulation leads to blood pressure elevation with renal and cardiovascular damage. The angiotensin receptors can be divided into two types: AT_1 and AT_2. The most of the classically recognized functions of the renin-angiotensin system are mediated by AT_1 receptors - release of aldosterone from the adrenal glomerulosa, vascular smooth muscle contraction, stimulation of hypothalamic thirst sensors, regulation of tubuloglomerular feedback, and stimulation of renal tubular sodium reabsorption (Crowley and Coffman, 2012). AT_2 receptors are characterized by the effects different from those of the AT_1 receptors, which include anti-inflammation, anti-fibrosis and anti-apoptosis (Namsolleck et al., 2014).

Endothelin also regulates cardiac output, central and peripheral nervous system activity, renal sodium and water excretion, systemic vascular resistance, and venous capacitance. Endothelin receptors ET_A and ET_B often have opposing actions - ET_A are particularly predominant in vascular smooth muscle and cardiomyocytes, while ET_B is found in endothelial cells and renal tubules (Kohan, 2011). Thus, ET_A mediate vasoconstriction whereas ET_B cause vasodilation.

Other vasoactive substances, playing an important role in the local regulation of arterial contraction are metabolites derived from arachidonic acid - vasoactive eicosanoids and prostaglandins. These substances are produced both in the vascular smooth muscle and also in the endothelium. The main vasoconstrictor prostanoid is thromboxane A_2. In most systemic vascular beds, the vasodilator prostanoids produced are prostacyclin (PGI_2) and prostaglandin E_2 (Sellers and Stallone 2008). In endothelium, nitric oxide and endothelial hyperpolarizing factor represent opposing forces to contractile stimuli (Furchgott and Zawadzki 1980; Ignarro et al., 1987; Chen et al., 1988). Nitric oxide, which is released from the endothelium, stimulates soluble guanylyl cyclase in vascular smooth muscle, producing cyclic guanosine monophosphate (cGMP), which interacts with cGMP-dependent protein kinases, that decrease intracellular calcium and cause vascular

relaxation (Tousoulis et al., 2012). An increase in the endothelial Ca^{2+} concentration and the consequent activation of endothelial SK_{Ca} and IK_{Ca} channels, which elicits the hyperpolarization of the endothelial cells is known as endothelial hyperpolarizing factor-mediated response. The endothelial hyperpolarization then spreads to the adjacent smooth muscle cells through myo-endothelial gap junctions and the efflux of K^+ through the endothelial SK_{Ca} and IK_{Ca} channels elicits the hyperpolarization of the surrounding myocytes by activating K_{IR} channels and/or the Na^+-K^+-ATPase. Several proposed mechanisms are reviewed by Busse et al. (2002). As local vasodilators inflammatory substances such as histamine and bradykinin may also function. Their systemic release does not significantly influence the total blood flow but their local elevation can evoke marked alterations of blood flow in distinct vascular beds (Richardson et al., 1977).

Blood vessels also respond to transmural pressure elevation with constriction – this is so called myogenic response. In contrast, blood vessels after pressure reduction dilate. Myogenic response of arteries is inherent to smooth muscle, independent of neural, metabolic, and hormonal influences. The most important physiological role of myogenic response is the establishment of basal vascular tone and the autoregulation of blood flow (Davis and Hill 1999).

1.3 Vascular smooth muscle contraction

Vascular smooth muscle (VSM) contraction is regulated by the activation of contractile proteins myosin and actin. Adrenergic vasoconstriction in small arteries involves Ca^{2+} entry through voltage-dependent channels and receptor-operated channels, as well as Ca^{2+} sensitization mechanisms mediated by protein kinase C, tyrosine kinase, and Rho kinase (Villalba et al., 2007). Binding of Ca^{2+} to calmodulin leads to the activation of myosin light chain kinase (MLCK) and phosphorylation of myosin light chain, which enables molecular interaction of myosin and actin. Energy released from ATP by myosin ATPase enables the cycling of the myosin cross-bridges with actin for contraction (Webb 2003). VSM contraction is also influenced by calcium sensitization that acts by altering the Ca^{2+} sensitivity of the contractile system by Rho/Rho kinase signalling pathway. In other words, Ca^{2+} sensitization reflects the level of inhibition of myosin light chain phosphatase, which dephosphorylates myosin light chain and leads to the relaxation of VSM. Thus, the contractile force is mainly determined by the amount of phosphorylated myosin light chain which is regulated by the balance between the activities of the MLC kinase and the MLC phosphatase (Somlyo & Somlyo, 2003).

Smooth muscle cell contraction is controlled by Ca^{2+} signalling pathways. Transmembrane Ca^{2+} entry is supported by agonist-activated non-voltage-gated Ca^{2+}-permeable ion channels that are activated either by PLC-mediated signalling cascade or Ca^{2+} store depletion. PLC-related channels are named receptor-operated calcium channels, while the others are referred as store-operated calcium channels, activated after intracellular Ca^{2+} store depletion. During the contraction-generation the primary drive for the rise in intracellular calcium (and thus for VSM contraction) is membrane depolarization, with the consequent opening of voltage-dependent calcium channels (McFadzean et al., 2002). Among various types of smooth muscles the mechanisms generating Ca^{2+} signals might vary. In general, inward Ca^{2+} current activates Ca^{2+} release from endoplasmatic reticulum, which in turn leads to the contraction of smooth muscle (Berridge 2008). However, in VSM the role of store-operated Ca^{2+} entry (to charge up stores of calcium in endoplasmatic reticulum) seems to play a minor role (Trebak et al., 2013). For the depolarization of smooth muscle cell membrane and vasoconstriction, inward Ca^{2+} current through receptor-

operated calcium channels (such as TRPC3) might be sufficient (Xi et al., 2008). α_1-adrenoceptor activation in VSM cells leads to the synchronous recruitment of individual VSM cells, followed by the development of asynchronous Ca^{2+} waves between these cells (Zang et al., 2001). After the removal of extracellular Ca^{2+} NE leads only to a short-term phasic α_1-adrenoceptor-induced contraction of isolated arteries, in agreement with the suggested importance of extracellular Ca^{2+} for contraction of VSM. Restoration of extracellular Ca^{2+} levels to physiological concentration during this α_1-adrenoceptor stimulation leads to the immediate restoration of tonic contraction (Paulis et al., 2007).

In vascular smooth muscle another signalling pathway is relevant during the processes of smooth muscle remodelling. Protein stromal interaction molecule 1 (STIM1) functions as Ca^{2+} store sensor located in the membrane of endoplasmic reticulum and the plasma membrane calcium release-activated calcium channel protein 1 (Orai1) functions as the store-operated channel. Activation of the STIM1/ORAI1 pathway after Ca^{2+} store depletion leads to the subsequent activation of gene transcription programs (Trebak 2012). Proliferative VSM cell phenotype is characterized by long-lasting intracellular Ca^{2+} oscillations. The switch from contractile to proliferative phenotype of VSM cells is accompanied by a change from voltage-dependent Ca^{2+} entry into voltage-independent Ca^{2+} entry (Misárková et al., 2016).

Another channels importantly contributing to the regulation of smooth muscle tone are K^+ and Cl^- channels. K^+ channels are dominant ion channels expressed in the plasma membrane of arteriolar smooth muscle cells and their currents contribute substantially to the membrane potential in VSM cells. Even small changes in membrane K^+ current produce significant changes in membrane potential, which regulates the activity of voltage-dependent Ca^{2+} channels. Due to of the K^+ electrochemical gradient, the opening of K^+ channels leads to K^+ efflux from cells and membrane hyperpolarization. This closes voltage-dependent Ca^{2+} channels, thus reducing intracellular Ca^{2+} and leading to vasodilatation. Conversely, the closure of opened K^+ channels causes membrane depolarization, opening of Ca^{2+} channels and leading to Ca^{2+}-dependent vasoconstriction. At least four different classes of K^+ channels are expressed in VSM cells: inward rectifier K^+ channels, ATP-sensitive K^+ channels, Ca^{2+}-activated K^+ channels, and voltage-activated K^+ channels (Jackson 2005). In contrast to K^+ channels, Cl^- channel activation result in

Cl^- efflux, leading to membrane depolarization and L-VDCC activation. Cl^- channels in VSM might be divided into five families: transmembrane protein 16/anoctamin, bestrophins, voltage-gated Cl^- channels, cystic fibrosis transmembrane conductance regulator, and ligand-gated Cl^- channels. The recently discovered expression of these families of Cl^- channels in VSM cells suggest that these channels might also contribute to the control of contractility and proliferation of VSM cells (Bulley et al., 2014).

1.3.1 Vascular smooth muscle cell signalling pathways

Activation of guanine nucleotide-binding proteins (G-proteins) and their respective receptors leads to the activation of PLC, which produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Biphasic increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) then follows - IP_3 causes the release of Ca^{2+} from intracellular stores, while DAG contributes to the membrane depolarization and continuous Ca^{2+} entry across the plasma membrane (Berridge, 2008).

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) are signal transducers that connect receptors of many signalling molecules to their effectors. The extracellular signals are received by G-protein-coupled receptors with seven transmembrane regions that enable the G-proteins to hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP). G proteins consist of three subunits (α, β, and γ). α-subunits have been divided into four groups by their sequence similarities and their classification also defines the G-protein (Neves et al., 2002).

Multiple G-protein-coupled pathways control VSM contraction. Norepinephrine signalling via the α_1-adrenoceptor, stimulates $G_{q/11}$-protein pathway which activates the membrane-bound PLC, cleaves PIP_2 into IP_3 and diacylglycerol. This process leads to the elevation of intracellular calcium $[Ca]_i$ – a key step required for the contraction of VSM cells. Another contracting mechanism – Rho/Rho kinase pathway is mediated by a family of guanine nucleotide exchange factors (GEFs), coupled with $G_{12/13}$-protein pathway. The G_S pathway produces cAMP from ATP by direct stimulation of the membrane-associated adenylate cyclase which activates protein kinase A, while G_I-protein pathway inhibits cAMP production from ATP by inhibiting

adenylate cyclase activity. β-adrenoceptor subtypes signal by coupling to the stimulatory G_S-protein pathway but in some circumstances they might also couple to inhibitory G_i-protein pathway (Bastin et al., 2011; Guimarães and Moura 2001).

Monomeric G-proteins of the Ras superfamily are also important for the VSM cell and its functions. These essential elements in the regulation of numerous cardiovascular functions comprise more than 100 members, structurally classified into five families: the Ras, Rho, Rab, Arf, and Ran. Hormones, cytokines, and growth factors activate Ras proteins through the stimulation of plasma membrane receptors. This activation involves receptors tyrosine kinase and specific GEFs. The most studied members of the Ras homologous (Rho) family of small GTPases are RhoA, Rac1, and Cdc42. Rho subfamily proteins act as molecular switches within extracellular factor-activated intracellular signalling pathways that regulate multiple cellular functions (Loirand et al., 2013).

Cytoplasmic signals produced by Ca^{2+} fluxes in vascular smooth muscle can be classified into five categories: Ca^{2+} waves, junctional Ca^{2+} transients, Ca^{2+} sparks, Ca^{2+} puffs, and L-type Ca^{2+} channel sparklets (Amberg et al., 2013). Ca^{2+} waves are produced by Ca^{2+} release via IP_3 and ryanodine receptors located in the sarcoplasmic reticulum. Ca^{2+} sparks are localized Ca^{2+} microdomains produced specifically by the opening of ryanodine receptors located in sarcoplasmic reticular membranes, while Ca^{2+} puffs are localized Ca^{2+} microdomains produced by the opening of sarcoplasmic reticulum IP_3 receptors. Ca^{2+} sparklets are distinct Ca^{2+} microdomains produced by plasmalemmal Ca^{2+}-permeable channels. Short-lived Ca^{2+} influx events evoked by sympathetic stimulation of vascular smooth muscle cells are defined as junctional Ca^{2+} transients. L-type voltage-dependent Ca^{2+} channels (L-VDCC) are the main source of Ca^{2+} influx in vascular smooth muscle cells. Adrenergic stimulation decreases the frequency of Ca^{2+} sparks, while it increases simultaneously the frequency of asynchronous propagating Ca^{2+} waves in which different sarcoplasmic Ca^{2+}-release channels are involved. IP_3 receptors are essential for adrenergically induced asynchronous Ca^{2+} waves and the associated steady vasoconstriction, while ryanodine receptors are not opened during adrenergic activation and IP_3 receptors are not essential for Ca^{2+} sparks (Lamont et al., 2004).

1.3.2 Transient receptor potential channels

Transient receptor potential (TRP) channels are defined by their molecular homology rather than by their ligand function or selectivity. Their functions are often unknown. All TRP channels consist of six-transmembrane polypeptide subunits that assemble as tetramers to form cation-permeable pores. Mammalian TRP channels comprise six related subfamilies – canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP) and mucolipin (TRPML) (Clapham et al., 2003). In vascular smooth muscle 10 types of TRP channels have emerging function: TRPC1, TRPC6, TRPC3, TRPC4/5 and also TRPP2/1 TRPV2, TRPM4 and TRPM7 (Beech et al., 2005; Early and Brayden 2015). Members of the TRPC subfamily are molecular candidates for non-selective cationic channels activated by G-protein-coupled receptors in various tissues, including smooth muscle. The role of TRP channels is also considered in vascular smooth muscle myogenic response. The increase in active force development as a result of stretch-induced Ca^{2+} influx occurs in part via a nifedipine-resistant pathway through non-voltage gated stretch-activated cation channels (Davis et al., 1992).

TRPC channel subunits form heterotetramers, especially TRPC1/4/5 and TRPC3/6/7 subfamilies. In VSM cells, TRPC1/4/5 might be involved in Ca^{2+} entry via store-operated channels (Flemming et al., 2003; Tai et al., 2008; Lindsey et al., 2008; Xu et al., 2006). TRPC3/C6/C7 are the candidates for receptor-operated channels that mediate Ca^{2+} entry stimulated by a G-protein-coupled receptors (Boulay et al., 1997). Receptor-activated opening of these channels is supported by the observations that diacylglycerol is able to activate these channels (Hofmann et al.,1999; Okada et al. 1999; Imai et al., 2012). In addition, TRPC6 channels can be negatively regulated by the NO–cGMP–PKG pathway which may serve as vital mechanism in blood pressure lowering and maintenance of local blood flow (Takahashi et al., 2008).

Protein kinase C activity causes smooth muscle depolarization and vasoconstriction by increasing the number of TRPM4 channels in the sarcolemma (Crnich et al., 2010). TRPP2 contributes to the cerebral artery myogenic response and also performs different functions in various vascular beds (Narayanan et al., 2013). TRPM4 channel is a mediator of pressure-induced membrane depolarization and arterial constriction in response to intraluminal pressure (Gonzales et al., 2010).

1.3.3 L-type voltage-dependent channels

L-type voltage-dependent Ca^{2+} channels (L-VDCC) mediate the influx of Ca^{2+} into the cell in response to membrane depolarization. These channels are protein complexes composed of pore-forming α_1 and auxiliary subunits modulating biophysical properties ($\alpha_2\delta$ and β). Similarly, other types of calcium channels such as N-type, P/Q-type, and the R-type are also built of such three subunits. In contrast, T-type channels seem to consist only of an α_1 subunit (Hofmann et al., 2014). There are four genes encoding $\alpha_2\delta$ subunits of calcium channel ($\alpha_2\delta$-1 to $\alpha_2\delta$-4), but in VSM cells only $\alpha_2\delta$-1 is expressed. Targeted blockade of $\alpha_2\delta$-1 demonstrated its essential function in channel trafficking and an important role of this subunits in the modulation of channel activity (Bannister et al. 2009).

Inactivation of the L-type $Ca_V1.2$ Ca^{2+} channel gene in mice reduced mean arterial blood pressure, phenylephrine- and angiotensin-II-induced BP changes, showing that these channels are the key players in the hormonal regulation of blood pressure and development of myogenic tone (Moosmang et al., 2003). In VSM cells the closure of L-type Ca^{2+} channels may also be caused by Ca^{2+} sparks, stimulating nearby big conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels that hyperpolarize the VSM cell cytoplasmic membrane. Thus L-VDCC channels contribute to global rise of cytosolic [Ca^{2+}], which in turn influences luminal SR calcium and thus Ca^{2+} sparks (Essin et al., 2007).

L-VDCC inhibitors can be divided into three classes with different chemical structures: phenylalkylamines (e.g. verapamil), benzothiazepines (e.g. diltiazem) and dihydropyridines (e.g. nifedipine). These compounds reduce blood pressure more effectively in hypertensive than in normotensive subjects. Michiels et al. (2014) demonstrated that L-VDCC inhibitors have a higher apparent affinity for non-inactivated open L-VDCC. The relationship of Ca^{2+}-dependent myosin light chain kinase (MLCK) and L-VDCC was studied by Martinsen et al., (2014). MLCK as the activator of smooth muscle contraction is involved in Ca^{2+} regulation of VSM in resistance arteries via the control of the transcription of $Ca_V1.2$ gene in vascular smooth muscle cells. Low concentrations of intracellular cAMP produce modest increases in Ca^{2+} channel activity, whereas cGMP and higher concentrations of cAMP result in the inhibition of L-VDCC activity in VSM cells. The observed similarities of effects induced by cGMP and high concentrations of cAMP suggest a

common mechanism, possibly involving the activation of cGMP-dependent protein kinase (Ishikawa et al., 1993). High blood pressure upregulates the L-VDCC α_1C subunit in VSMCs *in vivo* where membrane depolarization is a potential signal involved in this interaction that may contribute to the development of abnormal vascular tone (Pesic et al., 2004). Down-regulation of voltage-gated K^+ channels leads to membrane depolarization in VSM cells, which is partially counteracted by upregulation of BK_{Ca} channels (Cox and Rusch 2002). Transcription of L-VDCC in vascular smooth muscle cells might be controlled also by MLCK. MLCK protein has several binding domains, which, by interacting with particular targets, might be responsible for the alterations in Ca^{2+} responses and gene expression (Martinsen et al., 2014).

1.3.4 Ca^{2+} sensitization of vascular smooth muscle cells

Ca^{2+} sensitization of myosin contractile apparatus is the mechanism enhancing vascular contraction at a given level of cytosolic calcium. As already mentioned, this process is signalled via RhoA/Rho kinase pathway which inhibits the dephosphorylation of MLC by inactivation MLCP (Somlyo and Somlyo 2000; Shimokawa et al., 2016). MLCP is composed of a 38-kDa catalytic subunit of type 1 protein phosphatase (PP1c) and 110-kDa and 20-kDa non-catalytic subunits. The N-terminal region of the 110-kDa subunit binds to PP1c, while the C-terminal region binds to the 20-kDa subunit. These subunit interactions help to form the heterotrimeric holoenzyme of MLCP. The 110-kDa subunit also binds to myosin, targeting PP1c to the myosin filaments. This subunit is thus referred to as the myosin phosphatase target subunit 1 (MYPT1). CPI-17 is the endogenous inhibitory protein of the type 1 protein phosphatase and exerts its inhibitory effect on MLCP when it is phosphorylated (Hirano, 2007). Diverse signals converge on the active site of MLCP through the direct interaction with phosphorylated MYPT1 and CPI-17. The interaction between the active site of MLCP with the inhibitory phosphorylation sites is the determinant of Ca^{2+} sensitivity of VSM contraction (Khromov et al., 2009). RhoA-specific GEF (p63RhoGEF) selectively couples $G_{q/11}$ but not $G_{12/13}$, to RhoA activation in blood vessels and cultured VSM cells and thus mediates the physiologically important Ca^{2+} sensitization of force induced with $G_{q/11}$-coupled agonists (Momotani et al., 2011). Agonists acting via G_i-coupled receptors sustain

contraction dependent on CPI-17 and MLC_{20} phosphorylation by integrin-linked kinase (Huang et al., 2006). Specific signaling components such as proline-rich tyrosine kinase 2 and PDZ-RhoGEF are sufficient to activate Rho kinase pathway through Ca^{2+} signaling, necessary for constrictor responses (Ying et al., 2009). These two components of the inhibition of MLC phosphatase might have different time onset. During the onset of VSM contraction the initial rapid Ca^{2+} rise induces a rapid inhibition of MLC phosphatase by Ca^{2+}-dependent phosphorylation of the MLCP inhibitor protein CPI-17, associated with the activation of Ca^{2+}-induced MLC kinase to initiate a rapid MLC phosphorylation. During sustained contraction, slower RhoA/Rho kinase pathway involving MYPT1 is activated (Dimopoulos et al., 2007).

The RhoA/Rho kinase pathway activation induces translocation of RhoA to the plasma membrane within caveolae, where the interaction of RhoA with caveolin-1 leads selectively to the activation of a Rho kinase-dependent force development (Dubroca et al., 2006). In the absence of caveolin-1, protein kinase C-driven arterial contraction might be even increased (Shakirova et al., 2006). cAMP inhibits Ca^{2+}-mediated activation of the Rho kinase pathway in a manner independent of the Ca^{2+}-lowering effect of cAMP. The actions of cAMP on RhoA and MLCP appear to involve the inhibition of RhoA regulator phosphoinositide 3-kinase (Azam et al., 2007). Excitatory agonists might induce RhoA activation in an agonist-specific manner (Sakurada et al., 2001). Rho kinase pathway activation leads also to an increase in actin polymerization contributing to force generation in the myogenic response of skeletal muscle arterioles and regulates actin polymerization activated by α_1-adrenoceptors (Tsai et al., 2006; Moreno-Domínguez et al., 2013). Ca^{2+} sensitization was reported to be connected with L-VDCC opening. Depolarization-evoked sustained activation of RhoA/Rho kinase pathway and myocyte contraction might not depend only on the change in the membrane potential itself or on the release of Ca^{2+} from the SR, but they require also the simultaneous activation of L-VDCC and the downstream stimulation of a metabotropic pathway, leading to IP_3 synthesis and Ca^{2+} release (Fernández-Tenorio et al., 2011). Ca^{2+} sensitization contributes to myogenic control of arterial diameter in the cerebral vasculature and serotonin-evoked vasoconstriction in the presence of pressure-induced myogenic activation (Johnson et al., 2009; El-Yazbi et al., 2010). In cultured VSM cells, both IP_3-stimulated Ca^{2+} release and Rho kinase pathway are important, while either Ca^{2+}-influx or CPI-17 signalling is downregulated (Woodsome et al., 2006). Enhanced

RhoA/Rho kinase signalling was also demonstrated during hypertension at the level of small resistance mesenteric arteries in rats with angiotensin II-induced hypertension (Hilgers et al., 2007).

There are numerous approaches how to study calcium sensitization in the rat. They range from cellular studies over vascular myography up to blood pressure response to Rho kinase inhibition in living animals. The latter measurements based upon the use of various Rho kinase inhibitors are complicated by the influence of endogenous vasoconstrictors (angiotensin II, norepinephrine) or vasodilatators (NO) which augment or lower BP response to acute Rho kinase inhibition reflecting so called *actual calcium sensitization*. Moreover, each blood pressure decrease activates arterial baroreflex operation through which the activation of sympathetic nervous system leads to the increase of both cardiac output and systemic resistance, which diminish the observed blood pressure changes. In conscious rats, there are two possibilities how to evaluate the contribution of calcium sensitization to blood pressure maintenance in conscious rats (Zicha et al., 2014a). Rho kinase inhibitors can be administered either to intact animals or to animals in which certain endogenous vasoactive systems are blocked (Brunová et al., 2015). The data obtained in intact animals inform about the actual impact of calcium sensitization on BP maintenance (*actual calcium sensitization*), whereas the measurements in animals with blocked pressor systems (RAS, SNS) report on so called *basal calcium sensitization* (for details see Publications C and E).

1.4 Rat models of hypertension

Hypertension is a multifactorial disease involving complex interactions between genetically determined mechanisms and environmental factors. To study such a complex disease, different experimental models of hypertension have been developed. These models share many common features to human hypertension. The understanding of different animal models of hypertension represents an important step in the research on the human hypertension and development of new drug treatments. Among variety of animal hypertensive models the rats are by far the most often used research species. Selected rat models participating in our research will be described further in this chapter – for more complex reviews see Pinto et al., (1998), Lerman et al., (2005), Dornas and Silva (2011). Rat models of experimental hypertension may be divided into genetic and non-genetic forms of hypertension. Genetic models of hypertension are developed by selective breeding of animals with desired phenotype over several generations to fix the trait. The best example of such model is spontaneously hypertensive rat (SHR) with polygenic hypertension (Okamoto and Aoki 1963). Genetic models may also be created by single genetic intervention – for example by the introduction and over-expression of mouse Ren-2 gene in the rat (transgenic rats over-expressing mouse Ren2 gene; TGR) (Mullins et al., 1990) or by the knockout of gene(s) in systems involved in the regulation of vascular tone. These models are examples of specific forms of hypertension. Induced rat models of hypertension are caused for example by kidney artery stenosis (Leenen and de Jong 1971), mineralocorticoid administration (Garwitz and Jones 1982), high-sodium diet superimposed on the appropriate genetic background (Dahl 1972) or high-fructose diet inducing both insulin resistance and hypertension (Hwang et al., 1987).

1.4.1 Spontaneously hypertensive rats

The genetic hypertension in SHR is generally characterized by an increased activity of sympathetic nervous system (SNS) and by alterations in Ca^{2+} homeostasis in vascular smooth muscle cells (Head et al., 1989; Asano 1993; Pintérová et al., 2011). The initiation of hypertension development by neural factors is evident

because SHR hypertension could be prevented by sympatho-adrenal inactivation in early life (Lee et al., 1991; Korner et al. 1993), when hypertension becomes more severe non-neural mechanisms account for much of the rise in BP (Korner 2010). Our group previously confirmed enhanced activity of SNS contributing to the maintenance of hypertension in SHR through G_i-protein/cAMP-coupled pathway resulting in the increased calcium influx through L-VDCC (Pintérová et al. 2010). In contrast to major enhancement of vasoconstrictor systems in SHR, only a moderate augmentation of vasodilator systems was observed. Thus, the overall activity of vasodilator systems cannot match sufficiently the augmented vasoconstriction in this hypertensive strain. Behuliak et al. (2011) showed a considerable compensatory activation of vasodilator prostanoids and Ca^{2+}-activated K^+ channels in SHR, but this was not a case of NO bioavailability. Pintérová et al. (2014) showed increased activity of BK_{Ca} and voltage-gated potassium channels in SHR.

There is an intensive search for specific genes leading to hypertension in SHR. Arteries from SHR expressed higher mRNA and protein levels for α_{1C} subunit of L-VDCC than WKY arteries. Developed anomalous Ca^{2+}-dependent vascular tone might be attributed to the increased numbers of L-VDCC pores created by α_{1C} subunits (Pratt et al., 2002).

As mentioned above, calcium entry is mediated also through various receptor-operated channels which may be represented by certain subfamilies of TRP channels. A relatively great attention has been paid to the role of TRPC3 channels in hypertension, including their relationship to agonist-induced vascular contraction. Alterations of TRPC3 channel expression might lead to a greater smooth muscle depolarization, L-VDCC activation, and vascular contractility in SHR (Noorani et al. 2011). Liu et al. (2005) showed increased TRPC3 channel expression and increased TRPC3-related calcium influx in monocytes from SHR. Later, they also showed that increased store-operated and second messenger-operated calcium influx through TRPC3 channels in monocytes from SHR may be responsible for promoting vascular disease in this rat strain (Liu et al., 2007). TRPC3 together with increased TRPC1 and TRPC5 expression in mesenteric arterioles were suggested to be responsible for the increased vasomotion in SHR (Chen et al., 2010). Endothelin stimulates physical coupling of IP_3 receptors to TRPC3 channels in mesenteric artery myocytes, leading to vasoconstriction. Furthermore, the enhancement of IP_3 receptor coupling to TRPC3 channels augments ET-1-induced vasoconstriction in SHR (Adebiyi et al.,

2012). As mentioned before, Cl^- channels are also important for maintenance of normal vascular tone. Contribution of Cl^- channels to norepinephrine-induced contraction in SHR diminish with age, hypertension development, and/or NO synthesis inhibition and their impaired opening might be a part of pathological mechanisms (Líšková et al., 2014).

It should be noted that endothelial dysfunction also plays an important role in the pathogenesis of genetic hypertension in SHR (Watt and Thurston 1989; Tominaga et al., 1994). Although endothelial dysfunction is usually associated with decreased NO bioavailability (Cosentino et al., 1998; Hamilton et al., 2001), it was recently demonstrated that NO-independent component of endothelial relaxation is reduced in SHR arteries (Púzserová 2013; Púzserová 2014).

1.4.2 Ren-2 transgenic rats

Ren-2 transgenic rats (Ren-2 TGR) are characterized by severe hypertension, lethal for the homozygous rats (for review see Lee et al. 1996). This transgenic rat model of hypertension was created by the introduction of the murine Ren-2 gene into the germ line of Hannover Sprague Dawley rats. The expression of the transgene leads to fulminant hypertension in the resulting transgenic rat strain (Mullins et al., 1990). This hypertension model may be treated with ACE inhibitors (Lantelme et al., 1996), a direct renin inhibitors (Rakušan et al. 2010), but the exact mechanism underlying hypertension remains still elusive. The role of a locally activated renin angiotensin system in the blood vessel wall might be also involved in the pathogenesis of vascular hypertrophy in this rat strain (Brosnan et al., 1999).

The information of cell calcium handling and its relationship to VSM contraction in Ren2 TGR is very scarce, so that no detailed conclusions can be made at the moment. Nevertheless, our *in vivo* studies demonstrated increased BP response to acute L-VDCC blockade by nifedipine in both homozygous and heterozygous Ren2 TGR (Zicha et al. 2014b, Vaněčková et al. 2015), suggesting enhanced calcium influx through L-VDCC in resistance vessels of these hypertensive strains. Angiotensin II increases the expression of TRPC6, which represent another way for calcium influx as discussed before (Nijenhuis et al. 2011; Kunert-Keil et al. 2013).

Desensitization of soluble guanylyl cyclase to NO might lead to vascular dysfunction. Hypertension and an overactive renin–angiotensin system contribute to

desensitization of soluble guanylyl cyclase in hypertensive Ren-2 TGR rats. Decreased sensitivity of vascular soluble guanylyl cyclase to NO in Ren-2 TGR rats was only partially reversed by blood pressure reduction with L-VDCC blocker amlodipine but it was completely normalized after the inhibition of renin-angiotensin system (Jacke et al., 2000). Selective blockade of 20-HETE formation and/or soluble epoxide hydrolase activity, when applied chronically in young prehypertensive Ren-2 TGR, suppressed the development of hypertension and provided a substantial protection from the associated cardiac hypertrophy, proteinuria and glomerulosclerosis (Čertíková Chábová et al., 2011). Inhibition of platelet-derived growth factor receptor-β improved both heart and kidney function, suggesting that this inhibition might have broad blood pressure-independent protective effects against end-organ damage in Ren-2 TGR rats (Schellings et al., 2006).

1.4.3 Dahl rats

In the research of salt hypertension, Dahl et al. (1962) selected two contrasting outbred lines of Sprague Dawley rats – salt-sensitive (DS) and salt-resistant (DR) rats. The inbred strains – SS/Jr and SR/Jr (Rapp and Dene 1985) have been derived from the above original outbred strains. High salt intake in SS/Jr rats leads to hypertension, while high potassium intake attenuates salt hypertension development in young Dahl rats (Zicha et al., 2011). Salt hypertension in young SS/Jr rats is characterized by augmented sympathetic hyperactivity, relative NO deficiency, attenuated baroreflex as well as by a major increase of residual blood pressure indicating remodelling of blood vessels (for review see Zicha et al. 2012a). Salt-induced elevation of blood pressure of salt-sensitive Dahl rats might be due to the activation of both the sympathetic and arginine vasopressin systems (Bayorh et al., 1998). Endothelin-1 acts as a local mediator of vascular dysfunction and aortic hypertrophy in salt-induced hypertension of Dahl rats (Barton et al., 1998). Chronic blockade of endothelin A receptors diminished salt hypertension development in adult SS/Jr rats (Zicha et al. 2012b). Arterial relaxation in response to acetylcholine did not differ between the groups but the upregulation of endothelial hyperpolarizing factor appears to compensate for the loss of NO in the mesenteric arteries of salt hypertensive Dahl rats (Goto et al., 2012), but prostaglandin H_2 might be involved in vascular dysfunction of these rats (Ohya et al., 1997).

Genetical analysis suggested that Nos2 gene in the NO pathway possess mutations in the SS/Jr strain and contribute to salt-sensitive hypertension in these animals (Chen et al., 1998). Arteriolar responses to L-NAME and acetylcholine are impaired in salt hypertensive SS/Jr rats compared with normotensive ones, and this difference can be abolished by the inhibition of heme oxygenase (Johnson et al., 2003). Enhanced Ca^{2+} entry via L-VDCC is coupled to altered gene expression in arterial smooth muscle (Wellman et al., 2001). Increased norepinephrine responsiveness seen in aortic smooth muscle of hypertensive Dahl rats during the development of salt hypertension is the result of a decrease in beta-adrenergic responsiveness but not an increase in alpha-adrenergic responsiveness (Soltis et al., 1991). Generalized defect in vascular G-protein receptor kinase-2 protein expression in hypertension could be an important factor in the impairment of β-adrenergic-mediated vasodilation, which is characteristic for the hypertensive state (Gros et al., 2000). Cl^- handling and Ca^{2+}-dependent Cl^- channels seem to undergo modifications as a consequence of salt-induced hypertension. It is possible that the modified influence of NO on membrane potential may have a direct relationship to the observed changes in Cl^- handling in blood vessels of salt-resistant versus salt-sensitive Dahl rats (Parai et al., 2005).

The enhanced production of oxygen free radicals was found in the vasculature of hypertensive Dahl rats (Swei et al., 1997, Vaněčková et al., 2013). The reactive oxygen species influence gene expression of the 20-HETE pathway in the SS/Jr rat and their elevated levels stimulate production of 20-HETE, contributing to vascular dysfunction (Lukaszewicz et al., 2013). There is an up-regulation of Na^+/Ca^{2+} exchanger-1 and TRPC6 in endothelium-denuded mesenteric arteries from hypertensive Dahl rats kept on high-salt diet (Pulina et al., 2013). L-type Ca^{2+} channels in resistance arteries of salt hypertensive Dahl rats are available for opening near their altered resting potential (Ohya et al., 2000). Acute L-VDCC blockade lowers BP of salt hypertensive SS/Jr rats proportionally to their basal BP level (Kuneš et al., 2004; Zicha et al., 2011). Chronic treatment of SS/Jr rats with L-VDCC antagonists prevents salt hypertension development (Kazda et al., 1982). The effects of high salt intake might also influence the Na^+/Ca^{2+} exchange system in arterial smooth muscle. Ca^{2+} extrusion by the ATP-driven calcium pump is decreased in salt hypertensive DS rats, leading to an elevation in cellular calcium levels. Na^+/Ca^{2+} exchange might be increased in compensation for an increase in

cellular Ca^{2+} concentration in salt-sensitive Dahl rats on the high-salt diet (Ashida et al., 1992, Ashida et al., 1997).

2. Aims of the study

My thesis can be divided into the study of vascular smooth muscle (VSM) contraction and the study of VSM alterations in hypertensive rats. The study of VSM contraction was focused on detailed examination of Ca^{2+} influx through non-selective cationic channels in contracting arteries. Study of VSM alterations in spontaneously hypertensive rats (SHR) was focused on the characterization of enhanced contractile properties of their arteries. Finally, detailed examination of the role of Ca^{2+} sensitization in arteries of three rat models of hypertension (SHR, Ren-2 TGR, Dahl) have been performed.

Particular goals:

1. To describe the effects of most commonly used inhibitors of TRP channels on the contractility of isolated femoral arteries.
2. To characterize the enhanced contractility of isolated femoral arteries from SHR.
3. To describe the participation of Ca^{2+} sensitization and Ca^{2+} entry in arterial contractility in selected rat models of hypertension.

3. Methods

3.1 Animals

The animals were kept under standard conditions (Altromin diet and drinking water *ad libitum,* temperature 23±1 ºC, 12-h light-dark cycle). In some experiments chronic sympathectomy of SHR animals was induced by guanethidine administration (30 mg/kg/day i.p.) for 14 days. 12-week-old male Wistar rats were used in Publication A. In Publications B-E spontaneously hypertensive rats (SHR) and their normotensive controls (WKY) were studied. These animals were aged 14-16 weeks. In Publication E normotensive Hannover Sprague Dawley (HanSD) and heterozygous Ren-2 transgenic rats (TGR) aged 18 weeks were used. Salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl rats were aged 20 weeks. SS/Jr and SR/Jr were fed a low-salt diet (0.3 % NaCl) since weaning until the age of 12 weeks, when one-half of SS/Jr and SR/Jr rats was fed by high-salt diet (5% NaCl) for 8 weeks, whereas the remaining animals were fed a low-salt diet. All animals were provided by breeding facility of Institute of Physiology CAS, Prague. All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Physiology CAS and conform to European Convention on Animal Protection and Guidelines on Research Animal Use.

3.2 Isolation of blood vessels

Before the isolation of arteries the animals were anesthetized with isoflurane (3%) and euthanized by open-chest cardiac puncture. For the isolation of mesenteric arteries a part of intestine with feeding vasculature was dissected. Femoral arteries were dissected from hind limb vascular bed. Excised section was put to a Petri dish containing physiological saline solution and artery was dissected with a fine forceps at room temperature. Ring segments 2 mm in length of large femoral artery (diameter ≈1000 μm), small femoral artery (diameter ≈250 μm) or small mesenteric arteries (diameter ≈250 μm) were dissected. In all arteries the endothelium was removed by gentle rubbing of the vessel by hair.

3.3 Mounting of blood vessels

Arterial segments were placed in two- or four-channel Mulvany-Halpern isometric myographs (620-M or 510-A, DMT, Denmark) and incubated in a modified Krebs-Henseleit solution (mmol/l: 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 25 $NaHCO_3$, 1.18 KH_2PO_4, 2.5 $CaCl_2$, 2 g/l glucose, 37 °C, bubbled with 95 % O_2 and 5 % CO_2). Two short segments (≈ 3 cm) of a 40 μm stainless steel wire were inserted into the lumen of each artery and secured under the fixing screws. Stretching the vessel was avoided for all the time.

3.4 Normalization

Blood vessel was equilibrated for 30 min at 37 °C. It was stretched by a standardized procedure according to the manufacturer's protocol to the 90% of the internal circumference corresponding to diameter that the artery would have *in vivo* when relaxed and under a transmural pressure of 100 mmHg. After a normalization period, blood vessels were allowed to equilibrate for 30 min.

3.5 Measurement of responses

At the onset of each experiment two K^+-induced contractions separated by a washout were performed. Absolute values of arterial contraction were recorded in $mN.mm^{-1}$. Relative values were expressed as percentage of maximal K^+-induced contraction (% KPSS) or phenylephrine-induced contraction (% PE). Cumulative concentration-response curves were established for various agonists (phenylephrine in concentrations 10 nM – 30 mM; U-46619 in concentrations 1 nM – 1 μM; K^+ in concentrations 10 mM – 120 mM). To produce K^+-induced contraction (KPSS) NaCl was substituted in bathing solution for KCl (124 mM). Na^+-free solution was created by a replacement of 119 mM NaCl and 25 mM $NaHCO_3$ with 144 mM N-methyl-D-glucamine ($NMDG^+$).

3.6 Histochemical visualization of catecholamines

Histochemical measurement was performed by the protocol of de la Torre and Surgeon (1976). Briefly, glyoxylic acid solution, prepared fresh every day, consisted of 1% glyoxylic acid (Sigma), 236 mM KH_2PO_4 and 200 mM sucrose. Femoral arteries were cut longitudinally and dipped three times in the glyoxylic acid solution. Then they were mounted on glass slides and dried 5 min by air cooler, transferred to hot plate (80 °C) and heated for 5 min. Few drops of Mineral Oil (Sigma) was added and cover glass was added. The slides were again heated on hot plate (80 °C) for 90 s. Fluorescence was observed in Leica LMD6000 microscope with DAPI filter cube.

3.7 Data analysis

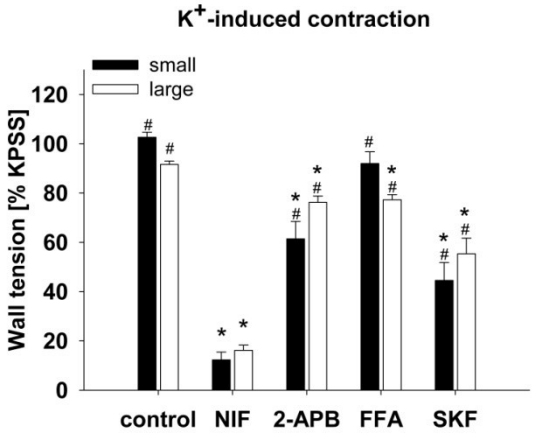
Force tracings were directly converted to numeric data in LabChart 7 (ADInstruments Ltd, Oxford, UK) and transferred to MS Excel, where they were corrected for vessel size. Data were analyzed and plotted with SigmaPlot 11.0 graphing program (Systat Software, Point Richmond, CA). Relative values were expressed as percentage of maximal K^+-induced contraction. Concentration-response data, calculated from the tracings, were analyzed by determining EC50 values from experimental data fitted to a four-parameter logistic function. Comparisons between groups and within groups were evaluated by one way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test. The differences were considered significant at $p<0.05$ level.

4. Results

4.1 Publication A: Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat

Opening of L-VDCC is a part of agonist-induced contraction of arteries isolated from normotensive Wistar rats. The increase of extracellular K^+ to 120 mM causes depolarization of VSM cells and opens L-VDCC without activation of signalling pathways. First, we studied the effects of selected inhibitors on this type of contraction (Fig. 1). In both small and large arteries K^+-induced contraction was almost completely blocked by L-VDCC inhibitor nifedipine. On the other hand, 2-APB, flufenamic acid and SKF-96365 (three different non-selective TRP inhibitors) attenuated K^+-induced contraction less in comparison with nifedipine. The effects of nifedipine or selected TRP channel blockers did not depend on vessel size (Fig. 1).

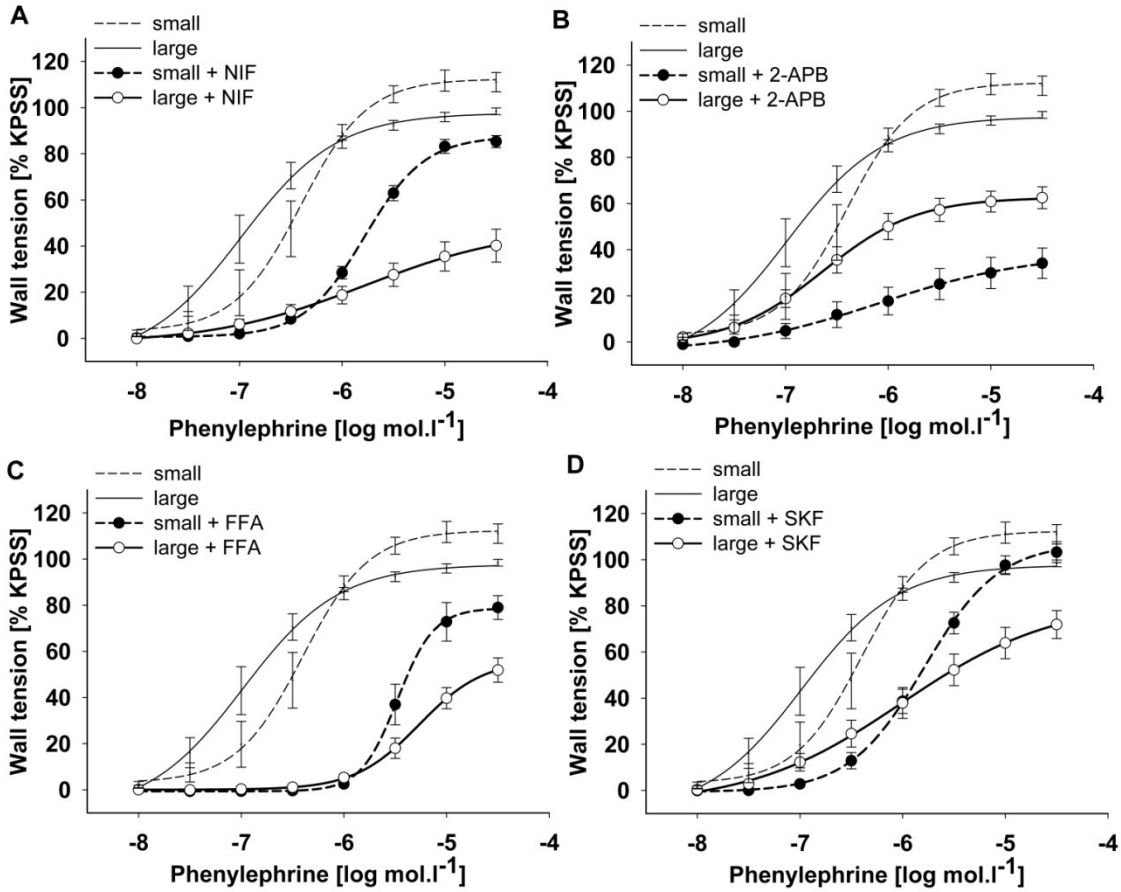
Figure 1. K^+-induced contraction in the presence of various blockers: NIF (Nifedipine, 1 μM), 2-APB (2-aminoethoxydiphenyl borate, 100 μM), FFA (flufenamic acid, 100 μM) and SKF (SKF-96365, 10 μM). Data are means ± S.E.M.; n=6. Significantly different (P<0.05): # from the contraction in the presence of L-VDCC blockade (NIF), * from control contraction. Adapted from Publication A.



Furthermore, the effects of selected blockers on arterial contraction induced by specific α_1-adrenergic agonist phenylephrine were studied. In both sizes of femoral

arteries nifedipine caused a shift of phenylephrine concentration-response curve to the right. Nifedipine had significantly greater effect on maximal contraction in large than in small arteries (Fig. 2A).

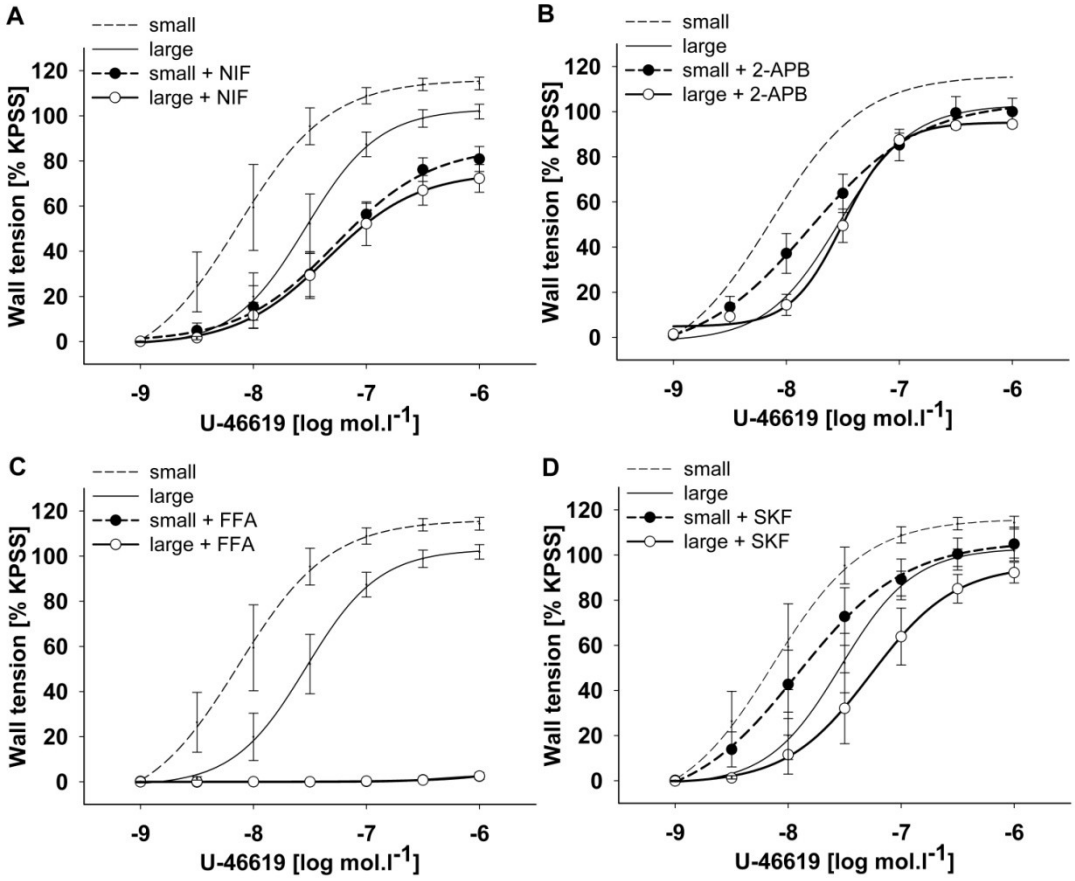
Figure 2. Relative concentration-dependent contraction of isolated small and large femoral arteries induced by phenylephrine (10 nM – 30 μM) in the presence of particular inhibitors: **A** - NIF (Nifedipine, 1 μM), **B** - 2-APB (2-aminoethoxydiphenyl borate, 100 μM), **C** - FFA (flufenamic acid, 100 μM) and **D** - SKF (SKF-96365, 10 μM). Data are mean ± S.E.M.; n=6. Adapted from Publication A.



On the other hand, 2-APB influenced contraction maximum more in small than in large arteries (Fig. 2B). Flufenamic acid lowered contraction maximum more in large than in small arteries and shifted both curves significantly to the right (Fig. 2C). SKF-96365 lowered contraction maximum only in large arteries (Fig. 2D). The important contribution of both types of channels (L-VDCC and TRP) to arterial contraction was confirmed when we combined nifedipine with 2-APB or FFA, respectively. These combinations almost completely prevented the adrenergic contraction in arteries of both diameters (see Publication A).

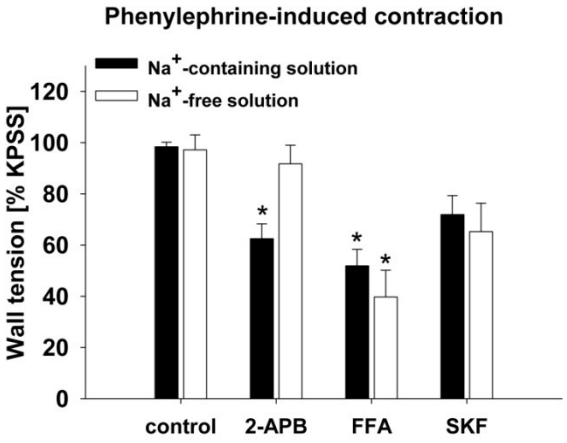
Contraction induced by thromboxane A_2 receptor agonist U-46619 was significantly attenuated by nifedipine in both sizes of arteries. FFA completely inhibited U-46619-induced contraction in both large and small femoral arteries, whereas 2-APB and SKF-96365 had no significant effects on this type of contraction (Fig. 3).

Figure 3. Relative concentration-dependent contraction of isolated small and large femoral arteries induced by U-46619 (1 nM - 1 μM) in the presence of particular inhibitors: **A** - NIF (Nifedipine, 1 μM), **B** - 2-APB (2-aminoethoxydiphenyl borate, 100 μM), **C** - FFA (flufenamic acid, 100 μM) and **D** - SKF (SKF-96365, 10 μM). Data are mean ± S.E.M.; n=6. Adapted from Publication A.



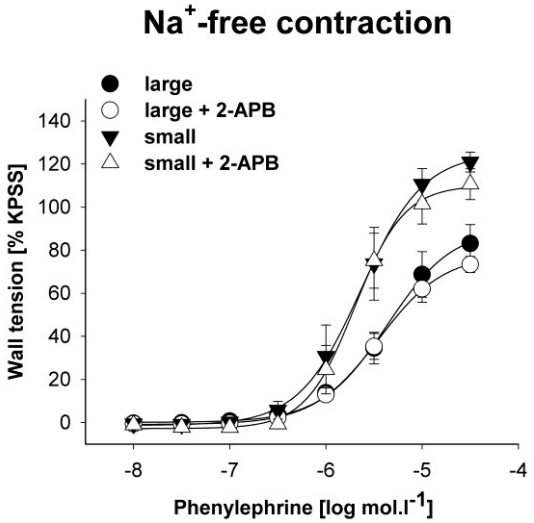
Thereafter we studied the effects of extracellular Na^+ absence on inhibiting properties of selected TRP inhibitors. In the absence of external Na^+ we observed the attenuation of inhibitory effects of 2-APB on phenylephrine-induced contraction, whereas the effects of FFA or SKF-96365 were not changed in comparison to their effects in Na^+-containing medium (Fig. 4).

Figure 4. Role of extracellular Na^+ on inhibitory effects of selected TRP inhibitors. (A) Maximal contractions of large femoral arteries induced by phenylephrine (30 μM) in Na^+-containing solution and in Na^+-free solution. 2-APB (2-aminoethoxydiphenyl borate, 100 μM), FFA (flufenamic acid, 100 μM) or SKF (SKF-96365, 10 μM). Significantly different (P<0.05): * from control. Data are mean ± S.E.M.; n=6. Adapted from Publication A.



Moreover, dose-response experiments indicated that in Na^+-free media 2-APB did not modify pharmacokinetic parameters of phenylephrine-induced contraction (Fig. 5). This experiment confirmed a loss of 2-APB inhibitory ability on the contraction of isolated arteries in the absence of extracellular Na^+.

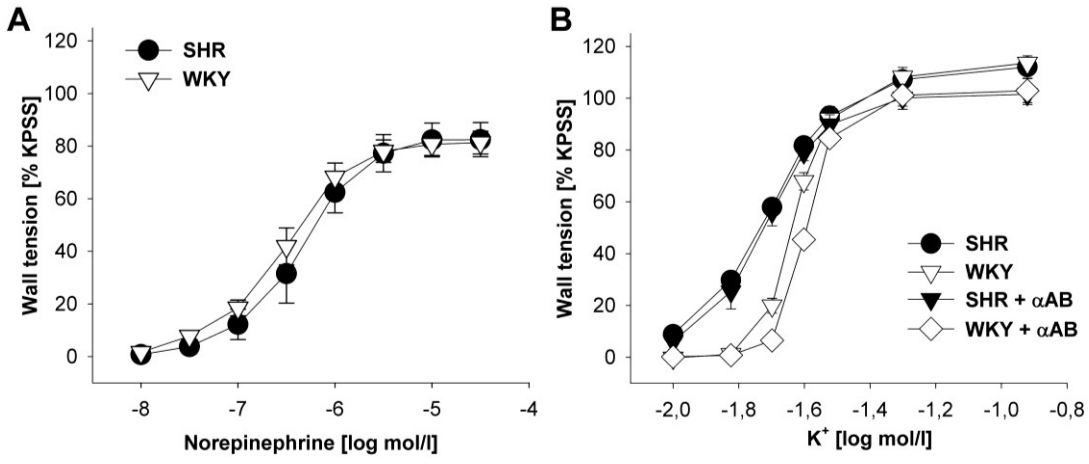
Figure 5. Dose-response contraction of large femoral arteries induced by phenylephrine (10 nM - 30 μM) in Na^+-free bathing solution. Measurements were performed in the presence of 2-APB (2-aminoethoxydiphenyl borate, 100 μM). Data are mean ± S.E.M.; n=6. Adapted from Publication A.



4.2 Publication B: Altered contractile responses of arteries from spontaneously hypertensive rat: The role of endogenous mediators and membrane depolarization

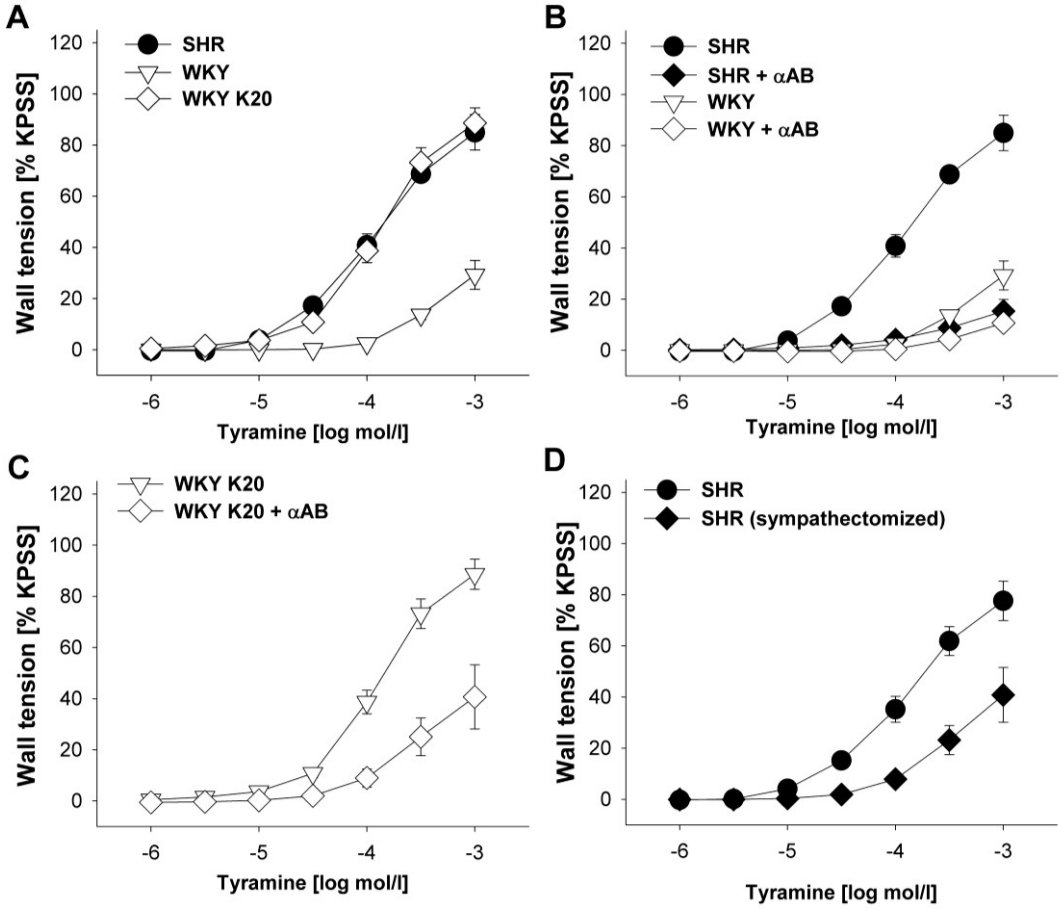
To measure the sensitivity of blood vessels from SHR and WKY rats to adrenergic agonist norepinephrine, concentration-response curves were determined in endothelium-denuded large femoral arteries. They were not significantly different (Fig. 6A). K^+-induced contractions of arteries isolated from SHR or WKY were performed in order to cause depolarization-induced opening of L-VDCC. These responses differed significantly at K^+ concentrations from 10-20 mM. We performed additional blockade of α-adrenergic receptors with phentolamine and prazosine (10 µM each) to assess the participation of endogenous norepinephrine release from axonal varicosities to K^+-induced contraction (Fig. 6B). This inhibition did not influence K^+-induced contraction, suggesting that in these arteries there was no significant release of endogenous norepinephrine during K^+-induced contraction.

Figure 6. Concentration-dependent contraction of femoral arteries isolated from SHR or WKY, stimulated by norepinephrine (A) or K^+ (B). K^+-induced contractions were also studied after the complete α-adrenoceptor blockade by prazosine (10 µM) plus phentolamine (10 µM) (αAB). Data are means ± SEM; n=6. Adapted from Publication B.



Tyramine is able to release endogenous norepinephrine from neuronal varicosities present in blood vessels (Miyahara and Suzuki 1986). Tyramine-induced contractions were significantly greater in femoral arteries isolated from SHR compared to WKY arteries (Fig. 7A).

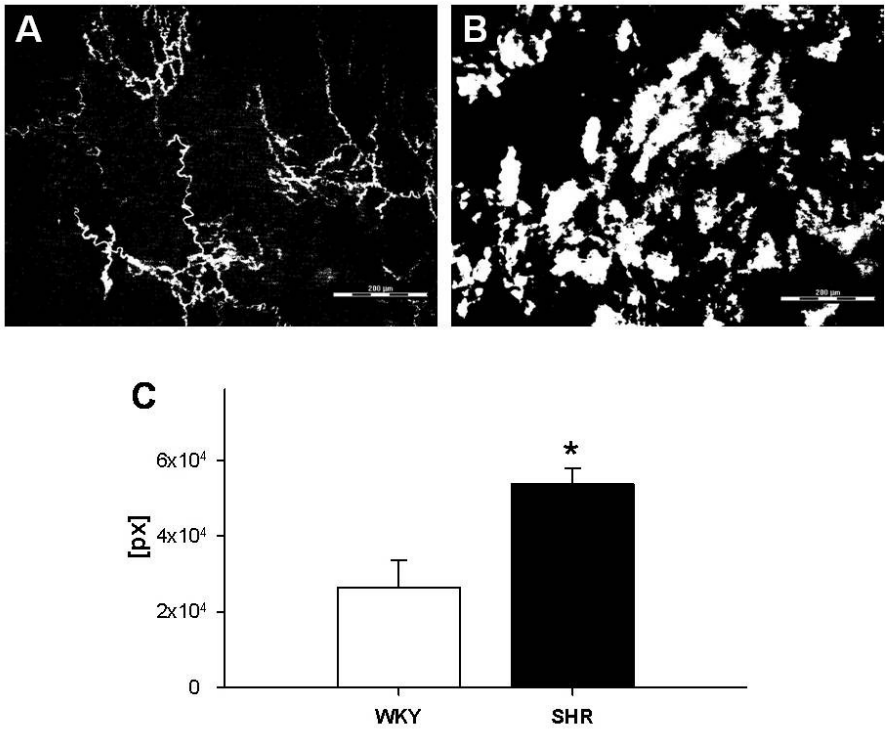
Figure 7. Tyramine-induced concentration-dependent contraction of femoral arteries isolated from SHR and WKY. (A) Contraction of SHR, WKY and WKY arteries with 20 mM K^+ in bathing solution (WKY K20). (B) the effect of α-adrenergic receptor blockade by prazosine (10 μM) + phentolamine (10 μM) (αAB). (C) the influence of α-adrenergic receptor blockade by prazosine (10 μM) + phentolamine (10 μM) (αAB) on WKY arteries pretreated with 20 mM K^+. (D) the effect of guanethidine-induced sympathectomy on SHR arteries. Data are means ± SEM; n=6. Adapted from Publication B.



When we evaluated tyramine-induced contraction in WKY vessels pretreated with increased non-contracting extracellular K^+ concentration (20 mM; K20) to induce a partial depolarization of their smooth muscle cells, tyramine-induced contractions were similar to those of SHR (Fig. 7A). To define the participation of endogenous norepinephrine, we measured tyramine-induced contraction in the presence of α-

adrenergic inhibitors (prazosine and phentolamine). There was a significant inhibition of contraction especially in SHR arteries (Fig. 7B). The α-adrenergic blockade also partially attenuated tyramine-induced contraction of WKY arteries pretreated with 20 mM K^+ (Fig. 7C). Furthermore, we studied the arteries isolated from SHR sympathectomized with guanethidine, which depleted catecholamines from neuronal varicosities. We observed a significant reduction of tyramine-induced contractions in these arteries (Fig. 7D), confirming that endogenous norepinephrine is participating in the enhanced tyramine-induced contraction in SHR. Catecholamines were not present in blood vessel wall of sympathectomized animals in contrast to untreated animals (Fig. 8).

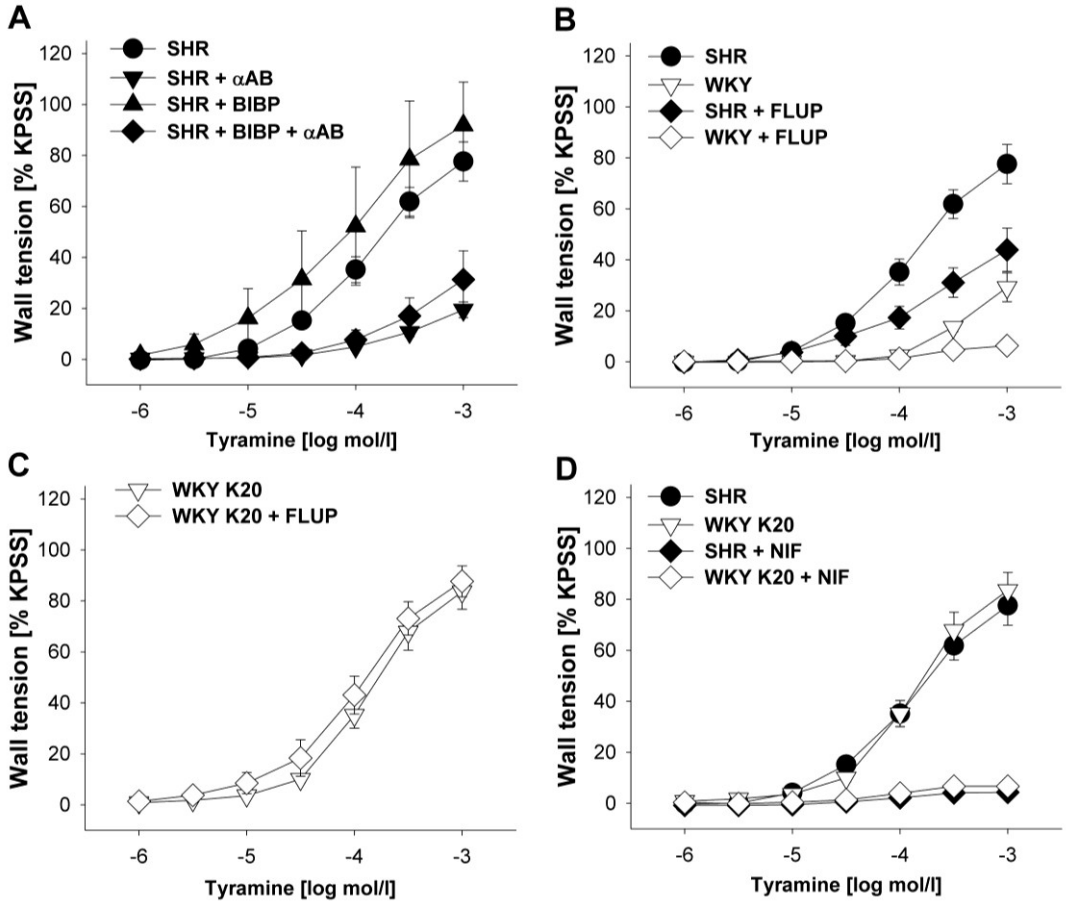
Figure 8. Vascular catecholamine content. (A) example of WKY femoral artery. (B) example of SHR femoral artery. (C) Quantification of catecholamine content. Scale bar=200 μm, n = 6. Significantly different (P<0.05) * from WKY femoral arteries. Adapted from Publication B.



Neuropeptide Y_1 receptor inhibitor BIBP-3226 did not affect tyramine contraction and its effects were not additive to the inhibition of α-adrenergic receptors by phentolamine and prazosine (Fig 9A). The opening of K^+ channels by their opener flupirtine (causing hyperpolarization of smooth muscle cells) reduced

tyramine-induced contraction in SHR and WKY arteries, but had no effect on the strain difference (Fig. 9B). There was no effect of flupirtine on tyramine-induced contraction in WKY arteries pretreated with 20 mM K^+ (Fig. 9C). Tyramine-induced contraction was completely abolished by the blockade of L-VDCCs with nifedipine in both SHR and WKY arteries (Fig. 9D).

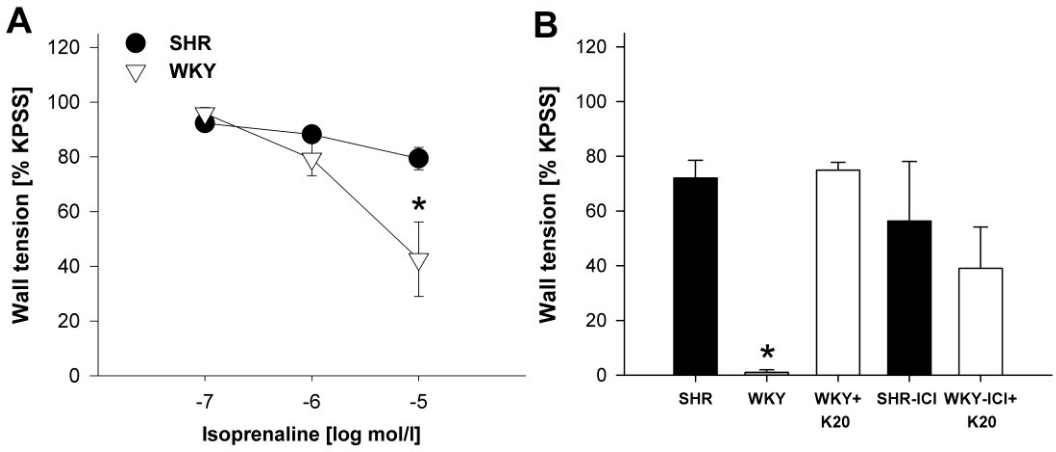
Figure 9. Tyramine-induced contraction of femoral arteries isolated from SHR or WKY. (A) Neuropeptide Y_1 receptor blockade by BIBP 3226 (10 µM), prazosine (10 µM) + phentolamine (10 µM) were used to inhibit α-adrenergic receptors (αAB). (B) K^+-channel opening effect by flupirtine (FLUP, 10 µM). (C) WKY arteries bathing solution with 20 mM K^+ (K20). (D) inhibition of L-type voltage-dependent Ca^{2+} channels by nifedipine (NIF, 1 µM). Data are means ± SEM; n=6. Adapted from Publication B.



The stimulation of β-adrenergic receptors by isoprenaline in phenylephrine-precontracted SHR and WKY arteries leads to a greater relaxation in WKY than SHR arteries (Fig. 10A). On the other hand, the inhibition of β-adrenergic receptors in

isolated endothelium-denuded arteries leads to their contraction, but only in those isolated from SHR. Arteries from WKY had to be pretreated with 20 mM K^+ in order to respond to propranolol similarly as SHR arteries (Fig. 10B). These contractions can be induced not only by a non-selective β-antagonist propranolol but also by a selective β_2-antagonist ICI-118,551 (Fig. 10B).

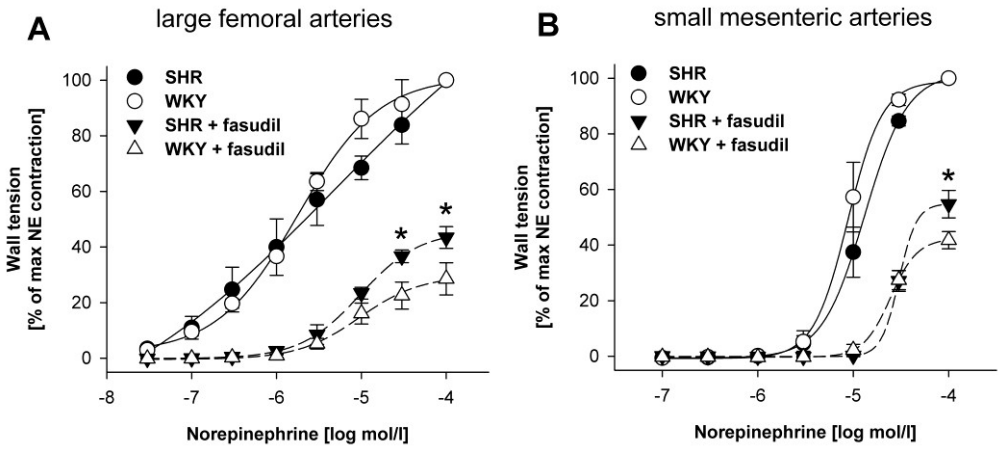
Figure 10. (A) Isoprenaline-induced relaxation of phenylephrine-precontracted femoral arteries isolated from SHR or WKY. (B) Contraction of femoral arteries isolated from SHR or WKY were elicited by β-blocker propranolol (column 1-3) or ICI-118,551(column 4-5) in concentration 10 μM each. Some WKY arteries were pretreated with 20 mM K^+ concentration (K20). Data are means ± SEM; n=6. * significantly different ($p<0.05$) from propranolol-induced contraction of SHR artery. Adapted from Publication B.



4.3 Publication C: Ca^{2+} sensitization and Ca^{2+} entry in the control of blood pressure and adrenergic vasoconstriction in conscious Wistar-Kyoto and spontaneously hypertensive rats

The measurement of changes in evolving adrenergic contraction after Rho kinase inhibition as well as the relaxation of adrenergic precontracted arteries by Rho kinase inhibiton were the goals of myography measurements in this work. Adult male rats were aged 14-16 weeks. Rho kinase inhibition by fasudil (HA-1077; 10 µM) attenuated norepinephrine-induced contractions of femoral or mesenteric arteries in both rat strains (Fig. 11).

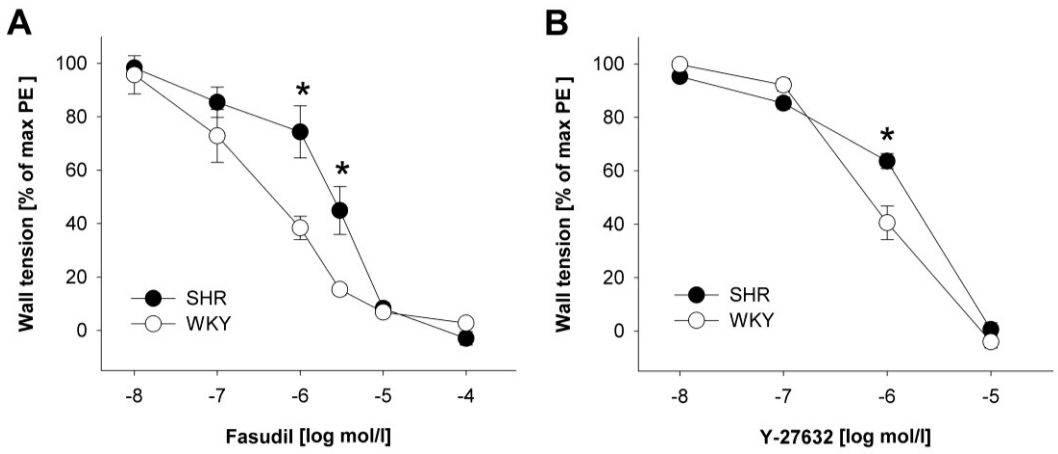
Figure 11. The effects of Rho kinase inhibition by fasudil (10 µM) on the development of norepinephrine-induced contractions of large femoral (A) and small mesenteric arteries (B) isolated from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data are mean ± SEM; n=5; * significantly different ($p<0.05$) from WKY. Adapted from Publication C.



The reduction of contractile force by Rho kinase inhibition was smaller in SHR than in WKY arteries. This difference was -56.6 ± 4.0% (SHR) vs. -71.4 ± 5.8% (WKY) in femoral arteries and -45.3 ± 4.9% (SHR) vs. -58.2 ± 3.1% (WKY) in mesenteric arteries.

Rho kinase inhibitor fasudil, administered in rising concentrations 10^{-8} – 10^{-4} mol/l caused less pronounced dose-dependent relative reduction of wall tension in large femoral arteries of SHR than those of WKY (Fig. 12A) precontracted with α_1-adrenergic agonist phenylephrine (PE). Similar results were measured with more potent Rho kinase inhibotror Y-27632 in concentrations 10^{-8} – 10^{-5} mol.l^{-1} (Fig. 12B). These data suggest a lower role of Rho kinase pathway in SHR.

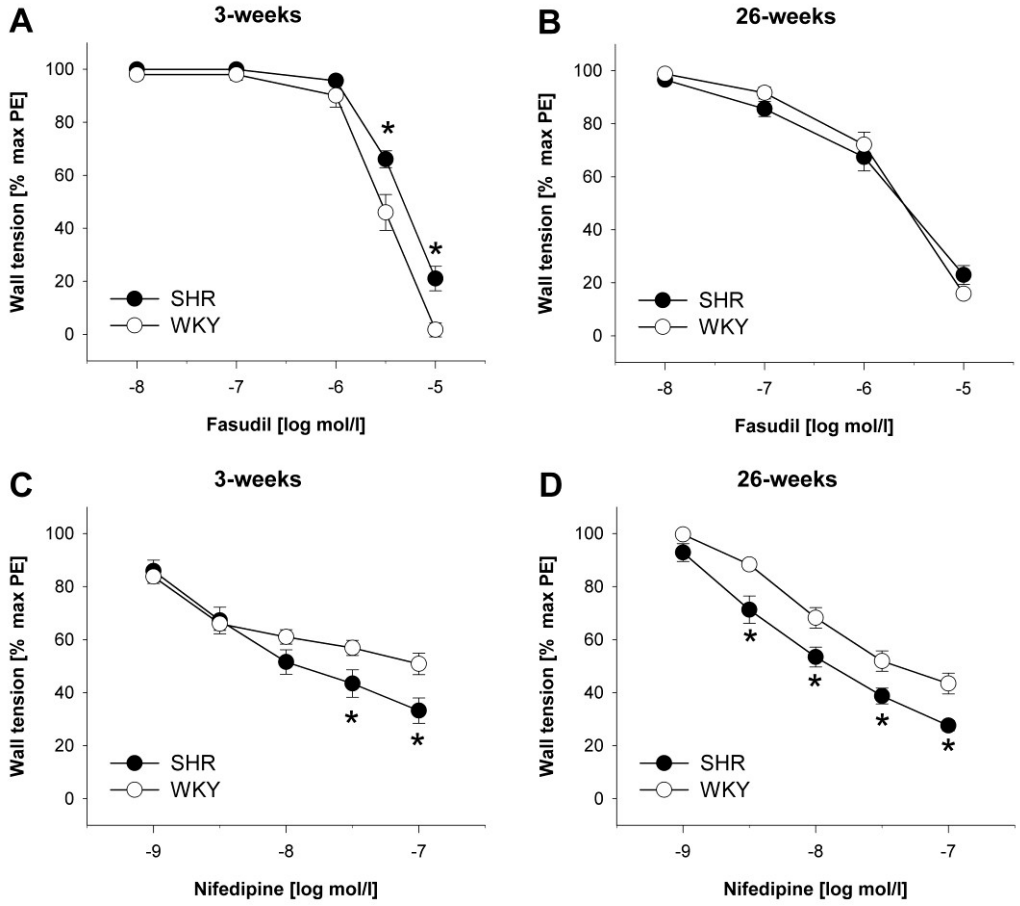
Figure 12. Relative values of dose-dependent relaxation effects of fasudil (A) and Y-27632 (B) on the femoral arteries (precontracted by 10 μM of phenylephrine - PE) of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Data are mean ± SEM; n=5; significantly different (* $p<0.05$) from WKY. Adapted from Publication C.



4.4 Publication D: Ontogenetic changes in contribution of calcium sensitization and calcium entry to blood pressure maintenance of Wistar-Kyoto and spontaneously hypertensive rats

Myography measurements in this work were aimed at search for differences in the participation of Rho kinase and L-type voltage-dependent channels (L-VDCC) in adrenergic contraction in both SHR and WKY rats. Both strains were studied at the age of 3 and 26 weeks. 3-week-old SHR were pre-hypertensive and hypertensive (26-week-old) rats were older than adult male rats in Publication C (14-16 weeks). Vascular effects of Rho-kinase inhibitor fasudil and L-VDCC inhibitor nifedipine were studied in femoral arteries precontracted with α_1-adrenergic agonist phenylephrine.

Figure 13. Dose-dependent relaxation effects of fasudil (A, B) or nifedipine (C, D) on the wall tension established in phenylphrine-precontracted femoral arteries isolated from 3- and 26- week-old WKY and SHR. Data are mean ± SEM; n=6-8; * significantly different ($p<0.05$) from WKY. Adapted from Publication D.

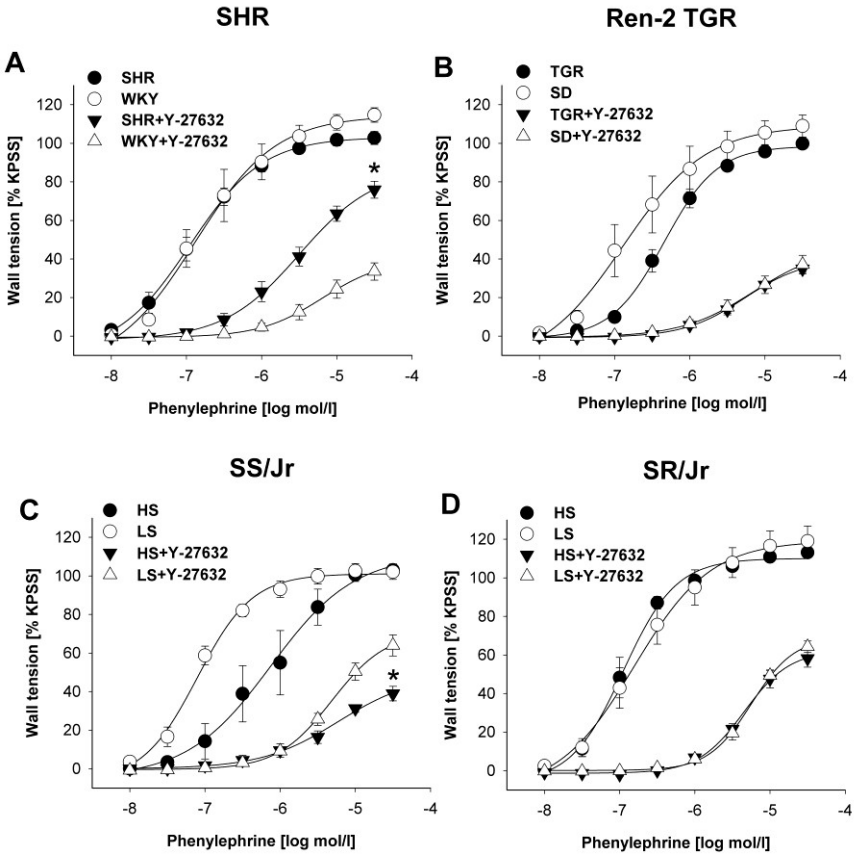


Fasudil-induced vascular relaxation (10^{-8} – 10^{-5} mol/l) was attenuated in arteries from prehypertensive 3-week-old SHR, but not those from hypertensive 26-week-old SHR (Fig. 13A, B). These data suggest a lower role of Rho kinase pathway in prehypertensive SHR, similarly as in 14-16 weeks old SHR from Publication C. Rho kinase participation in contractile force of arteries from 26-week-old hypertensive SHR seems to be similar as in WKY rats. Vascular relaxation elicited by increasing concentration of nifedipine (10^{-9} – 10^{-7} mol/l) was enhanced in both prehypertensive and hypertensive SHR (Fig. 13C, D). These data suggest enhanced role of Ca^{2+} influx through L-VDCC in both prehypertensive and hypertensive stage of SHR. While Rho kinase pathway may be able to compensate for enhanced contractile force in prehypertensive animals, it seems that in hypertensive stage (26 weeks) it plays a similar role as in WKY rats.

4.5 Publication E: Basal and activated calcium sensitization mediated by RhoA/Rho kinase pathway in rats with genetic and salt hypertension

In this work, comparison of changes after Rho kinase inhibition in evolving adrenergic contraction in three models of hypertension was performed. Phenylephrine-induced α_1-adrenergic contraction of large femoral arteries was studied in the presence of Rho kinase inhibitor (Y-27632).

Figure 14. The effects of Rho kinase inhibition by Y-27632 (10 μM) on the development of phenylepinephrine-induced contractions of femoral arteries from WKY rats and SHR (panel A), Ren-2 TGR (panel B) and Dahl salt-sensitive (SS/Jr; panel C) and salt-resistant (SR/Jr; panel D, next page) rats fed a low-salt (LS) or high-salt (HS) diet. Data are mean ± SEM; n=5; * significantly different ($p<0.05$) from control. Adapted from Publication E.



In 16-week-old SHR caused Rho kinase inhibition smaller shift in SHR arteries than in those from normotensive WKY (Fig. 14A). The contribution of Rho kinase

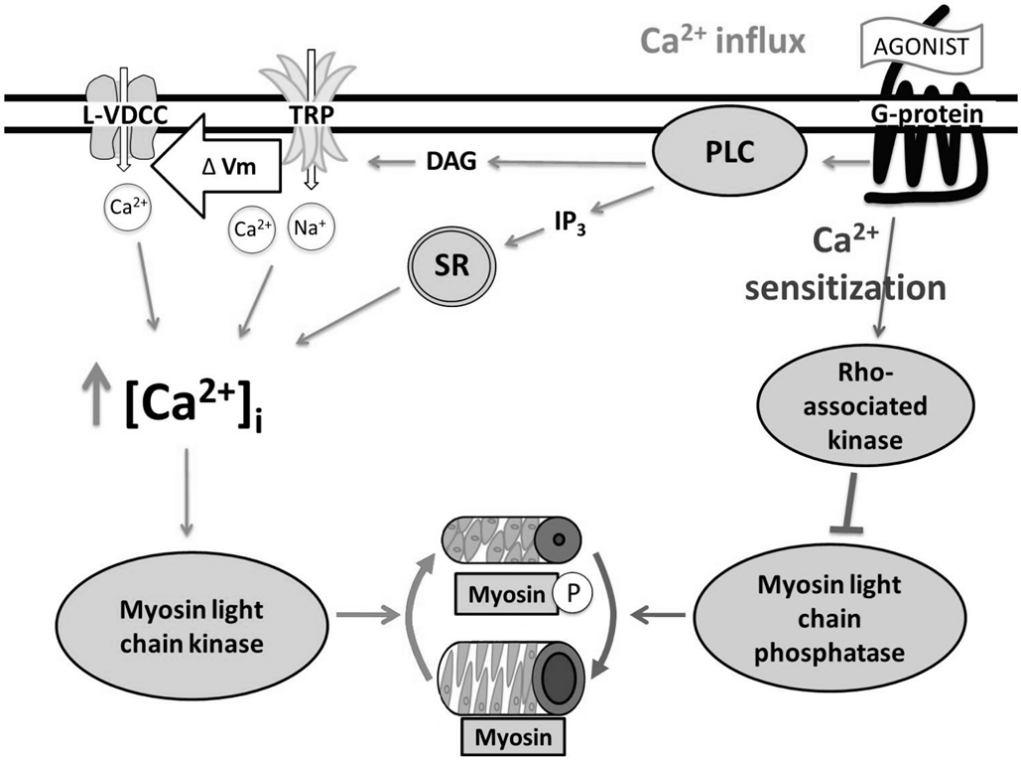
to arterial contractility is also relatively attenuated in phenylephrine concentrations of 18-week-old Ren-2 TGR compared to their HanSD controls (Fig. 14B), due to their rightward shift of arterial contractions under control conditions (more detailed data can be found in Publication E). On the contrary, Rho kinase inhibition elicited a greater attenuation of maximal contractile responses in femoral arteries of 20-week-old hypertensive Dahl salt-sensitive rats (SS/Jr) on high-salt diet (Fig. 14C), while there was no difference in 20-week-old Dahl salt-resistant rats (SR/Jr) on either high- or low-salt diet (Fig. 14D). In contrast indicating enhanced role of Rho/Rho kinase pathway role in hypertensive Dahl salt-sensitive rats.

5. Discussion

The thesis is focused on the pharmacological evaluation of the role of Ca^{2+} influx and Ca^{2+} sensitization in the contraction of isolated endothelium-denuded arteries. In normotensive rats, Ca^{2+} influx was studied by a comparison of three most commonly used non-selective inhibitors of TRP channels (Publication A). Furthermore, we found that a more detailed characterization of enhanced contractile properties of SHR arteries is necessary (Publication B). The role of Ca^{2+} sensitization was studied in both normotensive and hypertensive rats by the inhibition of Rho kinase either by fasudil (Publications C and D) or by a more selective inhibitor Y-27632 (Publication E).

The first aim of the thesis was to describe the effects of most commonly used inhibitors of TRP channels on the contractility of the isolated femoral arteries. In the Publication A we analyzed the effects of three broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF 96365) on K^+-induced and agonist-induced (adrenergic and thromboxane) contraction of resistance and conduit femoral arteries (measured in Na^+-containing or Na^+-free solutions) of normotensive Wistar rats. The effects of L-VDCC blocker (nifedipine) on arterial contractions were different from those of broad-range TRP channel inhibitors. Nifedipine had a more pronounced effect on K^+-induced contraction than all used TRP channel inhibitors, which is in agreement with the assumption that K^+-induced contraction opens directly L-VDCC but not receptor-operated channels such as TRP. The inhibitory effects of particular TRP channel inhibitors on α_1-adrenergic contraction elicited by phenylephrine were comparable to those of nifedipine in small and large arteries. The fact that nifedipine was not able to prevent completely the development of α_1-adrenergic contraction indicates the participation of additional mechanisms to L-VDCC in the generation of phenylephrine-induced contraction in contrast to K^+-induced contraction. This is in agreement with the assumption that VSM contraction is generated by the opening of non-selective cationic channels creating depolarization and consequently the opening of L-VDCC (Fig. 15). This view is compatible with general mechanism of vascular smooth muscle contraction as reviewed by Berridge (2008).

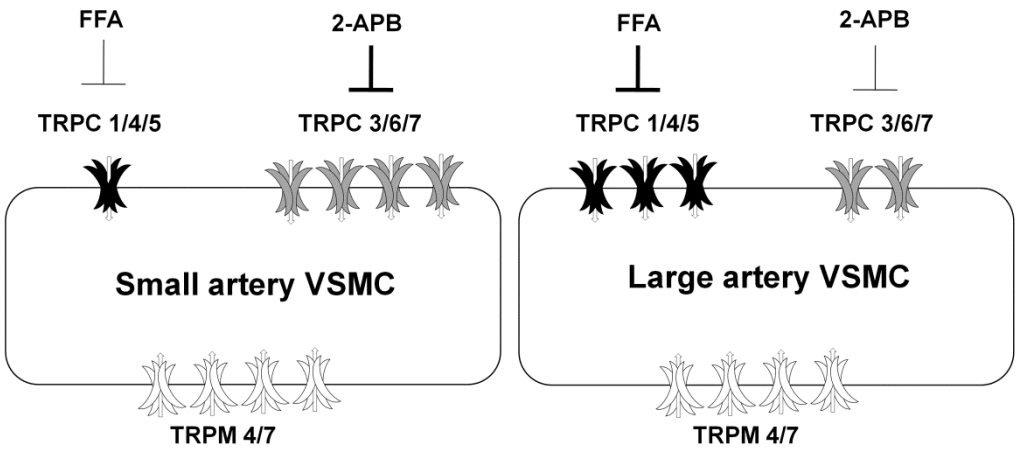
Figure 15. The schematic representation of VSM contraction. L-VDCC – L-type voltage-dependent calcium channel; TRP – transient receptor potential channel, Vm – membrane potential; DAG - diacylglycerol; PLC - Phospholipase C; G-protein - G protein–coupled receptor; SR – sarcoplasmatic reticulum; P – phosphorylation.



The different importance of L-VDCC and TRP channels for the contraction of large and small arteries was revealed by our study. We demonstrated that the adrenergic contraction of small femoral arteries is more dependent on 2-APB-sensitive TRP channels, while the participation of L-VDCC is more important in large femoral arteries. The relationship of TRP channels and L-VDCC is mutually complementary; the importance of TRP channels is increasing with the decrease in blood vessel diameter.

Different mRNA expression of TRP channel subunits and the differences in 2-APB and FFA effects found in small and large arteries suggest that there might be a connection between these two observations. On the basis of our results and the inhibitory effects of these compounds in previous studies (Lievremont et al., 2005; Jiang et al., 2012; Boulay et al., 1997; Shlykov et al., 2003), we suggested a possible relation of selected inhibitors (2-APB and FFA) to particular TRPC channels in small and large arteries as described in our scheme (Fig. 16).

Figure 16. The schematic representation of the relationship between TRP channel expression and the inhibitory effects of 2-APB and FFA on phenylephrine-induced contraction of small and large femoral arteries. The thickness of bar-headed lines reflects the extent of inhibitory effects of FFA and 2-APB on the contraction of small or large arteries. Adapted from Publication A.



Na^+ influx is assumed to mediate membrane depolarization. Therefore the effects of particular TRP channel inhibitors were studied also for their dependence on the presence of extracellular Na^+ in bathing solution. Isolated arteries in the Na^+-free solution preserved their ability to contract following adrenergic stimuli. This suggests that extracellular Na^+ is not crucial for phenylephrine-induced contraction, which is in agreement with the observation of norepinephrine-induced contractions in Na^+-free solution (Salomonsson et al., 2010). Our results surprisingly showed a major difference in the inhibitory effects of 2-APB and FFA on arterial contraction in the absence of Na^+. Only the inhibitory effects of 2-APB were dependent on the presence of extracellular Na^+. This significant Na^+-dependence of 2-APB effect is compatible with the role of non-selective TRP channels in membrane depolarization during α_1-adrenergic VSM contraction. Therefore the observed loss of inhibitory effect after Na^+ removal from bathing solution makes 2-APB an interesting pharmacological tool for future investigations.

The second aim of the thesis was to characterize the enhanced contractility of isolated arteries from SHR. In the Publication B we characterized the differences of endothelium-denuded arteries isolated from SHR and WKY rats, elicited by the addition of exogenous norepinephrine or by its tyramine-induced release from neuronal varicosities present in blood vessels.

The contractility of isolated arteries stimulated by adrenergic receptor agonist norepinephrine showed similar contractility of SHR and WKY arteries, what is in agreement with previous studies (Aoki et al. 1986). The opening of L-VDCC channels following membrane depolarization caused by increasing concentration of extracellular K^+ confirmed the earlier observations that L-VDCC are more prone to the opening in SHR (Aoki et al., 1986; Paulis et al., 2007). Further blockade of adrenergic receptors excluded the possibility that this difference in K^+-induced contraction of SHR and WKY arteries is dependent on endogenous catecholamines. The action of high extracellular K^+ seems not to affect neuronal endings in blood vessel wall.

Tyramine induces arterial contraction which stimulates the release of endogenous norepinephrine in isolated blood vessels (Miyahara and Suzuki 1986). Tyramine caused significantly greater contraction of SHR arteries when compared to that seen in WKY arteries. The pretreatment of WKY vessels with non-contractile K^+ concentration (20 mM) showed that tyramine induced a similar contraction in partially depolarized WKY arteries as in those of SHR. To confirm that tyramine-induced contraction is largely dependent on vascular norepinephrine content, we studied blood vessels of sympathectomized SHR rats. Our experiments showed considerably reduced contraction of arteries isolated from guanethidine-treated SHR. Stekiel et al. (1986) reported that local sympathectomy of *in situ* perfused vessels caused a hyperpolarization of small mesenteric vessels of SHR to the level of membrane potential measured in WKY vessels. Based on these results and on the histochemical analysis which revealed augmented presence of catecholamines in vascular wall of SHR, we suggest that enhanced SHR tyramine response might be caused by both partial depolarization of SHR cell membrane as well as augmented presence of norepinephrine in SHR vascular wall.

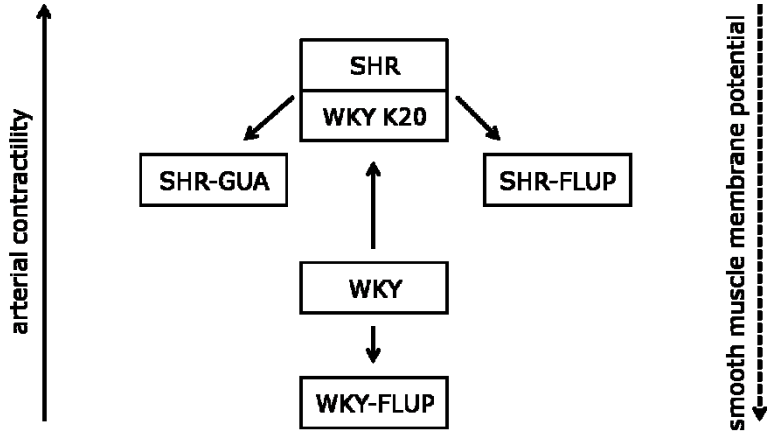
To describe better the enhanced tyramine contraction we studied the role of neuropeptide Y_1 by the blockade of its receptors as well as the contribution of K^+ channels opening. We did not detect any role of neuropeptide Y_1 in the generation of tyramine-induced contraction, but we revealed a partial contribution of closed K^+ channels to this type of arterial contraction. However, our experiments showed also a contribution of these channels in WKY arteries so that, the opening of these channels did not diminish the strain difference between tyramine-induced contractions of SHR and WKY arteries.

Defective β-adrenoceptor-mediated hyperpolarization was documented in SHR compared to WKY (Goto et al., 2001; Asano et al., 1988). Our experiments also confirmed the attenuated relaxation of phenylephrine-precontracted SHR arteries by β-adrenergic receptor agonist isoprenaline. Nevertheless, when we added propranolol (the inhibitor of β-adrenergic receptors) to endothelium-denuded arteries, it elicited a slow contraction of arteries isolated from SHR but not of those from WKY. This contraction was not observed in the presence of endothelium. Selective β_2-adrenergic inhibitor ICI-118,551 also caused such a contraction of SHR arteries. This apparent discrepancy between the enhanced contraction of SHR arteries after β-adrenoreceptor blockade and decreased vasodilatation induced by β-adrenoceptor stimulation in SHR suggests that none of these abnormalities can be explained by functional change of β-adrenergic receptors in SHR (Mallem et al., 2005). β-adrenoceptor signalling pathway operates via increasing of cAMP concentration (Delpy et al., 1996). The removal of small cAMP amount by β-adrenoceptor blockade in partially depolarized SHR vessels might elicit their contraction, whereas cAMP production caused by β-adrenoceptor stimulation might not oppose the enhanced contraction of SHR arteries as effectively as it does in more hyperpolarized WKY blood vessels. This explanation is compatible with the observation of propranolol-induced contraction in WKY arteries pretreated with 20 mM K^+.

The data on mRNA expression of α-adrenergic receptors are in line with the findings of Graham et al. (1982) that the density of α_2-adrenoceptors, but not that of α_1-adrenoceptors, was increased in SHR compared to WKY. Our data did not show attenuated expression of β-adrenergic receptors, as might be anticipated from the experiments studying β-adrenergic relaxation (Publication B).

Figure 17 shows that the enhanced contractility of SHR arteries was reduced by either sympathectomy or K^+ channel opening, whereas the low contractility of WKY arteries was augmented by moderate K^+-induced depolarization. These changes are compatible with the respective modification of membrane potential of vascular smooth muscle. Enhanced contractility of SHR arteries might be related to both increased presence of endogenous norepinephrine in vascular wall and also to altered vascular smooth muscle membrane potential.

Figure 17. The schematic representation of factors contributing to the changes of tyramine-induced arterial contraction in the studied groups of SHR and WKY rats. WKY K 20 – WKY arteries pretreated with non-contractile concentration of 20 mM K^+; SHR-GUA – arteries of SHR treated with guanethidine *in vivo*, SHR-FLUP – SHR arteries treated with flupirtine *in vitro*. Adapted from Publication B.



The third aim of this thesis was to describe the role of Ca^{2+} sensitization and Ca^{2+} entry in selected rat models of hypertension. Acute attenuation of Ca^{2+} sensitization by the inhibition of Rho kinase lowers BP (Uehata et al., 1997), the effects might be more pronounced in hypertensive than in normotensive rats (Mukai et al., 2001). In contrast, our results with measuring BP response of conscious rats indicated that actual BP maintenance as well as BP response to norepinephrine are less dependent on Rho kinase signalling in spontaneously hypertensive than in normotensive rats (Publications C and D). Our experiments confirmed the enhanced contribution of calcium entry through L-VDCC to the maintenance of high BP in SHR (Sonkusare et al., 2006). Experiments using isolated mesenteric or femoral arteries of SHR and WKY revealed that blockade of Rho-kinase pathway by fasudil attenuated the development of norepinephrine-induced vascular contraction in both rat strains, the relative fasudil effects being smaller in SHR than in WKY femoral arteries. Further *in vitro* experiments disclosed similar strain difference in the effects of Rho kinase inhibition on the established vascular contraction. A comparison of Rho kinase inhibitor fasudil with a more specific Y-27632 indicated that the *in vitro* relaxing effects of both drugs were similar in phenylephrine-precontracted femoral arteries. Fasudil-induced relaxations of arteries precontracted with phenylephrine

were more pronounced in WKY femoral arteries and similar results were measured with Y-27632. These data are in contrast to previous study (Nomura and Asano 2003), which is probably caused by different type of vascular constriction and different vascular bed.

In the study of prehypertensive SHR compared to age-matched WKY rats, we observed smaller contribution of Ca^{2+} sensitization but a greater role of Ca^{2+} influx in phenylephrine-induced contraction of femoral arteries. BP in young SHR rats is increasing from 3-weeks of age to hypertensive values of BP seen in animals aged 12 weeks (Albrecht 1974; Antonaccio et al., 1980). In our measurements blood pressure of 3-week-old SHR did not differ from that of age-matched WKY. Prehypertensive SHR and their blood vessels responded less to fasudil-induced Rho kinase inhibition than WKY, but their response to the blockade of L-VDCC by nifedipine was equal or even enhanced as compared to age-matched WKY. In animals with established hypertension, we also observed the enhanced role of Ca^{2+} entry through L-VDCC in addition to the lowered contribution of Rho kinase pathway. Thus, the importance of RhoA/Rho kinase pathway for BP and vascular tone control seems to be attenuated in SHR from prehypertensive stages, probably as a part of compensatory mechanisms to Ca^{2+} influx through L-VDCC to VSM which also elevated from prehypertensive stages (Lozinskaya and Cox 1997).

The contribution of calcium sensitization mediated by RhoA/Rho kinase pathway to BP maintenance *in vivo* was also attenuated in Ren-2 TGR. In contrast, the contribution of basal calcium sensitization to BP maintenance was much greater in salt-sensitive Dahl hypertensive rats than in their normotensive controls. To our knowledge, the observed changes of RhoA/Rho kinase pathway are the first reports in these rat strains. The decreased calcium sensitization in SHR and Ren-2 TGR might be a part of the compensation for pathological enhancement of calcium entry in genetically hypertensive rats (SHR and Ren-2 TGR). Experiments on endothelium-denuded femoral arteries (stimulated by phenylephrine) supported our *in vivo* findings of the attenuation of calcium sensitization in SHR and Ren-2 TGR, as well as its augmentation in salt sensitive Dahl rats. Our myography data confirm alterations of calcium sensitization described in vivo (described in Publications C-E).

6. Conclusions

In the thesis I have summarized the data concerning the mechanisms of vascular smooth muscle contraction, as well as the data related to the differences in contraction of isolated arteries from normotensive and hypertensive animals. The understanding of vascular smooth muscle contraction mechanisms in healthy individuals, as well as in hypertension might be important for future drug development. Calcium entry into the vascular smooth muscle cells enables vascular contraction and the study of selected non-selective TRP inhibitors contributed to the understanding of this mechanism in more detail. Among three non-selective TRP inhibitors used, 2-APB has been suggested as the most interesting. The observed inhibitory effects of 2-APB were dependent on the type of contraction stimulus and also on Na^+ presence in bathing solution. The enhanced contractility of arteries isolated from spontaneously hypertensive rats was studied in order to extend our understanding of the mechanisms causing hypertension in this model. The role of calcium sensitization was studied in three different models of hypertension in order to assess the role of this mechanism in different forms of hypertension. Both calcium entry and calcium sensitization contribute to the actual maintenance of blood pressure and adrenergic vasoconstriction in these three rat strains but calcium entry is more important in genetically hypertensive rats (SHR and Ren-2 TGR), in which the role of RhoA/Rho kinase pathway is attenuated. On the other hand, the role of calcium sensitization is enhanced in hypertensive salt-sensitive Dahl rats.

Calcium signalling plays a very important role in blood pressure maintenance. While voltage-dependent calcium channels are in focus of researchers for a long time and their inhibitors are widely used in hypertension treatment, in the present studies I attempted to describe some missing information about the TRP channels and calcium sensitization. For the future investigations, there are still many unanswered questions mainly about the role of specific TRP channels in blood pressure regulation. The role of TRP channels in SHR, Ren-2 TGR and Dahl rat models of hypertension is even less understood.

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8. Selected publications enclosed in full length

8.1 Publication A

Bencze M, Behuliak M, Vavřínová A, Zicha J. **Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat.** Eur J Pharmacol. 2015; 765: 533-540.

8.2 Publication B

Bencze M, Behuliak M, Vavřínová A, Zicha J. **Altered contractile responses of arteries from spontaneously hypertensive rat: The role of endogenous mediators and membrane depolarization.** Life Sci. 2016; 166: 46-53.

8.3 Publication C

Behuliak M, Pintérová M, Bencze M, Petrová M, Líšková S, Karen P, Kuneš J, Vaněčková I, Zicha J. **Ca^{2+} sensitization and Ca^{2+} entry in the control of blood pressure and adrenergic vasoconstriction in conscious Wistar-Kyoto and spontaneously hypertensive rats.** J Hypertens. 2013; 31: 2025-2035.

8.4 Publication D

Behuliak M, Vavřínová A, Bencze M, Polgárová K, Ergang P, Kuneš J, Vaněčková I, Zicha J. **Ontogenetic changes in contribution of calcium sensitization and calcium entry to blood pressure maintenance of Wistar-Kyoto and spontaneously hypertensive rats.** J Hypertens. 2015; 33: 2443-2454.

8.5 Publication E

Behuliak M, Bencze M, Vaněčková I, Kuneš J, Zicha J **Basal and activated calcium sensitization in three different forms of experimental hypertension**. Biomed Res Int. 2017, ID 8029728.