REGULATION OF CALCIUM INFLUX VIA VOLTAGE-DEPENDENT CALCIUM CHANNELS (L-VDCC) INTO THE VASCULAR SMOOTH MUSCLE OF GENETICALLY HYPERTENSIVE RATS

PhD Thesis

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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.

Signature: ...........................................

Date: ..............................................
I would like to thank all who have made this happen. First and foremost, I wish to express my deep and sincere gratitude to my supervisors, RNDr. Jaroslav Kuneš, DrSc and MUDr. Josef Zicha, DrSc, for their support, motivation, understanding and patience during the past five years. Their guidance and immense knowledge helped me during the whole time of my research and also writing of this thesis. The stimulating scientific discussions we had have been of great value for me. I also appreciate their precious time to read this thesis and to give me many constructive comments. They complemented each other perfectly and they were more than anything human. I could not have imagined having a better advisors and mentors for my Ph.D study.

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I owe my loving thanks to my fiancé and soul mate who was patiently waiting for me to finish this study. And last but not the least, I would like to thank my mother, who have encouraged and assisted me constantly throughout my life and without whose kindliness I would never attain this degree.
This work outlines my recent findings on pathophysiological mechanisms responsible for high blood pressure maintenance in genetic hypertension. It is generally accepted that this form of hypertension is characterised by abnormally elevated activity of sympathetic nervous system (SNS), which is associated with increased calcium influx to vascular smooth muscle cells via L-type of voltage-dependent calcium channels (L-VDCC) as well as with the enhanced sensitivity of contractile apparatus to the intracellular calcium mediated by RhoA/Rho-kinase pathway, both being regulated by vasoactive agonists. Our aims were to study the regulation of L-VDCC channels, to determine the role of “calcium sensitization” in hypertension and finally to evaluate which of these pathways is important in the maintenance of high blood pressure.

Using conscious spontaneously hypertensive rats and their normotensive controls, Wistar-Kyoto rats, we have confirmed the crucial role of the elevated sympathetic vasoconstriction in the maintenance of high blood pressure. We have revealed that high sympathetic tone is responsible for increased calcium influx via L-VDCC occurring in hypertension. Furthermore, our experiments based on the pertussis toxin-induced inactivation of inhibitory G proteins (G\textsubscript{i}) have demonstrated that the control of L-type calcium influx by SNS is mediated by G\textsubscript{i} protein-coupled pathway. The elimination of this pathway led to the attenuation of sympathetic vasoconstriction and to the decrease of blood pressure sensitivity to the nifedipine-induced blockade of L-VDCC in hypertensive rats. Moreover, the strong relationship between G\textsubscript{i} protein-coupled sympathetic vasoconstriction and L-VDCC was also confirmed by the results showing that both the inactivation of G\textsubscript{i} proteins and/or the blockade of L-VDCC caused a considerable rightward shift of norepinephrine dose-response curves, the effects being non-additive.

In addition, G\textsubscript{i} protein inactivation caused the augmentation of blood pressure responses to the blockade of BK\textsubscript{Ca}-dependent vasodilation suggesting that the activity of these channels is increased under these conditions. Since G\textsubscript{i} protein inactivation leads to cAMP overproduction, the increased blood pressure response to K\textsuperscript{+} channel blockade could indicate that membrane potential changes elicited by the activation of K\textsuperscript{+} channels (BK\textsubscript{Ca} and/or K\textsubscript{V}) could mediate the cAMP-induced closure of L-VDCC. We have confirmed this hypothesis and revealed that the inhibition of any of these K\textsuperscript{+} channels led to the diminution of cAMP overproduction-induced vasodilator effects in hypertensive rats, while in
normotensive rats the absence of one class of potassium channels can be compensated by the remaining K⁺ channel family pointing out the altered function of these K⁺ channels in hypertension.

It is evident that smooth muscle contractility is determined by both calcium influx through L-VDCC and RhoA/Rho-kinase-mediated calcium sensitization. Using Rho kinase inhibitor, fasudil, we have confirmed our hypothesis that blood pressure maintenance in hypertensive rats is more dependent on calcium influx through L-VDCC than on calcium sensitization.

Thus, we can conclude that augmented calcium influx into the vascular smooth muscle via L-VDCC is one of the most important factors responsible for the maintenance of high blood pressure. The increased calcium influx through these calcium channels is a result of elevated sympathetic activity mediated by Gᵢ protein-cAMP-coupled pathway. While the inhibition of cAMP pathway by Gᵢ proteins activation opens L-VDCC, the stimulation of this pathway leads to the inhibition of calcium influx via L-VDCC. The latter is mediated by the activation of BKCa and Kv channels, the activity of which seems to be different in normotensive and hypertensive rats. The altered function of these K⁺ channels in hypertension could be an interesting object of future studies. I hope that my present findings might contribute to the better understanding of pathophysiological mechanisms of genetic hypertension.
SOUHRN

Tato práce shrnuje moje současné nálezy týkající se patofyziologických mechanismů podílejících se na udržování vysokého krevního tlaku u genetické hypertenze. Je obecně uznáváno, že tato forma hypertenze je charakterizována abnormálně zvýšenou aktivitou sympatického nervového systému (SNS), která je provázena jak zvýšeným vstupem vápníku do cévních hladkosvalových buněk cestou napěťově-řízených vápníkových kanálů L typu (L-VDCC) tak i změnami citlivosti cév k intracelulárnímu vápníku zprostředkované RhoA/Rho kinázovou signální cestou. Cílem studie bylo prozkoumat regulaci vstupu vápníku cestou L-VDCC, určit úlohu RhoA/Rho kinázové cesty v hypertenze a zjistit, která z těchto cest je důležitá v udržování vysokého krevního tlaku.

Srovnáním bdělých SHR potkanů a jejich normotenzních kontrol (WKY potkanů) jsme ověřili klíčovou úlohu zvýšené sympatické vasokonstrikce při udržování vysokého krevního tlaku. Zjistili jsme, že vysoká sympatická aktivita u hypertenze je odpovědná za zvýšený vstup vápníku cestou L-VDCC. Pokusy založené na inaktivaci inhibičních G (G_i) proteinů pomocí pertusis toxinu ukázaly, že kontrola L-VDCC sympatickým nervovým systémem je zprostředkována cestou zahrnující G_i proteiny, jejíž vyřazení vede k oslabení sympatické vasokonstrikce a k poklesu odpovědí krevního tlaku na nifedipinovou blokádu L-VDCC u hypertenzních potkanů. Význam vazby sympatické vasokonstrikce, G_i proteinů a L-VDCC podtrhují nálezy, že inaktivace G_i proteinů podobně jako blokáda L-VDCC působí významným posunem křivky dávkové odpovědi na noradrenalin doprava, přičemž efekty obou zásahů nejsou aditivní.

Inaktivace G_i proteinů také zvyšuje odpověď krevního tlaku nastávající po blokádě vasodilatace závislé na vápníkem aktivovaných K^+ kanálech (BK_{Ca}), což ukazuje, že aktivita BK_{Ca} kanálů je zvyšená za těchto podmínek. Jestliže inaktivace G_i proteinů vede k nadprodukcí cyklického AMP (cAMP), zvýšená odpověď krevního tlaku na blokádu těchto kanálů naznačuje, že změny membránového potenciálu vyvolané aktivací K^+ kanálů (BK_{Ca} a K_V typu) mohou zprostředkovat uzavření L-VDCC kanálů působené cAMP. Při ověřování naší hypotézy jsme zjistili, že inhibice kteréhokoliv z těchto dvou typů K^+ kanálů vede u hypertenzních potkanů k oslabení vasodilatačních účinků vyvolaných nadprodukcí cAMP, zatímco u normotenzních potkanů může být nepřítomnost jednoho typu K^+ kanálů kompenzována činností druhého typu K^+ kanálů. To ukazuje porušenou funkci těchto K^+ kanálů u hypertenze.
Je zřejmé, že kontraktilita hladkého svalu je určována nejen vstupem vápníku skrze L-VDCC ale i citlivostí k vápníku ovlivňovanou RhoA/Rho kinázovou cestou. Použitím fasudilu, inhibitoru Rho kinázy, jsme ověřili naši hypotézu, že udržování vysokého tlaku u SHR potkanů je více závislé na vstupu vápníku cestou L-VDCC než na změnách citlivosti kontraktilního aparátu k vápníku.

Závěrem lze konstatovat, že zvýšený vstup vápníku do hladkého svalu cév cestou L-VDCC je jedním z nejdůležitějších faktorů pro udržování vysokého krevního tlaku. Zvýšený vstup vápníku skrze tyto vápníkové kanály je výsledkem zvýšené sympatické aktivity a je zprostředkován dráhou zahrnující G_i proteiny a tvorbu cAMP. Zatímco inhibice tvorby a účinků cAMP v důsledku aktivace G_i proteinů otevírá L-VDCC, stimulace této cesty vede ke snížení vstupu vápníku skrze L-VDCC. Tato inhibice vstupu vápníku je dána aktivací BK_{Ca} a K_V kanálů, jejichž aktivita se u normotenzních a hypertenzních potkanů liší. Změny funkce těchto draslíkových kanálů u hypertenze mohou být zajímavým tématem budoucích studií. Doufám, že moje současné výsledky mohou přispět k lepšímu porozumění patofyziologických mechanismů genetické hypertenze.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>AT₁</td>
<td>angiotensin II-type-1 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AV3V</td>
<td>third ventricle of the hypothalamus</td>
</tr>
<tr>
<td>BK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>large-conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>G protein</td>
<td>heterotrimeric guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>GDP, GTP</td>
<td>guanosinediphosphate, guanosine triphosphate</td>
</tr>
<tr>
<td>IK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>intermediate conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>K&lt;sub&gt;ir&lt;/sub&gt;</td>
<td>inward rectifier potassium channel</td>
</tr>
<tr>
<td>K&lt;sub&gt;V&lt;/sub&gt;</td>
<td>voltage-dependent potassium channel</td>
</tr>
<tr>
<td>L-NAME</td>
<td>nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>L-VDCC</td>
<td>L-type voltage-dependent calcium channel</td>
</tr>
<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>myosin light chain phosphatase</td>
</tr>
<tr>
<td>MND&lt;sub&gt;A&lt;/sub&gt;</td>
<td>(N)-methyl-D-aspartate</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide adenosine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus tractus solitarii</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
</tbody>
</table>
PKG - protein kinase G
PVH - paraventricular nucleus of the hypothalamus
PTX - pertussis toxin
RAS - renin-angiotensin system
RVLM - rostral ventrolateral medulla
SHR - spontaneously hypertensive rat
SKCa - small conductance calcium-activated potassium channels
SNS - sympathetic nervous system
TEA - tetraethylammonium ions
TRPV, TRPC - transient receptor potential vanilloid and transient receptor potential cation channels
VSMC - vascular smooth muscle cell
WKY - Wistar-Kyoto rat
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1. **INTRODUCTION**

1.1. **Arterial hypertension**

Arterial hypertension, or chronically elevated blood pressure, affects approximately 20-30% of adult population in the developed societies and, what is more serious, it belongs to the main risk factors responsible for the development of kidney failure and cardiovascular diseases (such as stroke, heart failure and myocardial infarction) (Carretero & Oparil, 2000). Although the hypertension is considered to be present when systolic blood pressure is consistently above 140 mm Hg while diastolic blood pressure is above 90 mm Hg, there is no specific level of blood pressure where cardiovascular and renal complications start to occur. These complications arising as a final result of chronic blood pressure elevation are often referred to as end-organ damages. Since high blood pressure usually occurs without any symptoms and can progress to the development of any complications mentioned above, the diagnosis of hypertension is important and the effort should be made to normalize blood pressure and to prevent these complications. Nowadays a considerable number of effective antihypertensive drugs is available, but the reliable blood pressure control is reached only in some hypertensive patients, most of them needing two or more drugs. Thus, hypertension and its concomitant risk factors remain uncontrolled in most patients. This is because the causes of high blood pressure development remain still unknown. The reason of blood pressure rise is clear only in 5-10% of cases. These patients have a secondary hypertension, i.e. the hypertension in these individuals develops as a consequence of a specific disorder of particular organs or blood vessels, such as the kidney (renal artery narrowing, renal damage), adrenal gland (adrenal gland hyperplasia or adenoma) or aorta (aortic coarctation). The second type of hypertension with an unknown cause is called primary, or essential hypertension, which is far more common (Oparil *et al.*, 2003; Korner, 2007).
1.1.1. Essential hypertension and its etiology

Approximately 90-95% of patients diagnosed with hypertension have essential hypertension which is, unlike secondary hypertension, a multifactorial disorder. The etiology of essential hypertension remains unknown and the disease is thought to evolve from the interaction of multiple genes with environmental factors, and bad lifestyle (stress, high salt intake, obesity, lack of regular exercise) during ontogenesis (Korner, 2007).

Although it is generally accepted that genetic factors play an important role in the development of essential hypertension, so far only little is known about the genes responsible for the onset of blood pressure rise. A variety of candidate genes have been identified, including those coding for components of renin-angiotensin system, sodium epithelial channels, catecholaminergic/adrenergic function, lipoprotein metabolism, hormone receptors and growth factors. Indeed, there are numerous studies showing the functional alterations of the sympathetic nervous (SNS) and renin-angiotensin system (RAS) in hypertensive state suggesting an important role of these two major vasoactive systems in the pathophysiology of essential hypertension (Laflamme et al., 1997; Antonaccio & Kerwin, 1981; Grassi et al., 1998a). Moreover, this can also be supported by the fact that blood pressure lowering of all widely used antihypertensive drugs (such as ACE inhibitors, AT\textsubscript{1} receptor antagonists and others) is mediated by the attenuation of the SNS function (Clough et al., 1982; Laflamme et al., 1997; Antonaccio & Kerwin, 1981). Since components of RAS are present not only in circulation but also in various tissues (such as blood vessel wall, heart, brain, kidney and other), RAS exerts autocrine and paracrine influences on local tissue function including facilitating effects on the central (Erdos et al., 2006) and peripheral SNS activity. Angiotensin II has been shown to facilitate neurotransmission at the nerve endings (de Jonge et al., 1982; Balt et al., 2003) and catecholamine release through AT\textsubscript{1} presynaptic mechanisms (Dendorfer et al., 1998), to decrease presynaptic reuptake (Raasch et al., 2004) as well as to affect postsynaptic \(\alpha\)-adrenoceptors (Richer et al., 1984; Eikenburg, 1984). Therefore, it is not surprising that in patients with essential hypertension an increased sympathetic outflow has been identified attributing to essential hypertension a neurogenic character (Grassi et al., 1998a).

Although numerous pathways involving endocrine factors, neural reflexes and vascular abnormalities are thought to contribute to essential hypertension, the final target of these effects is always the same, i.e. an increased vascular tone that is mediated by
enhanced calcium influx through voltage-dependent calcium channels. Therefore, the regulation of calcium influx through these channels and the associated calcium sensitization of contractile apparatus are the issue of the present thesis.

1.1.2. **Spontaneously hypertensive rats (SHR) as an experimental animal model of genetic hypertension**

In order to understand the pathophysiology of essential hypertension, many animal models with induced or genetic forms of experimental hypertension have been developed. These models share many features common to human hypertension and thus allow a pathophysiological analysis of the factors responsible for the development and maintenance of various types of the essential hypertension. The widely used model of genetic hypertension is the inbred spontaneously hypertensive rat (SHR) and its normotensive control Wistar-Kyoto (WKY) rat. The SHR was selectively bred for high blood pressure without any provocative dietary or environmental stimuli by Okamoto and Aoki (Okamoto & Aoki, 1963) in Kyoto, Japan. In SHR, mean arterial blood pressure is approximately 180-200 mm Hg as compared to 115-130 mm Hg in normotensive rats and is maintained at these high levels after 12 weeks of age (Zicha & Kunes, 1999). Moreover, the spontaneous development of high blood pressure in this rat strain is also accompanied by the development of characteristic hypertensive complications. It makes this strain indeed a suitable model which can replace some studies on human patients. Moreover, the SHR are characterized by an elevated activity of the sympathetic nervous system, both central and peripheral (Head, 1989; Judy & Farrell, 1979; Tsuda & Masuyama, 1991), which plays also a crucial role in the development and maintenance of hypertensive state in humans (Grassi et al., 2008; Grassi et al., 1998a; Schlaich et al., 2004).
1.2. **Neural mechanisms of blood pressure control**

1.2.1. **Role of central nervous system in the short- and long-term blood pressure control: regulation of sympathetic outflow**

Central nervous system (CNS) plays an essential role in the control and regulation of vasomotor tone and blood pressure level. The neurons involved in the regulation of cardiovascular functions are located mainly in the spinal cord, brainstem and hypothalamus. Limbic, cortical and midbrain structures are responsible for the rapid changes in sympathetic tone that relate to behaviour (Ross et al., 1984; Allen, 2002). The arterial pressure is regulated by feedback control systems, operating in both the short-term and long-term. These consist of autonomic nerves, a collection of afferent and efferent neurons linking the CNS with visceral effectors, and circulatory hormones as their effector mechanisms. The activity of sympathetic nervous system, one of the efferent arms of the autonomic nervous system, has a dominant role in both short-term and long-term blood pressure regulation. The abnormalities in the background activity of sympathetic vasomotor tone, set by a core network of neurons, are one of the main factors responsible for the development and maintenance of various forms of neurogenic hypertension (Guyenet, 2006).

1.2.1.1. **Short-term blood pressure control**

The autonomic nervous system receives continuous information from the baroreceptors, pressure-sensitive nerve endings situated in the carotid sinus and aortic arch. Their activation by increased blood pressure or electrical stimulation inhibits sympathetic vasomotor activity. Baroreceptor afferent fibres terminate within the nucleus tractus solitarii (NTS), located in caudal medulla, and excite second-order neurons via glutamatergic synapses (Li & Pan, 2010; Hayward et al., 2002). Microinjection of glutamate receptor antagonists into the NTS leads to the blood pressure increase, whereas L-glutamate or NMDA (N-methyl-D-aspartate) produce a decrease in sympathetic outflow and blood pressure (Li & Pan, 2010; Sander & Victor, 1999). The second-order glutamate-sensitive NTS neurons conveying baroreceptor signals then project to and excite (via glutamate synapse) neurons within caudal ventrolateral medulla (CVLM) and subsequently
neurons within rostral ventrolateral medulla (RVLM) (Guyenet, 2006). The RVLM contains tonically active neurons that provide a major source of tonic excitatory drive to the preganglionic neurons in the spinal cord and control sympathetic nerve discharges to the heart and blood vessels as well as a reflex regulation of blood pressure (Dampney et al., 2002). Most of these RVLM vasomotor neurons are C1 adrenaline-synthesizing neurons containing the enzymes tyrosine hydroxylase and phenylethanolamine N-methyltransferase (Ross et al., 1984). The synaptic inputs to RVLM neurons are excitatory or inhibitory and are generally mediated via glutamate or GABA B (GABA, γ-aminobutyric acid) receptors, respectively (Guyenet, 2006). The experiments of Ross et al. (Ross et al., 1984) have demonstrated that stimulation of this region by glutamate or electrical stimulation produces an elevation of arterial pressure attributable to sympathetic vasomotor fibre excitation, since the response was almost entirely blocked by cervical spinal cord transaction, pharmacological blockade of α- and β-adrenoceptors or destruction of sympathetic nerves with 6-hydroxydopamine (6-OHDA). On the other hand, GABA microinjection into the RVLM produced a marked and dose-dependent reduction of arterial pressure (Ross et al., 1984).

Vasomotor sympathetic nerve discharge is also influenced by the paraventricular nucleus of the hypothalamus (PVH) via direct connections with lower brainstem (NTS and RVLM) and spinal cord. The PVH is currently seen as a key hypothalamic integrative centre for circulatory control. Allen (Allen, 2002) has shown that inhibition of this region by the GABA A receptor agonist leads to a reduction in sympathetic nerve discharge and arterial blood pressure. His study indicates that the PVH exerts a powerful, tonic effect on the control of sympathetic vasomotor tone under basal conditions and that this effect is enhanced in SHR rats.

1.2.1.2. Long-term blood pressure control

Although it was previously believed that the ability of neural system to control arterial pressure is limited only to the detection and correction of rapid short-term changes of arterial pressure, the present evidences propose that cardiovascular homeostasis depends not only upon hormonal regulation but also upon the sympathetic nervous system (Dampney et al., 2002). Since baroreceptors are able to reset in time to the prevailing level of arterial pressure, they cannot provide a sustained negative feedback signal to the long-term regulation of arterial pressure in the face of sustained stimuli. Indeed, it was shown
that surgical removal of arterial baroreceptors does not chronically affect arterial pressure or the pathogenesis of experimental hypertension. Thus, it is apparent that the long-term basal level of sympathetic activity is regulated independently of arterial baroreceptor input (Osborn, 2005). However, the anteroventral region of the third ventricle of the hypothalamus (AV3V) was detected as a region that plays a crucial role in the long-term regulation of sympathetic activity and the pathogenesis of hypertension. The AV3V includes structures (referred to as the lamina terminalis), which are able to sense plasma concentrations of several hormones related to arterial pressure and body fluid composition. These signals are integrated and the information is transmitted to the PVH, which sends excitatory projections to the sympathetic premotor neurons in the RVLM (Osborn, 2005).

1.2.1.3. Modulatory effects of central nitric oxide and angiotensin II in the regulation of sympathetic outflow

Regulation of sympathetic nerve activity can be centrally modulated not only by glutamate and GABA but also by other systems such as neuronally produced nitric oxide or brain renin-angiotensin system. It should be mentioned that both systems also have an important role in the peripheral regulation of vascular contractility.

Nitric oxide produced in the NTS and the RVLM was shown to be an inhibitory modulator of central sympathetic outflow being specifically implicated in the modulation of synaptic transmission related to the excitatory NMDA receptors (Morimoto et al., 2000). Thus, nitric oxide produced in these regions can amplify the baroreception and contribute to the inhibition of central sympathetic outflow. Stimulation of NMDA receptors by presynaptically released glutamate causes calcium channel opening and subsequent calcium/calmodulin-dependent activation of nitric oxide synthase. The resultant production of nitric oxide potentially modulates the postsynaptic neurons. Since nitric oxide is a retrograde messenger, it also induces production of cGMP in presynaptic neurons and thus modulates glutamate release from the presynaptic terminals, the glutamate-gated channels and glutamate reuptake (Sander & Victor, 1999).

The brain renin-angiotensin system has an important role in the regulation of sympathetic nervous activity and arterial blood pressure. Central angiotensin II enhances sympathetic outflow, blunts the sensitivity of the baroreflex and stimulates secretion of vasopressin via its action at various hypothalamic and medullary areas. Almost all these central actions of angiotensin II are mediated by AT1 receptors which have been found in
vasomotor regulatory areas such as hypothalamus, including lamina terminalis and PVH as well as in RVLM and NTS. These central angiotensin II-induced modulations of sympathetic nerve activity have been shown to be mediated by NAD(P)H-oxidase-dependent production of superoxide in the hypothalamus (Erdos et al., 2006).

1.2.2. Sympathetic nervous system in blood pressure control

Sympathetic nervous system (SNS) is one of the major pressor systems involved in the regulation of blood pressure (Vanhoutte, 1981) and its enhanced efferent control of vascular smooth muscle is present in various forms of human essential as well as experimental hypertension (Head, 1989; de Champlain, 1990; Judy et al., 1979). Indeed, Judy and Farrel (1979) have demonstrated that SNS is hyperactive in the SHR compared to that of the WKY.

SNS consists of cholinergic neurons located within the CNS innervating peripheral ganglia that can control cardiovascular targets containing the noradrenergic motor neurons. Sympathetic nerves traverse in the adventitial layer of blood vessels. They have varicosities releasing norepinephrine that acts on the underlying smooth muscle and endothelial cells to regulate vascular tone (Kanagy, 2005). While Yamaguchi and Kopin (1980) have shown that there is a similar amount of catecholamines released by equivalent magnitudes of sympathetic nerve stimulation in pithed SHR, Westfall et al. (1984) have demonstrated the alterations in the in vitro evoked release of norepinephrine from blood vessels of hypertensive rats. A histofluorescent and electron microscopic analysis of the sympathetic innervations have revealed that SHR vessels had a greater density of sympathetic innervations compared to normotensive rats (Cassis et al., 1985). Furthermore, Magee and Schofield (1992) have shown that physiological frequencies of preganglionic activity are more effectively transmitted through sympathetic ganglia from SHR compared with normotensive rats. Besides, higher interstitial norepinephrine concentrations reported in SHR may depend not only on enhanced release but also on diminished reuptake/metabolism or both (Cabassi et al., 1998).
1.2.2.1. Adrenoceptors in the control of vascular contractility

The regulation of vascular contractility by sympathetic nervous system is mediated via the activation of one or more of the nine known subtypes of adrenergic receptors. The major subdivision of these receptors distinguishes between α- and β-adrenoceptors. Both classes are not homogenous and different subtypes of these adrenoceptors have been described. Alpha-adrenoceptor subtypes have been classified on a morphological basis as pre- and postsynaptic; and on the pharmacological basis as α₁- and α₂-subtypes. Both α₁- and α₂-adrenoceptors were found to be present post- as well as presynaptically (Kobinger, 1984). Postsynaptic receptors are localized on vessels of the effector organs. Beta-adrenoceptors are divided into β₁- and β₂-adrenoceptors. While β₁-adrenoceptors are present only in certain vascular smooth muscle cells (e.g. coronary), β₂-adrenoceptors are present in most vascular cells, where they cause a relaxation of vascular smooth muscle by reducing an intracellular calcium concentration through cAMP-dependent mechanism (Orlov et al., 1996). This adrenoceptor diversity and their different location are responsible for the fact that single sympathetic mediator can produce different physiological responses. Thus, the vasoconstrictor action of catecholamines is mediated by α-adrenoceptors, while catecholamine stimulation of β-adrenoceptors leads to increases in contractile force and heart rate (β₁-adrenoceptors) as well as to vascular smooth muscle relaxation (β₂-adrenoceptors) (Vanhoutte, 1981). Both adrenoceptor types are metabotropic receptors coupled to respective G proteins and G protein-coupled pathways, but this will be described in more detail in the section 1.3.2.2. focused on particular G protein pathways.

1.2.2.1.1. Central α-adrenoceptors

Central α₂-adrenoceptor family participates in the regulation of the cardiovascular system at many levels. Although all three subtypes of α₂-adrenoceptors (α₂A-, α₂B- and α₂C-adrenoceptors) are expressed within the central cardiovascular control centres, the α₂A-adrenoceptor is a predominant subtype (Kanagy, 2005) exerting a sympathoinhibitory function (Makaritsis et al., 1999).
1.2.2.1.2. Peripheral adrenoceptors

In addition to the effects on sympathetic efferent traffic, cardiovascular function is also regulated by peripheral presynaptic and postsynaptic adrenoceptors.

a. Presynaptic adrenoceptors and their effects on norepinephrine release

Presynaptic adrenoceptors have been shown to modulate the release of sympathetic neurotransmitters by either facilitating or attenuating this function during sympathetic stimulation. The presynaptic α2A-adrenoceptors have a dual role. Those located at the soma and dendrites of the neuron determine the frequency of the nerve impulses travelling along the axons, while those located on the varicose terminal decrease the amount of norepinephrine released per nerve impulse (Johansson, 1984). Thus, norepinephrine released in the synaptic cleft can suppress its further release by stimulation of these adrenoceptors. This is an important negative feedback mechanism in noradrenergic transmission because the attenuation of α2A-adrenoceptor-mediated inhibition of neurotransmitter release can lead to a hypertensive state (Tsuda & Masuyama, 1991).

On the other hand, the activation of presynaptic β2-adrenoceptor subtype facilitates the norepinephrine release from sympathetic nerve terminals and exerts a positive feedback modulation of norepinephrine release (Esler et al., 2001). Westfall et al. (1984) have demonstrated that stimulation of β-adrenoceptors produced a similar degree of enhancement of the field-stimulation-induced norepinephrine release in vessels obtained from SHR and WKY.

However, there are also other factors that can modulate norepinephrine release such as dopamine inhibiting the stimulation-evoked norepinephrine release, which is attenuated in SHR (Tsuda & Masuyama, 1991), as well as angiotensin II having facilitatory effect on norepinephrine release, which is significantly enhanced in SHR (Westfall et al., 1984).

b. Postsynaptic adrenoceptors

The postsynaptic sympathetic control of vascular peripheral resistance is mediated by vasoconstrictor α-adrenoceptors (Gavras & Gavras, 2001; Villalobos-Molina et al., 1999) and vasodilator β-adrenoceptors (Borkowski & Porter, 1983). The response evoked
by catecholamines is determined by the relative activation of these two receptor subtypes. The postsynaptic $\alpha_1$- and $\alpha_2$-adrenoceptors on vascular smooth muscle cell contribute to increased peripheral vascular resistance and to the control of blood pressure. Duka et al. (2000) reported that as much as 68% of the adrenergically induced vasoconstriction is mediated by postsynaptic $\alpha_1$-adrenoceptors, more specifically $\alpha_{1D}$-adrenoceptors alterations of which may have a role in the pathogenesis of hypertension in SHR (Villalobos-Molina et al., 1999). The rest of the catecholamine-induced vasoconstriction is attributed to $\alpha_2$-adrenoceptors (Duka et al., 2000). Vascular smooth muscle cells express three $\alpha_2$-adrenoceptor subtypes the activation of which leads to vasoconstriction. However, the distribution of individual subtypes varies between vascular beds, between species and between large and small vessels. $\alpha_{2A}$-adrenoceptors appear to be expressed exclusively in large arteries, whereas $\alpha_{2B}$-adrenoceptors in small arteries and thus play an important role in the regulation of total peripheral vascular resistance (Kanagy, 2005). Although no significant hemodynamic effects of $\alpha_{2C}$-adrenoceptors are known (Duka et al., 2000), they probably play an important role in cold-induced vasoconstriction and also contribute to cerebral blood flow by regulating the tone of carotid artery (Kanagy, 2005).

On the other hand, the activation of $\alpha_2$-adrenoceptors on endothelial cells increases production of nitric oxide as well as other calcium-driven vasodilators and hence promotes smooth muscle relaxation. However, specific receptor subtype mediating this effect is not defined yet (Kanagy, 2005).

In addition to increased $\alpha$-adrenergic vasoconstriction, a reduced $\beta$-adrenoceptor-mediated membrane hyperpolarization and vasodilation is suggested to be another determinant of elevated sympathetically mediated vascular smooth muscle tone in hypertension. Although some authors have really found a decreased $\beta$-adrenoceptor-mediated relaxation in arteries from SHR compared to those from WKY (Dawes et al., 1997), there were also reported contrary data showing an increased $\beta$-adrenoceptor-mediated vasodilation in SHR rats (Deragon et al., 1978; Carvalho et al., 1987). The latter data are supported by the results of Bucher et al. (Bucher et al., 1984) demonstrating increased endogenous $\beta$-adrenergic stimulation of the cyclic AMP system in SHR. These discrepancies could be explained by parallel enhancement of $\beta$-adrenoceptor expression accompanied by enormous increase in $\alpha$-adrenoceptor expression in SHR compared to WKY rats (Oliver et al., 2009). Thus, this imbalance between the opposing adrenoceptor-mediated effects leads to a relative attenuation of $\beta$-adrenergic function.
1.3. **Vascular mechanisms of blood pressure control**

1.3.1. **Membrane potential in hypertension**

Although neural and humoral factors influence both central and peripheral sites to regulate arterial blood pressure, the final common pathway for the control of vascular reactivity, and ultimately peripheral vascular resistance, is located at the level of membrane and intracellular processes of the vascular smooth muscle cell (VSMC). Since membrane potential changes of few millivolts cause significant changes in blood vessel diameter (Nelson & Quayle, 1995), knowledge of the membrane processes responsible for vascular smooth muscle cell activation seems to be crucial for understanding of the complete scope of blood pressure regulation. Indeed, the relevance of the VSMC membrane potential in hypertension was indicated by the proportionality between the magnitude of membrane potential and vascular smooth muscle contractile force (Harder et al., 1981). These and other experiments (Stekiel et al., 1986) have also shown that the in situ membrane potential of small mesenteric vessels (i.e. arteries as well as veins) in SHR with established hypertension is relatively depolarized compared to normotensive WKY (-43 mV in mesenteric arteries from WKY and -38 mV from SHR rats; see also Table 1). Although there were no significant differences in two-week-old WKY compared to age-matched SHR, the changes became obvious in 12- to 16-week-old animals (Harder et al., 1981). These differences of membrane potential were demonstrated to be independent of high systolic blood pressure as well as of structural changes that occur in the vessel as a result of the blood pressure elevation (Campbell et al., 1981). The experiments with arteries transplanted into the anterior eye chamber have proved that there is a neural or humoral trigger occurring at an early age that determines membrane properties of arterial VSMC which determine exaggerated responsiveness to vasoconstrictor agents in SHR. The interrelationship between nerve and smooth muscle cells called trophic phenomena may be defined as a long-term interaction affecting or regulating VSMC membrane properties (Abel & Hermsmeyer, 1981; Campbell et al., 1981). It appears that the sympathetic nervous system of the SHR has abnormal trophic influence that is primarily responsible for the development of the altered membrane properties characteristic for this hypertensive strain (Abel & Hermsmeyer, 1981).
1.3.1.1. Membrane potential and increased neural input

Harder et al. (1981) have revealed an interesting fact that the membrane potential of small mesenteric vessels of hypertensive SHR differs markedly from normotensive WKY when measured in vivo but not when measured in vitro (Table 1). This difference seen in vivo can be abolished if neural input is blocked by tetrodotoxin or if α-adrenoceptors are blocked by phenoxybenzamine (Willems et al., 1982) suggesting an important role of sympathetic nervous system in the regulation of VSMC membrane potential. Indeed, reduced transmembane potential in small resistance vessels of the SHR is dependent on an intact supraspinal pathway and thus a functional adrenergic innervation (Willems et al., 1982). Harder et al. (1981) have also shown that the targets of an increased sympathetic activity are both the precapillary resistance as well as the postcapillary capacitance vessels. Furthermore, both vessel types of SHR were selectively hyperpolarized in situ either by neural blockade with tetrodotoxin or by chemical sympathectomy with 6-OHDA to the membrane potential level measured in the respective WKY vessels (Stekiel et al., 1986). These observations again support the role of increased sympathetic neural tone in vascular smooth muscle of SHR with established hypertension. Increased neural influence contributing to the maintenance of hypertension can be mimicked by the alterations in synthesis, release, reuptake and metabolism of norepinephrine (Harder & Hermsmeyer, 1983) as well as by the strong α-adrenergic input to small blood vessels in SHR which, as mentioned above, masks an endogenous hyperpolarizing β-adrenergic influence present in both WKY and SHR (Willems et al., 1982).

Table 1. Membrane potential values of small mesenteric veins of normotensive WKY and hypertensive SHR (Harder et al., 1981)

<table>
<thead>
<tr>
<th></th>
<th>In situ (mV)</th>
<th>In vitro (mV)</th>
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<tr>
<td></td>
<td>Tetrodotoxin</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>WKY</td>
<td>-49</td>
<td>-48</td>
</tr>
<tr>
<td>SHR</td>
<td>-34</td>
<td>-45</td>
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</table>
1.3.1.2. Membrane potential and ion transport

If agents that act on membrane ion transport cause depolarization or hyperpolarization of sufficient magnitude, they induce vascular contraction or relaxation, respectively. It has been shown that arterial smooth muscle cells from the SHR have elevated ion transport capacity increasing the electrogenic component of the resting membrane potential by 5 to 7 mV over those of WKY (Hermsmeyer, 1976). Stekiel et al. (1986) have suggested that the enhanced sympathetic neural input in small mesenteric vessels of SHR increases the permeability of vascular smooth muscle membrane to Na\(^+\) and K\(^+\), thus causing an elevation of electrogenic Na\(^+\)-K\(^+\) pump activity. One of the reasons for increased membrane permeability in vascular smooth muscle cells might be the existence of transient receptor potential channels, namely their TRPM family, which represents a group of non-selective cation channels (Early & Brayden, 2010). TRPM4 channel is highly selective for monovalent cations (such as Na\(^+\)) and it requires high levels (>1 μM) of intracellular calcium for its activation. This channel is exclusively activated by calcium released from sarcoplasmic reticulum following IP\(_3\) receptor stimulation. TRPM4 activation enables a short-lasting Na\(^+\) influx leading to membrane depolarization (Gonzales et al., 2010). This explains why agonist-induced stimulation of G protein-coupled receptors (G\(_{q/11}\)) causes a rapid nifedipine-insensitive phasic contraction (due to Ca\(^{2+}\) release from sarcoplasmic reticulum), which is followed by membrane depolarization (due to TRPM4 activation) leading to prolonged nifedipine-sensitive tonic contraction of vascular smooth muscle (Paulis et al. 2007). In addition, Na\(^+\) influx can cause not only depolarization of plasma membrane, but also reversal of the Na\(^+\)-Ca\(^{2+}\)-exchanger resulting in the calcium influx into the VSMC (Poburko et al., 2006).
1.3.2. **Heterotrimeric G proteins**

Heterotrimeric guanine nucleotide-binding proteins (G proteins) serve as ubiquitous signal transducers and regulators of cellular signalling in all eukaryotic cells. They are attached to the cell surface plasma membrane and transfer signals from membrane-bound G protein-coupled receptors to intracellular effectors such as adenylyl cyclase, phospholipase and ion channels (Neves et al., 2002). Thus extracellular stimuli, such as hormones, neurotransmitters, chemokines, and other local mediators, can be transduced into respective intracellular signalling pathways and produce appropriate cellular responses.

G proteins consist of three subunits, G_α, G_β, and G_γ. Under physiological conditions, G_β and G_γ subunits are tightly associated (non-covalently) and form G_βγ complex (Clapham & Neer, 1993). Currently, there are 20 known G_α, 6 G_β and 11 G_γ subunits. The G_α subunits are classified into subfamilies by its sequence homology and the downstream signal. These include four major families - G_q/11, G_s, G_i/o and G_12/13 (Neves et al., 2002).

1.3.2.1. **Mechanism of G protein action**

G proteins became active on binding GTP but their intrinsic ability to hydrolyze GTP converts them to inactive GDP-binding form. Only G_α subunits bind and hydrolyze GTP. G proteins remain in inactivated state until the GDP is exchanged for the GTP in response to agonist activation of G protein-coupled receptors. Binding of GTP activates the G_α subunit and causes this subunit to dissociate from G_βγ complex and hence activate the downstream effectors. The activated state lasts until GTP is hydrolyzed to GDP by the intrinsic GTPase activity of G_α subunit. Subsequent reassociation of G_α with G_βγ complex turns off signal transduction and primes the system to respond to a new stimulus (Clapham & Neer, 1993). Initially, G_βγ complex was considered to act as a binding partner for the G_α subunit which suppresses spontaneous signalling and provides a membrane anchor for the G_α subunit due to its hydrophobic character. However, recent evidences indicate that G_βγ complex also plays an important role in intracellular signalling (Milligan & Kostenis, 2006).

In addition to the regulation of G protein activity by extracellular stimuli, the activity of G_α subunit can also be modulated by extraneous factors such as bacterial toxins which modify the properties of G proteins. The G_sα subunit can be specifically ADP-ribosylated by cholera toxin (exotoxin of *Vibrio cholerae*) and thus irreversibly activates G_s proteins mediating the activation of adenylyl cyclase. On the other hand, G_iα subunit can be ADP-
ribosylated by pertussis toxin (exotoxin of *Bordetella pertussis*) at its COOH-terminal region preventing thus the interaction of G₁ protein with the receptor. This results in the inhibition of adenylyl cyclase activity (Birnbaumer *et al.*, 1991). The G₁βγ complex plays an important role in ADP-ribosylation of G₁α subunit by pertussis toxin. It was revealed that this can happen only when G₁α subunit is associated with G₁βγ complex (Graf *et al.*, 1992). Thus, cholera and pertussis toxins are invaluable tools for the study of the role of Gₛ and Gᵢ proteins.

### 1.3.2.2. G protein pathways

As mentioned above, the Gₛ subunits are divided into four subfamilies, G₉/₁₁, Gₛ, G₁₂/₁₃ and G₁₂/₁₃, on the basis of gene sequence similarity. This classification serves to define both receptor and effector coupling specificity, except when a signal is transferred through G₁βγ complex.

Gₛ pathway stimulates adenylyl cyclase activity leading to accumulation of cAMP and to a subsequent activation of cAMP-dependent protein kinase (Milligan & Kostenis, 2006). This pathway mediates vasodilator action of β-adrenergic stimulation and has an important role in the regulation of calcium influx into vascular smooth muscle cells through L-type of voltage-dependent calcium channels (Orlov *et al.*, 1996) (Fig.1).

In contrast to Gₛ protein family, G₁₂/₁₃ pathway mediates the inhibition of adenylyl cyclase activity leading to a decrease of cAMP levels (Anand-Srivastava, 1992). Many important hormones and neurotransmitters, including norepinephrine, acetylcholine, dopamine and serotonin, use this pathway to evoke their physiological responses (Neves *et al.*, 2002). Activation of Gᵢ proteins is also involved in the regulation of the α₂-adrenoceptor-mediated arterial contraction (Li & Triggle, 1993) leading to the opening of L-type of voltage-dependent calcium channels through cAMP-dependent mechanism (Pinterova *et al.*, 2010).

G₉/₁₁ pathway is activated by calcium-mobilizing hormones and stimulates PLC-β to produce the intracellular messengers, inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ triggers the release of calcium from intracellular stores, and DAG recruits protein kinase C to the membrane and activates it (Wickman & Clapham, 1995).

Activation of G₁₂ and/or G₁₃ proteins is associated with stimulation of the low-molecular-weight G proteins of Rho (Ras homologous) family, belonging to Ras superfamily. G₁₂ protein has been reported to directly interact with a GTPase-activating protein for Ras, RasGAP; while G₁₃ activates a guanine nucleotide exchange factor for Rho.
(RhoGEF) (Neves et al., 2002) leading to the activation of Rho-mediated pathway involved, among others, in calcium sensitization of the vascular smooth muscle (Uehata et al., 1997). This pathway will be discussed in the chapter 1.3.5.1.

1.3.2.3. G proteins in hypertension

The altered activity of adenylyl cyclase and its responsiveness to various hormonal stimuli has been shown to be implicated in the pathogenesis of various forms of hypertension (Amer, 1975). Anand-Srivastava (1992) and Thibault et al. (1992) have shown that the level of $G_{sat}$ expression is unaltered, whereas the expression of $G_{ia-2}$ and $G_{ia-3}$ subunits is enhanced at protein as well as mRNA levels in SHR aorta. This enhanced expression of genes for $G_i$ proteins results in the decreased levels of cAMP, which precedes the development of high blood pressure in this strain (Marcil et al., 1997). Later reports confirmed the suggestion that altered $G_i$ protein-mediated pathway contributes to the development (Li & Anand-Srivastava, 2002) as well as to the maintenance (Kost, Jr. et al., 1999) of high blood pressure in SHR. $G$ proteins seem to play an important role also in the pathogenesis of human essential hypertension (Siffert, 2003). The enhanced cellular signalling by $G_i$ proteins was disclosed in lymphoblasts (Siffert et al., 1995) and skin fibroblasts (Pietruck et al., 1996) of hypertensive patients. This enhanced $G$ protein activation in a subgroup of patients with essential hypertension is coupled with C825T polymorphism in $G_i$ protein $\beta 3$ subunit gene (Siffert et al., 1998).

In various forms of experimental hypertension including SHR, $G_i$ proteins can also be up-regulated by superoxide radicals and angiotensin II (Lappas et al., 2005; Marcil & Anand-Srivastava, 1995; Anand-Srivastava, 1997). Indeed, Pandey and Anand-Srivastava (1996) have reported that one of the possible mechanisms by which captopril lowers blood pressure in SHR may be due to its ability to modulate the levels of $G$ proteins and adenylyl cyclase activity.

1.3.3. Ion channels and vascular tone

Regulation of contractile activity of vascular smooth muscle cells is dependent on the complex interplay of vasodilator and vasoconstrictor stimuli of diverse origin and all of these signals are integrated to determine the contractile activity of the smooth muscle cells
and hence a vascular tone. Ion channels in the plasma membrane of vascular smooth muscle as well as overlying endothelial cells play a central role in this process (Jackson, 2000).

1.3.3.1. Calcium channels in vascular smooth muscle cells: L-type of voltage-dependent calcium channels

Although it has been described that vascular smooth muscle cells contain both types of the voltage-dependent calcium channels, i.e. L-type as well as T-type (Benham et al., 1987), the role of dihydropyridine-sensitive L-type voltage-dependent calcium channels (L-VDCC) appears to be predominant (Hughes, 1995). Much of the calcium that activates the contractile apparatus in smooth muscle enters the cell during periods of depolarization through these channels (Sanders, 2001). Moreover, many studies attribute the increased vascular tone of SHR to the elevated calcium entry into smooth muscle cells through L-VDCC (Sonkusare et al., 2006; Cox & Rusch, 2002). Nelson et al. (1988) have shown that L-VDCC can be activated by norepinephrine. Thus, the calcium influx through L-VDCC is necessary for norepinephrine-induced tonic contraction of arteries in vitro and decisive for blood pressure level in vivo (Paulis et al., 2007).

1.3.3.1.1. Molecular structure and physiological properties of L-type of voltage-dependent calcium channels

Voltage-dependent calcium channels of vascular smooth muscle cells are multi-subunit protein complexes composed of a central pore-forming \( \alpha_{1C-b} \) subunit and additional \( \beta \) subunit and \( \alpha_2\delta \) complex. Large \( \alpha_1 \) subunit confers most functional properties to the calcium channel, including voltage-sensing, calcium permeability, calcium-dependent inactivation and inhibition by dihydropyridine calcium channel blockers. The structure of the \( \alpha_{1C-b} \) subunit includes four repeating segments (I, II, III, IV) each composed of 6 transmembrane spanning domains (S\(_1\)-S\(_6\)) (Sonkusare et al., 2006). Signalling molecules, such as protein kinase A and protein kinase C, which are the important regulators of L-type calcium channels, can bind to intracellular domains of \( \alpha_{1C-b} \) subunit to modify a gating of these channels. Other subunits are necessary for the regulation of pore formation, gating and kinetics of the channel (Sanders, 2001). The intracellular \( \beta \) subunit modulates the
availability of α₁C subunits at the surface membrane (Keef et al., 2001). The function of α₁C-b subunit also depends on the association with the α₂δ complex, where the δ subunit is anchored in the membrane and the extracellular α₂ subunit interacts with the α₁C-b subunit (Sonkusare et al., 2006; Sanders, 2001).

Voltage-dependent calcium channels exist in at least three functional states: resting, inactivated and open. The resting and inactivated states are non-conducting forms of the channel. At negative membrane potentials, calcium channels reside primarily in the resting state, which can be easily turned into the open state by membrane depolarization. However, this cannot be readily reached from the inactivated state (Nelson & Worley, 1989). Thus, membrane depolarization causes a large increase in calcium influx by increasing the open-state probability and then calcium influx decreases as inactivation progresses during maintained depolarization (Sonkusare et al., 2006). Moving the channels into the inactivated state increases the affinity of these channels for dihydropyridine calcium channel blockers. Thus, the action of dihydropyridine calcium channel blockers seems to be voltage-dependent (Nelson & Worley, 1989). Moreover, the inactivation of these calcium channels seems to be both voltage- and calcium-dependent (Sonkusare et al., 2006).

1.3.3.1.2. Calcium channel blockers

Regulation of calcium influx through voltage-dependent calcium channels is an important mean of controlling the contractile state of smooth muscles. These channels are highly susceptible to the blockade by 1,4-dihydropyridine calcium channel antagonists, such as nifedipine, nitrendipine and others. Dihydropyridine calcium channel blockers are more effective in the lowering of blood pressure and peripheral resistance in hypertensive compared with normotensive rats (Ishii et al., 1980; Kazda et al., 1985; Takata et al., 1983). This could be attributed to the differences in the control of transmembrane calcium influx of vascular smooth muscle cells between hypertensive and normotensive animals (Orlov & Postnov, 1980). Van Meel et al. (1983) suggested a similar type of interaction for the calcium entry blockers in vivo and in vitro, i.e. inhibition of transmembrane influx of extracellular calcium. The potency of calcium entry blockers to depress the α₂-adrenoceptor-mediated increase in diastolic pressure in vivo was linearly correlated with the activity of the substances to inhibit contraction after K⁺-depolarization in vitro. This is in agreement with our recent findings (Paulis et al., 2007).
1.3.3.1.3. Alterations of calcium channels in hypertension

Several studies indicated increased calcium channel activity in hypertensive compared with normotensive rats (Matsuda et al., 1997; Ohya et al., 1993; Ohya et al., 1998) contributing significantly to tonic force maintenance during hypertensive state (Matsuda et al., 1997; Paulis et al., 2007). Ohya et al. (1993; 1998) have shown that enhanced amplitude of the whole-cell current in SHR rats can be attributed to the increased number of single L-VDCC openings without evidence of altered single-channel conductance or open-time distribution. Thus, the increased number of functional calcium channel proteins rather than altered channel properties may account for elevated L-VDCC current in the vascular smooth muscle cells of the SHR (Ohya et al., 1998). Indeed, Pratt et al. (2002) have provided the first evidence of increased expression of the pore-forming α1C subunits of the L-VDCC in SHR arteries. This L-VDCC upregulation is promoted by high blood pressure and even short-term rises of intravascular pressure are capable to increase the expression of α1C subunits (Pesic et al., 2004). Membrane depolarization has been demonstrated to be a stimulus associated with elevated blood pressure that promotes L-VDCC expression at the cell membrane (Sokusare et al., 2006). However, these findings suggest not only quantitative differences in calcium channel expression in SHR but also differences in the regulation of these channels by intracellular factors (Ohya et al., 1993).

1.3.3.1.4. Regulation of calcium influx through L-VDCC

As mentioned above, L-VDCC channels are crucial for excitation-contraction coupling of vascular smooth muscle cells. These channels are regulated by various second messengers belonging to dilatation as well as contraction promoting pathways, which ultimately control the contractile state of vascular smooth muscles. In many cases, alterations in calcium influx are regulated by modulations of membrane potential that are mediated by the activation of potassium or chloride channels. Two major antagonistic players in the regulation of blood pressure, sympathetic nervous system and nitric oxide, elicit their actions via the control of calcium influx through L-VDCC. While nitric oxide dilates resistance vessels by direct and/or indirect closure of L-VDCC (Lewis et al., 2005) through cGMP signalling pathway (Tewari & Simard, 1997), the stimulation of vessels by norepinephrine increases L-type calcium current (Nelson et al., 1988; Benham & Tsien, 1988) through G, protein-mediated mechanism resulting in the decrease of cAMP level (Li
& Triggle, 1993; Li & Anand-Srivastava, 2002). While low doses of cAMP enhance L-type calcium channel current (Taguchi et al., 1997; Ruiz-Velasco et al., 1998), higher levels of intracellular cAMP lead to its inhibition (Liu et al., 1997a; Ishikawa et al., 1993). This could be explained by the fact that low cAMP concentrations are excitatory due to the stimulation of PKA, whereas higher cAMP levels lead to cross-over activation ofPKG inhibiting L-VDCC activity (Ruiz-Velasco et al., 1998). A possible pathway through which cAMP/PKA inhibit calcium entry through L-VDCC could be the activation of large-conductance Ca\(^2+\)-dependent potassium channels (BK\(_{Ca}\)) (Sadoshima et al., 1988; Scornik et al., 1993) leading to a hyperpolarization of cell membrane (Ousterhout & Sperelakis, 1987).

1.3.3.2. Potassium channels

The crucial role of potassium channels in the vasculature consists in their ability to set membrane potential and hence to determine and to regulate the vascular tone by controlling L-type calcium influx into vascular smooth muscle as well as into endothelial cells (Nelson & Quayle, 1995). Since relatively little is known about potassium channels localized in the endothelium, this chapter will be focused on the properties and roles of potassium channels expressed in vascular smooth muscle cells.

1.3.3.2.1. Potassium channels in vascular smooth muscle cells

Vascular smooth muscle cells appear to express at least four different types of potassium channels. These include calcium-activated (K\(_{Ca}\)), voltage-dependent (K\(_V\)), ATP-sensitive (K\(_{ATP}\)) and inward rectifier (K\(_{ir}\)) potassium channels. The expression of these four channel classes has been reported to vary among vascular beds as well as with vessel size.

- Large-conductance calcium-activated potassium channels (BK\(_{Ca}\))

The dominant K\(_{Ca}\) channels expressed by smooth muscle cells are large-conductance, BK\(_{Ca}\) channels, with a single-channel conductance of 200-240 pS (Jackson, 2000). These channels are activated by increased intracellular calcium concentration as well as by membrane depolarization (Nelson & Quayle, 1995). Under the low intracellular calcium,
the BK$_{Ca}$ channels behave as a pure voltage-dependent potassium channels (Ledoux et al., 2006). BK$_{Ca}$ are composed of pore-forming Slo1 (or $\alpha$) subunits and Slo$\beta_1$ (or $\beta_1$) subunits that modulate the calcium sensitivity of the channel. Slo$\beta_1$ subunit, however, appears to be uniquely expressed in smooth muscle cells and its co-expression with Slo1 subunit results in BK$_{Ca}$ channels with increased calcium sensitivity (Amberg et al., 2003).

BK$_{Ca}$ channels do not contribute to resting membrane potential under normal conditions. However, they are opened and play an important negative feedback role during active agonist-induced vasoconstriction due to membrane depolarization and elevated calcium influx. These channels exist in signalling complexes with L-VDCC channels, protein kinases (PKA, PKC, PKG), phosphatases and other signalling proteins (Jackson, 2005). A number of vasodilators, including nitric oxide (Fukami et al., 1998; Hamaguchi et al., 1992), prostacyclin (Clapp et al., 1998), epoxides of arachidonic acid (EETs) (Li et al., 1997) or $\beta$-adrenoceptor-mediated relaxation (Tanaka et al., 2003), activate BK$_{Ca}$ channels either directly or by activation of protein kinases (through cAMP or cGMP pathway).

Pharmacologically, they can be blocked by millimolar concentrations of tetraethylammonium ions (TEA); toxins such as charybdotoxin (non-selective), iberiotoxin (highly selective); and indoles such as paxilline. Conversely, compounds such as NS 1619 and NS 004 activate these channels (Nelson & Quayle, 1995).

- **Small conductance calcium-activated potassium channels (SK$_{Ca}$)**

Some smooth muscles express also small conductance K$_{Ca}$ (SK$_{Ca}$) channels, which have a single-channel conductance of 10 pS. They require calmoduline for calcium sensitivity and are blocked by apamin. However, their physiological function in smooth muscles has not been well studied (Jackson, 2005).

- **Voltage-dependent potassium channels (K$_V$)**

Another ubiquitous class of potassium channels expressed in vascular smooth muscle cells are voltage-sensitive potassium channels that contribute to resting membrane potential and vascular tone. K$_V$ channels are thought to have a basic structure very similar to that of L-VDCC channels (Nelson & Quayle, 1995). They are composed of pore-forming KV$_\alpha$ subunits and accessory KV$_\beta$ subunits (Jackson, 2005). K$_V$ channels activate in response to
membrane depolarization and participate in the negative feedback regulation of membrane potential along with BK$_{Ca}$ channels. They are also activated by vasodilators acting via cAMP signalling pathway such as adenosine or prostacyclin. Conversely, vasoconstrictors close K$_V$ channels through signalling pathway involving protein kinase C and calcium ions. Pharmacological blockers of K$_V$ channels are 4-aminopyridine, correilide, and agitotoxin-2 (Jackson, 2000; Jackson, 2005).

- **ATP-sensitive potassium channels (K$_{ATP}$)**

  These channels also appear to play a role in the mechanisms of action of both vasodilators and vasoconstrictors. They have been implicated in the vasodilation induced by adenosine, prostacyclin, calcitonin-gene-related-peptide and nitric oxide. They are closed when intracellular ATP concentration increases, however, they can also be regulated by other intracellular signals including ADP, H$^+$ and Ca$^{2+}$. K$_{ATP}$ channels may be activated by protein kinase A and cGMP-dependent protein kinase. Conversely, activation of protein kinase C and elevation of intracellular calcium by vasoconstrictors such as norepinephrine, vasopressin, endothelin and angiotensin II close these channels. K$_{ATP}$ channels can also be blocked by sulfonylureas like glibenclamide and opened by activators such as pinacidil and cromakalim (Jackson, 2005).

- **Inward rectifier potassium channels (K$_{ir}$)**

  These potassium channels conduct potassium ions into the cells at the membrane potentials negative to the potassium equilibrium potential. However, at more positive potentials, outward K$^+$ current is limited (Jackson, 2000). These channels are blocked by Ba$^{2+}$ ions at micromolar concentrations and are activated by increases in extracellular K$^+$. K$_{ir}$ channels can be also activated by endothelial-derived hyperpolarizing factor (EDHF), bradykinin, protein kinases or by G proteins (Jackson, 2005).

  Properties and function of K$_{ir}$ channels vary among vascular beds as well as with vessel size (Jackson, 2005). The expression of K$_{ir}$ channels is more abundant in the smooth muscle of autoregulatory vascular beds such as the coronary and cerebral circulations. In the systemic circulation, the expression of the K$_{ir}$ channel increases as the diameter of the artery decreases (Haddy et al., 2006). In the coronary and cerebral microcirculation, smooth
muscle $\text{K}_{ir}$ channels serve as sensor for increases in extracellular $\text{K}^+$ from 5 mM to 8-15 mM, leading to membrane hyperpolarization and vasodilation (Jackson, 2005). However, very little is known about their role in the regulation of resting membrane potential and vascular tone (Jackson, 2000).

1.3.3.2.2. Endothelial cell potassium channels

Although endothelial cells also express a similar complexity of potassium channels as vascular smooth muscle cells including $\text{K}_{\text{ATP}}$, $\text{K}_V$, $\text{K}_{ir}$ and $\text{K}_{\text{Ca}}$ channels, relatively little is known about these channels. They play an important role in endothelial cell signalling through the regulation of endothelial cell membrane potential. Due to the absence of voltage-dependent calcium channels, the endothelial membrane hyperpolarization by activation of potassium channels results in calcium influx into endothelial cells through transient receptor potential channels (TRPC and TRPV) (Jackson, 2005). The increase of intracellular calcium results in vascular relaxation through generation of endothelium-derived vasodilator factors including nitric oxide, prostacyclin and EDHF (Ledoux et al., 2006). Moreover, endothelial cells are electrically coupled to one another by gap junctions as well as to overlying smooth muscle cells by myoendothelial gap junctions. Thus, the changes in endothelial cell membrane potential can be transmitted to smooth muscle cells in order to modulate vascular tone (Jackson, 2005; Ledoux et al., 2006).

Endothelial cells express at least two classes of calcium-activated potassium channels: small conductance ($\text{SK}_{\text{Ca}}$) and intermediate conductance ($\text{IK}_{\text{Ca}}$) channels. Both channel types have smaller single-channel conductance as compared with $\text{BK}_{\text{Ca}}$ channels (10 pS for $\text{SK}_{\text{Ca}}$ and 30-80 pS for $\text{IK}_{\text{Ca}}$) (Jackson, 2005). Distinct from $\text{BK}_{\text{Ca}}$ channels, these channels are not voltage-activated and their calcium sensitivity is ascribed to the association with the calcium-binding protein, calmodulin. They are insensitive to iberiotoxin and TEA. However, $\text{SK}_{\text{Ca}}$ channels can be blocked by apamin and tetrabutylammonium ions and $\text{IK}_{\text{Ca}}$ channels are blocked by charybdotoxin, clotrimazole and its analogues TRAM 34 and TRAM 39. Both channels mediate an agonist-induced hyperpolarization of endothelial cells and play a major role in vasodilation induced by endothelium-derived vasodilators (acetycholine, bradykinin or histamine).

Physiological function of endothelial $\text{K}_V$ channels has not been established yet. They may participate in membrane potential oscillation and negative feedback regulation of membrane potential. Endothelial $\text{K}_{ir}$ may act as a sensor for elevated extracellular
potassium and provide a hyperpolarizing signal to alter vessel function. In addition, they have the potential to act as amplifier of hyperpolarization initiated by the opening of other potassium channels (Jackson, 2005).

1.3.3.2.3. Potassium channel alterations in hypertension

Defects in potassium channel function may lead to vasoconstriction and hence these alterations may be involved in pathogenetic mechanisms of hypertension (Nelson & Quayle, 1995). Indeed, the abnormalities of potassium channel function were reported in vessels from hypertensive animals. It was shown that under the experimental conditions which reflect physiological ones, BK\textsubscript{Ca} currents are larger and KV currents are smaller in VSMCs from hypertensive animals (Cox, 2002). One of the mechanisms responsible for larger BK\textsubscript{Ca} current during hypertension appears to be increased expression of BK\textsubscript{Ca} channel proteins which probably occurs as a negative feedback response to the increased vascular tone (Liu et al., 1997b). Despite this increased expression, there are major changes in BK\textsubscript{Ca} channel unit composition affecting the calcium sensitivity of BK\textsubscript{Ca} channels and hence leading to increased vascular tone (Amberg et al., 2003). Considering these results, it seems that regardless of the increased BK\textsubscript{Ca} currents reported during hypertension, the altered function of BK\textsubscript{Ca} channels is insufficient to oppose the effects of vasoconstrictors.

It seems that a decreased function of KV channels in VSMCs from hypertensive animals can also lead to depolarization and hence to increased vascular tone (Jackson, 2000). In hypertension, a functional down-regulation of KV channels was demonstrated, although the expression of the channel protein was increased. This functional down-regulation can be related to increased intracellular calcium observed in hypertensive animals (Cox & Rusch, 2002). Although K\textsubscript{ATP} channels appear to be also down-regulated in hypertension, the direct evidence is missing (Jackson, 2005).
1.3.4. Role of intracellular calcium in hypertension

Although the development of genetic hypertension evolves from the interaction of multiple factors of diverse origin involving endocrine factors, neural reflexes and vascular abnormalities, one hallmark is an anomalous vascular tone arising from altered calcium influx. Calcium is a fundamental second messenger in vascular smooth muscle cells and the increase of intracellular concentration is the primary mechanism mediating activation-contraction coupling. The increase in cytoplasmic free calcium occurs as a result of both intracellular calcium release and extracellular calcium influx. Agonist stimulation of smooth muscle results in the activation of phospholipase C, which increases the production of second messenger inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ promotes the release of calcium stores in the sarcoplasmic reticulum with the subsequent increase in intracellular free calcium. Although the role of intracellular calcium release is very important at least in the development of phasic contraction, in the absence of extracellular calcium influx the tonic component of smooth muscle contraction cannot be sustained (Paulis et al., 2007). However, exaggerated calcium influx into VSMCs through L-VDCC contributes to increased contractility and promotes the rise of blood pressure leading to the blood pressure elevation in various forms of experimental hypertension (Kunes et al., 2004). As described before, the altered calcium entry into VSMCs might be caused by up-regulation of voltage-dependent calcium channels (Pratt et al., 2002), depolarized membrane potential (Pesic et al., 2004) and/or abnormal calcium channel properties (Asano et al., 1995).

1.3.5. Molecular mechanisms of vascular smooth muscle contraction

Vascular smooth muscle contraction is initiated by increases in intracellular calcium concentrations that are regulated by the above mentioned receptor-mediated pathways in response to specific stimuli. The primary target protein in the initial rise of intracellular calcium is a calcium binding protein, calmodulin. The binding of calcium to calmodulin initiates a conformational change in the calmodulin molecule leading to a subsequent interaction with myosin light chain kinase (MLCK) and hence its activation (Lee et al., 2004). MLCK phosphorylates the light chain of myosin, enabling the cycling of myosin cross-bridges with actin. Thus, the contractile activity of vascular smooth muscle cells is determined by the phosphorylation level of myosin light chain (Hilgers & Webb, 2005). However, its phosphorylation state can be regulated not only by the calcium/calmodulin-
dependent MLCK, but also by dephosphorylation-mediated myosin light chain phosphatase (MLCP) promoting smooth muscle relaxation (see Fig. 1). The role of MLCP will be discussed in the following section.

1.3.5.1. Calcium sensitization and RhoA/Rho-kinase signalling in vasculature

In addition to fluctuations of intracellular calcium, contractility of vascular smooth muscle is also regulated by so-called “calcium sensitization”. Since the increase of cytosolic calcium concentration following agonist stimulation is only temporary, the contractile response has to be maintained by calcium-independent mechanisms leading to the increase of myofilament calcium sensitivity. Calcium sensitization of the contractile proteins is signalled by the RhoA/Rho-kinase pathway to inhibit the dephosphorylation of the light chain by inactivation of myosin light chain phosphatase (MLCP) (Hilgers & Webb, 2005).

Various vasoactive agonists activate specific heterotrimeric G protein-coupled receptors and lead not only to increases in intracellular calcium via G_{q/11} or G_{i} proteins, but also activate G_{12/13} proteins that are coupled to RhoA/Rho-kinase signalling pathway via guanine nucleotide exchange factors (RhoGEFs) (Wirth, 2010) (Fig. 1). RhoA is a small monomeric G protein which is like other heterotrimeric G proteins active when contains bound GTP. In vascular smooth muscle cells, RhoA occurs as inactive cytosolic or active plasma membrane bound form which is required for calcium sensitization. The cytosolic, inactive, form of RhoA is associated with guanine nucleotide dissociation inhibitor. This complex is activated by RhoGEFs that stimulate nucleotide exchange on RhoA, followed by RhoA dissociation and translocation to the plasma membrane (Somlyo & Somlyo, 2000). Indeed, a translocation of RhoA to the cell membrane has been shown to occur during agonist activation (Carter et al., 2002).

The calcium-sensitizing effector of RhoA is Rho-kinase, a serine/threonine kinase, which plays an important role not only in the regulation of vascular smooth muscle cell contraction but also in other cellular functions such as proliferation, adhesion and migration. Rho kinase activation leads to phosphorylation and thus deactivation of the MLCP regulatory subunit resulting in the accumulation of phosphorylated myosin light chain at constant intracellular calcium concentrations (Nagumo et al., 2000). Pharmacological inhibitors of Rho-kinase, such as fasudil, block Rho kinase activity by
competing with the ATP-binding site on the enzyme (Nagumo et al., 2000). Rho-kinase inhibition induces relaxation of isolated segments of blood vessels contracted with many different agonists and lower blood pressure in hypertensive animal models (Uehata et al., 1997). There have been identified two different isoforms of Rho kinase encoded by two different genes, Rho kinase I and II with the similarity of 92%. Both isoforms are expressed in vascular smooth muscle and in heart (Wirth, 2010).

1.3.5.1.1. Crosstalk between nitric oxide and RhoA/Rho-kinase signalling

RhoA/Rho-kinase pathway appears to have an impact on NO-signalling and vice versa. Recent evidences suggest that NO may protect against the enhanced contractile and proliferative effects of RhoA/Rho-kinase. Sauzeau et al. (2000) have revealed that RhoA-mediated calcium sensitization and contraction are inhibited in smooth muscle cells by the NO/cGMP signalling pathway, via phosphorylation and hence inactivation of RhoA. They suggested that the consequent inhibition of RhoA-induced calcium sensitization and actin cytoskeleton organization contributes to the vasodilator action of nitric oxide. Furthermore, Bolz et al. (2003) suggested that NO dilates resistance arteries by activating the MLCP in a cGMP-dependent manner. Indeed, some studies (Wu et al., 1996; Lee et al., 1997) have demonstrated that MLCP is indirectly activated by cGMP. However, while endogenous NO may regulate at least RhoA activation, chronic inhibition of NO production causes RhoA desensitization (Carter et al., 2002). On the other hand, stimulated RhoA/Rho-kinase activity has negative effects on endothelial NO synthase expression (Takemoto et al., 2002) and thus even amplifies the increased VSMC contractility induced by RhoA/Rho-kinase signalling (Wirth, 2010).

1.3.5.1.2. Role of RhoA/Rho kinase pathway in hypertension

Alterations of RhoA/Rho-kinase pathway promote the development of hypertension. Changes in RhoA/Rho-kinase signalling are proposed to contribute to the increased peripheral vascular resistance in hypertensive state (Uehata et al., 1997). Direct evidence for the involvement of Rho kinase in hypertension was given by Mukai et al. (2001). They have demonstrated in blood vessels from SHR that there is a significantly greater mRNA expression and activity of Rho kinase compared to blood vessels from
normotensive WKY. Moreover, they have also shown that Rho kinase is substantially involved in functional and structural alterations of hypertensive blood vessels. Long-term blockade of Rho kinase suppressed vascular lesion formation such as medial hypertrophy and perivascular fibrosis in SHR. However, the extent of the contribution of altered RhoA/Rho-kinase pathway to hypertension development and/or maintenance remains to be investigated.

Figure 1. Molecular mechanisms of agonist-induced vascular smooth muscle cell contraction and calcium sensitization mechanism involving RhoA/Rho-kinase signalling pathway. For more detailed description, refer to the text. Ang II - angiotensin II, cGK - cGMP-dependent protein kinase, IP$_3$ - phosphatidylinositol 1,4,5-trisphosphate, MLCK - myosin light chain kinase, MLCP - myosin light chain phosphatase, NE - norepinephrine, PLC - phospholipase C, ROCC - receptor-operated calcium channels, VDCC - voltage-dependent calcium channels
2. AIMS OF THE STUDY

As described in the previous section, the abnormality in the modulation of nifedipine-sensitive L-VGCC of the vascular smooth muscle cells, which is caused mainly by increased activity of sympathetic nervous system, increases vascular tone and hence contributes to hypertension. Therefore, the aims of my PhD. thesis were:

- to determine the role of nifedipine-sensitive sympathetic vasoconstriction in the maintenance of high blood pressure in SHR
- to test the hypothesis that the increased activity of sympathetic nervous system in SHR and its effects on L-VGCC channels are mediated by G\textsubscript{i} protein-coupled pathway

Since one of the discussed mechanisms by which the sympathetic nervous system modulates the calcium entry through L-VGCC are the changes in cAMP levels mediating a modulation of certain potassium channel activities, the next aims were:

- to determine whether cAMP pathway itself contributes to the control of calcium influx through L-VGCC
- to find out whether a possible way by which cAMP/PKA pathway inhibits calcium influx through L-VGCC is the activation of large-conductance Ca\textsuperscript{2+}-activated (BK\textsubscript{Ca}) and/or voltage-dependent potassium (K\textsubscript{V}) channels leading to a hyperpolarization of the cell membrane

However, it seems that the contractility of vascular smooth muscle is regulated not only by fluctuations of intracellular calcium levels but also by alterations in the myofilament sensitivity to calcium mediated by the RhoA/Rho-kinase pathway. Thus, the final aim was:

- to establish how the inhibition of the RhoA/Rho kinase pathway can modify catecholamine-induced vasoconstriction in normotensive and hypertensive rats
3. MATERIAL AND METHODS

3.1. Animals

All experiments were carried out in conscious 12-16-week-old males of spontaneously hypertensive (SHR) rats and their normotensive controls, Wistar-Kyoto (WKY) rats. The animals were obtained from the colony of the Institute of Physiology AS CR, which is based upon breeding pairs from Charles River. They were housed under controlled conditions (temperature 23±1°C, 12-h light-dark cycle) and fed a standard pellet diet (ST-1). Tap water and food were available ad libitum. All studies were performed in conscious animals because the whole body response to particular vasoactive drugs reflects the changes of peripheral resistance just in those vascular beds, which play a dominant role in blood pressure control. All procedures and experiments were approved by the Ethical Committee of the Institute of Physiology AS CR and were performed according to European Convention on Animal Protection and Guidelines on Research Animal Use.

3.2. Blood pressure measurement

One day prior to the experiments, polyethylene catheters were filled with heparin, plugged and inserted into the left carotid artery (PE-50, for blood pressure measurement) and left jugular vein (PE-10, for drug application) of SHR and WKY rats under the ether anaesthesia. Then they were tunneled under the skin and exteriorized in the interscapular region. After the surgery, animals were housed one per cage with water and food ad libitum. After 24-h recovery, the individual rats were placed in small plexiglass cages. The measurements were performed between 8 and 12 h a.m. to reduce circadian variations in blood pressure. Systolic, diastolic and mean arterial blood pressure was monitored and recorded by PowerLab system (ADInstruments Ltd, Bella Vista, NSW, Australia). All animals were allowed to stabilize for a period of 30 min before any experimental protocol was performed.
3.3. Experimental protocols

Since the present work was aimed to answer a number of questions covering an extensive issue, it is divided into the three studies, each of them being described in detail in the following sections. It should be mentioned that each study begins with a short introduction (written in bold italic font) to help reader to understand the design and methods used to achieve our aims.

Study 1: Contribution of $G_i$ proteins to SNS hyperactivity-induced augmentation of calcium influx through L-VDCC in hypertension

This series of experiments was designed to test the hypothesis that nifedipine-sensitive blood pressure component is a considerable part of sympathetic vasoconstriction and that increased activity of SNS in SHR is mediated by $G_i$ protein/cAMP pathway. Therefore, we examined whether $G_i$ protein inactivation by pertussis toxin (PTX) influences the maintenance of the vasoactive balance between main vasoconstrictor (RAS, SNS) and vasodilator systems (nitric oxide and endothelial hyperpolarizing factor, EDHF, by many studies recognized as the activation of potassium channels leading to membrane hyperpolarization) as well as whether PTX elicits alteration of blood pressure responses to exogenous norepinephrine injection. In order to compare the influence of chronic $G_i$ protein inactivation with that of acute L-VDCC blockade by nifedipine on norepinephrine-induced vasoconstriction, we determined norepinephrine dose-response curves in untreated and PTX-treated rats prior to and after the acute nifedipine administration. Further attention was paid whether PTX treatment alter blood pressure changes induced by acute L-VDCC blockade with nifedipine.

SHR and WKY rats were randomly allocated to two experimental groups: untreated and PTX-treated animals. PTX (10 µg/kg b.w.) was injected to the right jugular vein 48 hours prior to the experiments.
- **Nifedipine-sensitive blood pressure component as a considerable part of sympathetic vasoconstriction**

  To evaluate the role of nifedipine-sensitive blood pressure component in sympathetic vasoconstriction which is represented by pentolinium-induced blood pressure changes, the separate animal groups were subjected to the acute blockade of L-VDCC (nifedipine, 0.4 mg/kg b.w.) followed by SNS blockade with pentolinium (5 mg/kg b.w.) or *vice versa*.

- **Effect of G\textsubscript{i} protein inactivation by pertussis toxin on the balance between particular vasoactive systems**

  After the stabilization period, the blockers of particular vasoactive systems were intravenously administered into the jugular vein. First, angiotensin converting enzyme inhibitor (captopril, 10 mg/kg b.w.) and 15 min later ganglionic blocker (pentolinium, 5 mg/kg b.w.) were injected to block renin-angiotensin system (RAS) and sympathetic nervous system (SNS), respectively. Since ganglionic blockade is accompanied not only by great blood pressure fall but also by the elimination of sympathetic baroreflex component, this allowed us to determine the full extent of blood pressure increases elicited by acute NO synthase blockade following the injection of N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg b.w.). Twenty minutes later, blood pressure levels became stabilized and the blocker of large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (BK\textsubscript{Ca}), tetraethylammonium (TEA 16.5 mg/kg b.w.), was injected to reveal the contribution of these channels to vasoactive balance. After the next 10 min when blood pressure response to TEA disappeared and blood pressure levels became stabilized, the direct donor of NO, sodium nitroprusside (20 µg/kg b.w.), was injected to reach maximal vasodilation. Thus we obtained so called “residual blood pressure”, which was considered as a parameter that reveals possible alterations of fully dilated resistance vessels dependent on the changes in the structure and/or basal tone of resistance vessels (see Fig. 2). The contribution of respective vasoactive systems to blood pressure maintenance was calculated from the absolute changes of mean arterial pressure (MAP) elicited by the above-mentioned blockers.
Figure 2. Scheme of the sequential blockade of particular vasoactive systems in SHR and WKY rats. CPT: captopril injection (10 mg/kg b.w.), PENTO: pentolinium injection (5 mg/kg b.w.), L-NAME: L-NAME injection (30 mg/kg b.w.), TEA: tetraethylammonium injection (16.5 mg/kg b.w.) and SNP: sodium nitroprusside injection (20 µg/kg b.w). Residual BP: the minimal blood pressure obtained after maximal vasodilation induced by direct NO donor injection, sodium nitroprusside.

- **Effect of G_i protein inactivation by pertussis toxin on the blood pressure changes elicited by nifedipine injection**

  In separate groups of WKY and SHR rats, the influence of PTX on nifedipine-sensitive blood pressure component was investigated. Following 30-min stabilization period, increasing cumulative doses of nifedipine (0.025, 0.05, 0.1, 0.2 and 0.4 mg/kg b.w.) were administered to untreated as well as to PTX-treated animals at 10-min intervals. The magnitude of nifedipine-sensitive blood pressure component was obtained from a rapid nifedipine-induced blood pressure fall (after blood pressure stabilization). The possible effects of 50% ethanol in the nifedipine solution on blood pressure were tested in the additional group of animals.

- **Effects of pertussis toxin and nifedipine on blood pressure responses to norepinephrine**

  Before norepinephrine administration, we performed a sequential blockade of RAS by captopril (10 mg/kg b.w.) and SNS by ganglionic blocker, pentolinium (5 mg/kg b.w.),
to avoid the influence of these two major vasoactive systems. Thereafter, the animals of untreated groups were injected with norepinephrine in the increasing non-cumulative doses of 0.01-20 µg/kg b.w. PTX-treated animals of both rat strains were subjected to the same procedure, however, norepinephrine was administered at the doses of 0.01-80 µg/kg b.w. because of the decreased sensitivity to this agonist induced by G protein inactivation. The subsequent blockade of L-VDCC by nifedipine (0.4 mg/kg b.w.) was performed in all animal groups to determine the contribution of these channels to the norepinephrine-induced contractions. This was followed by further non-cumulative administration of norepinephrine in the doses of 0.1-40 µg/kg b.w. in untreated rats and 0.1-160 µg/kg b.w. in PTX-treated animals. To exclude the possibility of adrenoceptor desensitization evoked by high norepinephrine doses, control animals were subjected to two series of increasing non-cumulative doses of norepinephrine (0.01-20 µg/kg b.w.) administered one after another without the use of nifedipine between them. To test the effects of ethanol vehicle on blood pressure responses to norepinephrine, additional test groups were injected with norepinephrine doses as mentioned above and this was followed by 50 % ethanol injection, after which a further series of norepinephrine doses was applied. In all groups, blood pressure levels stabilized after pentolinium injection were considered as initial blood pressure levels from which peak MAP changes following particular norepinephrine doses were read. Under the conditions of acute L-VDCC blockade by nifedipine, blood pressure levels stabilized after nifedipine injection were used as the initial levels. The norepinephrine-induced blood pressure increments obtained in this way were used to construct dose-response curves from which the half-maximal blood pressure responses, ED50 values and slopes of the curves were calculated for individual rats (Fig. 3).
Figure 3. The evaluation of dose-response curves of untreated SHR (curve A) and PTX-treated SHR (curve B) constructed from the peak BP values induced by particular doses of non-cumulatively administered norepinephrine, which were read from the respective baseline pressure levels stabilized after pentolinium injection (dotted lines). The hatched areas represent confidence limits at 5% level.
Study 2: Role of large-conductance Ca\(^{2+}\)-activated (BK\(_{Ca}\)) and voltage-dependent (K\(_V\)) potassium channels in cAMP-induced vasodilatation

One of the possible pathways through which the SNS regulates calcium influx via L-VDCC is supposed to be the modulation of cyclic nucleotide levels (at least cAMP) resulting in the activation of certain potassium channels. To confirm this hypothesis, the overproduction of cAMP was elicited. Since the use of forskolin or phosphodiesterase-resistant analogues of cAMP is not suitable for the in vivo experiments, we induced the cAMP overproduction by β-adrenoceptor stimulation with isoprenaline.

- Effect of isoprenaline-induced cAMP overproduction on blood pressure responses to nitric oxide synthase blockade with L-NAME

Since L-VDCC channels are also closed by NO through cGMP-dependent pathway (Lewis et al., 2005), the extent of isoprenaline-induced attenuation of blood pressure response to NO synthase blockade was considered to reflect the degree of L-VDCC inhibition elicited by cAMP overproduction. First, the blockade of RAS and SNS was performed as described in detail above (see Study 1). Then, the infusion of isoprenaline (6 µg/kg/h i.v.) was started before or after NO synthase blockade by L-NAME (30 mg/kg i.v.) as seen in Fig. 4 (panel b and c). To test whether isoprenaline effects were mediated by the stimulation of β-adrenoceptors, the antagonist of β-adrenoceptors (propranolol, 1 mg/kg i.v.) was administered.

- Effect of BK\(_{Ca}\) and K\(_V\) channel blockade on isoprenaline-induced changes of blood pressure responses to L-NAME

The role of BK\(_{Ca}\) and K\(_V\) channels was determined using a continuous infusion of tetraethylammonium (TEA, 100 mg/kg/h i.v.) and 4-aminopyridine (4-AP, 4 mg/kg/h i.v.), respectively. To block only BK\(_{Ca}\) channels, TEA was used in the concentrations below 1 mM. To exclude the influence of TEA and 4-AP infusion *per se* on L-NAME-induced blood pressure responses, the control groups of animals were subjected to TEA or 4-AP infusion before L-NAME injection. To evaluate the effect of these potassium channel
inhibitors on isoprenaline-induced changes of blood pressure responses to L-NAME, the infusions were started before or after L-NAME injection (Fig. 4 panel e and f).

Figure 4. Study designs used to evaluate the role of BK$_{Ca}$ and KV channels in cAMP-induced vasodilatation. Control group was subjected to the sequential blockade of renin-angiotensin system by captopril (CPT 10 mg/kg i.v.), sympathetic nervous system by pentolinium (PENTO, 5 mg/kg i.v.) and NO-synthase by L-NAME (30 mg/kg i.v.) (panel a). The influence of cAMP overproduction was assessed by isoprenaline infusion (6 μg/kg/h i.v.) started before (panel b) or after L-NAME administration (panel c). Propranolol (PRO) was used to test whether the effect of isoprenaline is caused by β-adrenoceptor stimulation. To establish the role of BK$_{Ca}$ and KV channels in the effects of isoprenaline, the infusion of tetraethylammonium (TEA, 100 mg/kg/h i.v.) or 4-aminopyridine (4-AP, 4 mg/kg/h i.v.) was used in control animals (panel d) as well as in animals subjected to isoprenaline infusion as described above (panel e and f). The horizontal lines represent time axis (in minutes).
Study 3: Role of calcium sensitization in hypertension

Although the elevation of intracellular calcium concentration is a major trigger of vascular smooth muscle contraction, the contractile state is also maintained and regulated by RhoA/Rho-kinase signalling pathway, which is modulated by certain vasoconstrictors and vasodilators. This set of experiments was designed to detect the contribution of Rho kinase pathway to high blood pressure maintenance in hypertensive rats. Furthermore, we compared the effects of Rho kinase inhibition by fasudil to that of L-VDCC blockade by nifedipine on norepinephrine dose-response curves, to find out whether the modulation of calcium sensitization also plays such an important role in catecholamine-induced vasoconstriction as calcium entry through L-VDCC.

- **Role of RhoA/Rho-kinase pathway in the maintenance of blood pressure**

  Following 30-min stabilization period, Rho kinase inhibitor, fasudil, was cumulatively administered (0.5, 1, 2, 4 and again 2 mg/kg b.w.) to both WKY and SHR in order to determine the role of RhoA/Rho-kinase pathway in the blood pressure maintenance.

- **Effect of Rho kinase inhibition by fasudil on norepinephrine-induced vasoconstriction**

  In this series of experiments, norepinephrine dose-response curves were determined in WKY and SHR rats before and after Rho kinase inhibition. After the blockade of RAS by captopril (10 mg/kg b.w.) and SNS by pentolinium (5 mg/kg b.w.), norepinephrine was injected in the increasing non-cumulative doses of 0.005-10 µg/kg b.w. (for more details see the description in Study 1). Subsequently the blockade of Rho kinase by fasudil (2.5 mg/kg b.w. or 10 mg/kg b.w.) was performed, followed by another set of non-cumulative administration of norepinephrine at the doses of 0.01-80 µg/kg b.w. Finally, a separate group of animals was subjected to the norepinephrine doses (0.5-10 µg/kg b.w.) following both fasudil (10 mg/kg b.w.) and nifedipine (0.4 mg/kg b.w.) administration to uncover a possible additive effect of both inhibitor combination.
3.4. **Drugs**

All drugs were purchased from Sigma St. Louis, USA. Each drug except nifedipine was dissolved in saline solution and given as an intravenous bolus in a volume of 1 ml/kg b.w. Nifedipine was dissolved in 50 % ethanol, and the total volume of nifedipine injected was 0.4 ml/kg b.w. Isoprenaline, TEA and 4-AP were infused in a volume of 1 ml/kg/h.

3.5. **Statistical analysis**

Results were expressed as means ± SEM. The differences between groups were evaluated by one-way analysis of variance (F test) followed by post-hoc least significance test (LSD). The differences were considered significant at P<0.05 level. The data on the parameters of norepinephrine dose-response curves were evaluated by means of three-way fixed Analysis of Variance (ANOVA) with grouping factors: strain, PTX-treatment and nifedipine. The logarithmic transformation was applied on ED\(_{50}\) data to stabilize cell variances.
4. RESULTS

4.1. Study 1: Contribution of G_i proteins to SNS hyperactivity-induced augmentation of calcium influx through L-VDCC in hypertension

- Nifedipine-sensitive blood pressure component as a considerable part of sympathetic vasoconstriction

The aim of this experiment was to determine which part of calcium influx through L-VDCC is caused by sympathetic stimulation and which part of sympathetic vasoconstriction is mediated by nifedipine-sensitive mechanisms. The basal values of mean arterial blood pressure (MAP) were significantly elevated in SHR compared to WKY (173 ± 2 mm Hg and 114 ± 1.7 mm Hg, respectively, n=12, P<0.01). Acute closure of L-VDCC channels by nifedipine caused significantly greater blood pressure decreases in SHR compared to WKY (Table 2). Blockade of SNS elicited by ganglionic blocker, pentolinium, reduced blood pressure responses to nifedipine by 90% and 75% in WKY and SHR, respectively, thus cancelling the above mentioned strain difference (Table 2). Injection of pentolinium caused a considerable blood pressure fall in both rat strains, which was again significantly greater in SHR (Table 2). These blood pressure changes were significantly attenuated by previous nifedipine injection, which also caused the loss of the strain difference in blood pressure decreases to pentolinium administration (Table 2).

Table 2. Blood pressure changes elicited by NIFEDIPINE (0.4 mg/kg b.w.) injection prior to and after pentolinium administration, and blood pressure changes elicited by PENTOLINIUM (5 mg/kg b.w.) injection prior to and after nifedipine administration in WKY and SHR rats.

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<tr>
<td></td>
<td>Before Pentolinium</td>
<td>After Pentolinium</td>
<td>Before Nifedipine</td>
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<tr>
<td>WKY</td>
<td>-20 ± 4</td>
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<td>-54 ± 4</td>
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<tr>
<td>SHR</td>
<td>-39 ± 6**</td>
<td>-10 ± 3</td>
<td>-69 ± 5*</td>
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Values are means ± SEM. *P< 0.05, **P<0.01 vs. WKY, #P<0.05, ## P<0.01 vs. blood pressure falls induced by the first inhibitor alone. n=6 in each group
Effect of G\textsubscript{i} protein inactivation by pertussis toxin on the balance between particular vasoactive systems

Likewise in the previous experiment, basal blood pressure levels were significantly higher in SHR than in age-matched WKY rats (Fig. 5A). \textit{In vivo} pretreatment of both rat strains with PTX caused a significant decrease of baseline blood pressure levels. The effect was substantially greater in SHR as compared to WKY (Fig. 5A). The administration of angiotensin-converting enzyme inhibitor, captopril, induced a moderate blood pressure decrease which was similar in both strains (Fig. 5B). However, ganglionic blockade induced by pentolinium was followed by a major blood pressure fall which was significantly greater in SHR than in WKY (Fig. 5C). Pretreatment with PTX led to a substantial augmentation of blood pressure responses to captopril, which were more pronounced in SHR compared to WKY (Fig. 5B). Augmented vasoconstrictor role of angiotensin II was compensating for a significantly attenuated role of SNS as evidenced by reduced blood pressure responses to pentolinium in both rat strains treated with PTX (Fig. 5C). Nitric oxide synthase blockade with L-NAME caused similar blood pressure rises in untreated WKY and SHR rats. However, PTX treatment resulted in a considerable reduction of L-NAME-induced blood pressure responses, the effect being greater in WKY rats (Fig. 5D). Blockade of BK\textsubscript{Ca} channels with TEA elicited similar blood pressure increases in both rat strains (Fig. 6A). PTX treatment significantly augmented blood pressure responses to TEA in WKY as well as in SHR rats. This effect of PTX treatment was, however, greater in hypertensive rats (Fig. 6A). While the residual blood pressure level was substantially higher in untreated SHR as compared with WKY, PTX pretreatment significantly reduced the level of residual blood pressure in SHR but not in WKY rats. Thus, blockade of G\textsubscript{i} proteins with PTX eliminated strain differences in the values of residual blood pressure levels (Fig. 6B).
Figure 5. Basal values of mean arterial pressure (MAP) (panel A) and MAP changes induced by sequential blockade of renin-angiotensin system with captopril (panel B), sympathetic nervous system with pentolinium (panel C) and NO-synthase inhibition with L-NAME (panel D) in untreated as well as in PTX-treated WKY and SHR rats. Values are means ± SEM, * P<0.05, ** P<0.001 effect of PTX treatment, # P<0.01 vs. respective WKY (effect of genotype). n=8 in each group.

Figure 6. MAP changes induced by tetraethylammonium (Δ TEA, 16 mg/kg i.v.) following the blockade of RAS by captopril, SNS by pentolinium and NO synthase by L-NAME in untreated and PTX-treated WKY and SHR (panel A). Residual MAP values reached after sodium nitroprusside injection to untreated as well as PTX-treated WKY and SHR subjected to previous blockade of RAS by captopril, SNS by pentolinium and NO synthase by L-NAME (panel B). Values are means ± SEM, * P<0.05, ** P<0.001 effect of PTX treatment, # P<0.05 vs. respective WKY (effect of genotype). n=6-8 in each group.
Effect of Gi protein inactivation by pertussis toxin on the blood pressure changes elicited by nifedipine injection

Acute cumulative administration of L-VDCC blocker, nifedipine, to untreated rats elicited dose-dependent blood pressure decreases, which were significantly more pronounced in SHR than in WKY (Fig. 7). PTX pretreatment attenuated the nifedipine-induced blood pressure responses only in SHR, not in WKY. It should be noted that basal blood pressure of PTX-treated SHR was decreased to the blood pressure level of untreated SHR that was observed after the maximal nifedipine dose (Fig. 7). Ethanol vehicle caused only a small blood pressure reduction compared to nifedipine injection (data not shown).

Figure 7. Blood pressure changes induced by increasing cumulative doses of nifedipine (administered in doses 0.025, 0.05, 0.1, 0.2 and 0.4 mg/kg b.w.) in untreated as well as in PTX-treated WKY and SHR rats. Values are means ± SEM, * P< 0.001 effect of PTX treatment, # P<0.05, ## P<0.001 vs. respective WKY. n=5 in each group
- **Effects of pertussis toxin and nifedipine on blood pressure responses to norepinephrine**

This series of experiments was designed to compare the responsiveness of WKY and SHR to exogenous norepinephrine as well as to determine the influence of *in vivo* pretreatment with PTX and/or acute application of nifedipine on these blood pressure responses. The values of baseline MAP in particular studied groups were similar to those observed in the previous experiments (114 ± 3.4 mm Hg in untreated WKY, 104.8 ± 2.6 mm Hg in PTX-treated WKY, 173.2 ± 8.3 mm Hg in untreated SHR, 117.2 ± 5.3 mm Hg in PTX-treated SHR). In untreated rats of both strains, intravenously injected norepinephrine produced dose-response curves, which started from the different baseline blood pressure levels (Fig. 8A). This can also explain the differences in the maximal blood pressure levels reached after the maximal norepinephrine doses (Fig. 8A). However, it should be noted that except for the different starting blood pressure level the shapes of both curves were very similar and there were no significant differences in the slopes of the curves, half-maximal blood pressure responses or in the values of ED$_{50}$ between WKY and SHR (Table 3).

Blockade of calcium influx through L-VDCC by nifedipine shifted norepinephrine dose-response curves to the right in both strains (i.e. ED$_{50}$ values were increased 4 times in SHR and 10 times in WKY rats, Table 3, Fig. 8A). This nifedipine-induced reduction of vascular sensitivity to norepinephrine was not associated with any significant changes of half-maximal blood pressure responses and slopes of the curves in none of the rat strain (Table 3).

The pretreatment of SHR and WKY rats with PTX increased ED$_{50}$ values of norepinephrine dose-response curves approximately twenty times compared to untreated groups (Fig. 3, Table 3). It is noteworthy that half-maximal blood pressure responses induced by norepinephrine were significantly augmented in PTX-treated animals of both genotypes compared to those of untreated ones. This augmentation was similar in both strains (Table 3). The effect of L-VDCC blockade by nifedipine in PTX-treated rats of both strains (Fig. 8B) was negligible compared to untreated animals (Fig. 8A). Nifedipine did not significantly alter the particular parameters of these curves except for a further increase of ED$_{50}$ values in PTX-treated SHR (Table 3). The minimal effect of nifedipine in PTX-treated rats (Fig. 8B) indicates that the inactivation of $G_i$-proteins really affects calcium influx through L-VDCC.
It should be mentioned that we did not notice any signs of adrenoceptor desensitization evoked by high norepinephrine doses used in our experiments or any major influence of ethanol vehicle on norepinephrine-dose responses (data not shown).

Figure 8. The effect of acute L-VDCC blockade by nifedipine (NIF, 0.4 mg/kg b.w.) on the dose-response curves obtained after non-cumulative administration of norepinephrine (0.01-160 μg/kg b.w.) in untreated (panel A) and PTX-treated (panel B) rats. Norepinephrine was injected after previous blockade of RAS by captopril and SNS by pentolinium as described in detail in the text. The curves represent mean values of respective rat groups. — — WKY and — ▲ — SHR: WKY and SHR rats before nifedipine injection; - -○- - WKY NIF and - -△- - SHR NIF: WKY and SHR rats after nifedipine injection. $n = 5$ in each group
Table 3. ED\textsubscript{50}, half-maximal blood pressure responses and slopes of dose-response curves for norepinephrine in untreated and PTX-treated animals of both genotypes measured before and after acute nifedipine (NIF) administration.

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<th>PTX-treated groups</th>
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<tr>
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<tr>
<td>(\text{ED}_{50}) (\text{mg/kg})</td>
<td>0.2 ± 0.02</td>
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<td>0.8 ± 0.06 (***)</td>
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<td>Half-maximal blood pressure (\text{mm Hg})</td>
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<td>50 ± 2</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Slope (\text{mm Hg/log dose})</td>
<td>54 ± 3</td>
<td>46 ± 4</td>
<td>64 ± 4</td>
<td>73 ± 6</td>
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Values are means ± SEM. Statistical analysis was performed with three-way ANOVA. \(*P<0.05, ~**P<0.01, ~***P<0.001\) effect of PTX treatment, \(*P<0.01\) vs. respective WKY (effect of genotype), \(***P<0.001\) effect of nifedipine. \(n=5\) in each group.
4.2. Study 2: Role of large-conductance Ca\textsuperscript{2+}-activated (BK\textsubscript{Ca}) and voltage-dependent (K\textsubscript{V}) potassium channels in cAMP-induced vasodilatation

This study was designed to determine whether vasodilating effect of cAMP overproduction affects calcium influx through L-VDCC by changes of membrane potential resulting from the modulation of potassium current through K\textsubscript{V} and/or BK\textsubscript{Ca} channels.

- **Effect of isoprenaline-induced cAMP overproduction on blood pressure responses to nitric oxide synthase blockade with L-NAME**

Since L-VDCC are modulated by both NO and norepinephrine (through cAMP and cGMP pathway, respectively), the extent of isoprenaline-induced attenuation of blood pressure response to NO synthase blockade was considered to reflect L-VDCC inhibition elicited by cAMP overproduction. Isoprenaline infusion, which started just after stabilization period (i.e. before the administration of any other substances), caused a decrease of baseline blood pressure levels in both rat strains, which was, however, substantially more pronounced in SHR compared to WKY rats ($\Delta$ isoprenaline -54±7 mm Hg in SHR and -9±3 mm Hg in WKY, Fig. 9). It should be noted that all depressor blood pressure responses induced by isoprenaline infusion were accompanied by maximal increase of heart rate (Fig. 9, Fig. 10).

The overproduction of cAMP elicited by isoprenaline infusion prevented blood pressure rises induced by subsequent acute inhibition of NO synthase by L-NAME. This was seen when the infusion started just after a stabilisation period (Fig. 9) or after the preceding blockade of renin-angiotensin and sympathetic nervous system (Fig. 11). Moreover, isoprenaline infusion almost completely abolished the fully developed blood pressure response to L-NAME (Fig. 10). These isoprenaline effects were completely reversed by $\beta$-adrenoceptor blockade with propranolol injection while isoprenaline infusion continued (Fig. 9 - 11).
Figure 9. Blood pressure and heart rate records in WKY (A) and SHR rats (B). After a stabilization period, the animals were subjected to isoprenaline infusion (start indicated by black arrow). This was followed by the blockade of renin-angiotensin system with captopril (CPT) and sympathetic nervous system with pentolinium (PENTO) to eliminate the influence of these two systems. Subsequently, the blocker of NO-synthase, L-NAME, was injected. To test whether isoprenaline effects are mediated by β-adrenoceptor-coupled pathway, the antagonist of β-adrenoceptors, propranolol (PRO, 1 mg/kg i.v.) was administered.
Figure 10. Blood pressure and heart rate records in WKY (A) and SHR rats (B). After a stabilization period, the animals were subjected to sequential blockade of renin-angiotensin system with captopril (CPT) and sympathetic nervous system with pentolinium (PENTO) to eliminate the influence of these two systems. This was followed by the blockade of NO-synthase with L-NAME. When blood pressure became stabilized, the infusion of β-adrenoceptor agonist, isoprenaline, was started (start indicated by black arrow). To test whether isoprenaline effects are mediated by β-adrenoceptor-coupled pathway, the antagonist of β-adrenoceptors, propranolol (PRO, 1 mg/kg i.v.) was administered.
Figure 11. Effect of isoprenaline infusion on blood pressure responses to NO synthase blockade with L-NAME and elimination of this effect by propranolol, β-adrenoceptor blocker, in WKY and SHR rats subjected to previous blockade of RAS by captopril and SNS by pentolinium. Values are means ± SEM, ** P<0.001 vs. respective L-NAME group, * P<0.001 vs. respective L-NAME + Isoprenaline group. n=5-10 in each group.

- **Effect of BK<sub>Ca</sub> and K<sub>V</sub> channel blockade on isoprenaline-induced changes of blood pressure responses to L-NAME**

This experiment was designed to determine the role of BK<sub>Ca</sub> and K<sub>V</sub> channels in isoprenaline-induced changes of blood pressure responses to L-NAME. Blockade of BK<sub>Ca</sub> channels with TEA infusion had no significant effect on L-NAME-induced blood pressure changes in any of the rat strains (Fig. 12A). On the other hand, TEA infusion restored blood pressure responses to L-NAME during isoprenaline infusion and diminished the isoprenaline-induced prevention of blood pressure rise following acute L-NAME injection (Fig. 12A). However, these blood pressure responses were completely restored only in SHR rats. In WKY, the restoration was not observed and blood pressure responses to L-NAME were not significantly different from those found during the isoprenaline infusion (Fig. 12A). Moreover, there were no significant differences in blood pressure responses to L-NAME between the WKY groups subjected to previous isoprenaline infusion or to combined isoprenaline and TEA infusions (Fig. 12A).
Similar effects were also elicited by the blockade of \( K_V \) channels with 4-AP. Contrary to TEA, the blockade of \( K_V \) channels *per se* slightly decreased blood pressure responses to L-NAME in both rat strains (Fig. 12B). The infusion of 4-AP restored blood pressure responses to L-NAME during isoprenaline infusion, abolishing thus the isoprenaline-induced prevention of blood pressure rise to acute L-NAME injection (Fig. 12B). However, similarly to TEA, this effect of 4-AP was seen only in SHR. In normotensive rats, infusion of 4-AP had no significant effect on isoprenaline-induced prevention of blood pressure rises elicited by L-NAME (Fig. 12B).

In the additional experiments performed on WKY, we attempted to elucidate why the blockade of \( B_{KC_a} \) or \( K_V \) channels has no effect on isoprenaline-induced attenuation of blood pressure responses to L-NAME in WKY compared to SHR (Fig. 12). We supposed that this could be caused by the fact that one class of \( K^+ \) channels can replace the missing effect of another \( K^+ \) channel family that was inhibited. Therefore, we used the combination of TEA and 4-AP infusions in WKY rats. As seen in Figure 13, simultaneous infusion of TEA and 4-AP caused a restoration of blood pressure responses to L-NAME during isoprenaline infusion. The blood pressure responses restored after the combination of both potassium channel blockers were not significantly different from the control L-NAME-induced increases (Fig. 13).
Figure 12. Influence of BK$_{Ca}$ channel blockade by tetraethylammonium (TEA, panel A) and Kv channel blockade by 4-aminopyridine (4-AP, panel B) on blood pressure responses to acute NO synthase blockade with L-NAME and the influence of these potassium channel blockers on isoprenaline-induced effect. The experiments were performed in WKY and SHR rats subjected to previous blockade of RAS by captopril and SNS by pentolinium. Values are means ± SEM, *P<0.05, **P<0.001 vs. respective L-NAME group, #P<0.05, #P<0.001 vs. respective SHR, *P<0.001 vs. respective L-NAME + Isoprenaline group. n=5-10 in each group

Figure 13. Effects of BK$_{Ca}$ channel blockade by tetraethylammonium (TEA), Kv channel blockade by 4-aminopyridine (4-AP) and their combination (TEA + 4-AP) on isoprenaline-induced inhibition of blood pressure responses to L-NAME in WKY rats subjected to previous blockade of RAS by captopril and SNS by pentolinium. Values are means ± SEM, **P<0.001 vs. L-NAME group, *P<0.001 vs. L-NAME + Isoprenaline group. n=4-8 in each group
4.3. Study 3: Role of calcium sensitization in hypertension

- Role of RhoA/Rho-kinase pathway in the maintenance of blood pressure

The aim of this experiment was to determine the contribution of Rho kinase pathway to blood pressure maintenance in WKY and SHR using Rho kinase inhibitor, fasudil. Baseline MAP levels in both genotypes were similar to those measured in the previous experiments (108 ± 4 mm Hg in WKY, 183 ± 3 mm Hg in SHR). Acute cumulative administration of fasudil elicited dose-dependent blood pressure decreases in both rat strains, however the effect was more pronounced in SHR compared to WKY (Fig. 14A). When these fasudil-induced pressure responses are compared with blood pressure decreases elicited by nifedipine (Fig. 14B), it is evident that WKY rats are more sensitive to Rho kinase inhibition by fasudil than to L-VDCC blockade by nifedipine (Fig. 15). On the contrary, in SHR rats, L-VDCC blockade by nifedipine caused significantly greater blood pressure fall than fasudil (Fig. 15). Moreover, SHR rats were more sensitive to the lowest nifedipine doses (Fig. 14B) while significant differences in fasudil-induced blood pressure responses between the two rat strains were seen only at the highest fasudil doses (Fig. 14A).

Figure 14. Dose-dependent blood pressure changes elicited by increasing cumulative doses of fasudil (0.5, 1, 2, 4 and again 2 mg/kg b.w., panel A) and nifedipine (0.025, 0.05, 0.1, 0.2 and 0.4 mg/kg b.w., panel B) in intact WKY and SHR rats. Values are mean ± SEM, * P<0.05 vs. WKY. n=4 in each group
Figure 15. A comparison of blood pressure decreases elicited by submaximal doses of fasudil (9.5 mg/kg b.w.) and nifedipine (0.75 mg/kg b.w.) in WKY and SHR rats. Values are mean ± SEM, * P<0.05, ** P<0.01 vs. SHR, # P<0.5 vs. respective Fasudil group. n=4 in each group

- **Effect of Rho kinase inhibition by fasudil on norepinephrine-induced vasoconstriction**

To determine the contribution of calcium sensitization to catecholamine-induced vasoconstriction, the effect of Rho kinase inhibition by fasudil on norepinephrine dose-response curves was evaluated. Baseline MAP values resembled those reported in previous experiments (113.5 ± 2 mm Hg in WKY and 179 ± 3 mm Hg in SHR). Norepinephrine produced dose-response curves which started from the different baseline blood pressure levels (Fig. 16A). In this study, SHR rats were more sensitive to norepinephrine compared with WKY, but there were no differences in the slopes of these curves or half-maximal blood pressure responses (Fig. 17A).

Rho kinase blockade by low fasudil doses (2.5 mg/kg b.w.) decreased the starting blood pressure level in SHR but not in WKY (Fig. 16A) and shifted norepinephrine dose-response curves to the right in both strains (Fig. 17A). Thus, dose-response curve of SHR after fasudil was almost identical to that of WKY before fasudil administration (Fig. 17A). Moreover, fasudil application decreased half-maximal blood pressure responses to norepinephrine in WKY, but not in SHR (Fig. 17A).

High dose of fasudil (10 mg/kg b.w.) caused even more pronounced rightward shift of norepinephrine dose-response curves in both strains (Fig. 16B). Contrary to low fasudil doses, high doses increased half-maximal norepinephrine-induced blood pressure responses in WKY but this might be caused by the absence of the highest norepinephrine doses (Fig. 17B). We did not use too high norepinephrine doses due to the discomfort of WKY after
the combination of high fasudil and high norepinephrine doses. This effect of fasudil was not evident in SHR rats (Fig. 17B).

Combined blockade of L-VDCC by nifedipine and Rho kinase by fasudil caused an additional rightward shift of norepinephrine dose-response curves, which was more pronounced in SHR rats (Fig. 18). Thus, after the combined blockade the dose-response curves of both rat strains became identical, indicating the different contribution of calcium influx and calcium sensitization in both rat strains. However, likewise in previous experiment with high fasudil doses, the discomfort of the experimental animals after the combination of fasudil and nifedipine did not allow us to obtain sufficient data for the construction of full dose-response curves neither in WKY nor in SHR rats (Fig. 18). A comparison of Figure 17B and Figure 18 suggests that norepinephrine-induced vasoconstriction is highly dependent on L-VDCC opening in SHR whereas Rho kinase-mediated sensitization of contractile apparatus to calcium plays a greater role in normotensive rats.

Figure 16. The effect of Rho kinase blockade by low (2.5 mg/kg b.w., panel A) and high (10 mg/kg b.w., panel B) fasudil doses (FAS) on the norepinephrine dose-response curves obtained after non-cumulative administration of norepinephrine (0.01-80 μg/kg b.w.) in WKY and SHR rats subjected to previous blockade of RAS by captopril and SNS by pentolinium. The curves represent mean values of respective rat groups. —●— WKY and —▲— SHR: WKY and SHR rats before fasudil injection; - -●- WKY FAS and - -▲- SHR FAS: WKY and SHR rats after fasudil injection. n =4 in each group
Figure 17. The analysis of the effects of low (2.5 mg/kg b.w., panel A) and high fasudil (10 mg/kg b.w., panel B) doses on norepinephrine dose-response curves in WKY and SHR rats. The curves represent mean values of respective rat groups. — — WKY and — — SHR: WKY and SHR rats before fasudil injection; - - - - WKY FAS and - - - - SHR FAS: WKY and SHR rats after fasudil injection. $n = 4$ in each group
Figure 18. The analysis of the effect of L-VDCC blockade by nifedipine on norepinephrine dose-response curves under the conditions of previous Rho kinase inhibition by high fasudil dose (10 mg/kg b.w.) in WKY and SHR rats. The curves represent mean values of respective rat groups.

- - ● - WKY FAS 10 and - - ▲ - SHR FAS 10: WKY and SHR rats after fasudil injection;
- - ○ - WKY FAS 10 + NIF and - - △ - SHR FAS 10 + NIF: WKY and SHR rats after both fasudil (10 mg/kg b.w.) and nifedipine (0.4 mg/kg b.w.) administration. \( n = 4 \) in each group.
5. DISCUSSION

5.1. Role of sympathetic vasoconstriction and calcium influx in hypertension: their relationship

Numerous studies have provided evidence that blood pressure rise in various forms of human essential hypertension as well as in spontaneously hypertensive rats (SHR) is initiated and sustained by the increased activity of sympathetic nervous system (SNS) (Head, 1989; de Champlain, 1990; Judy et al., 1979). The enhanced activity of SNS was also confirmed by the present study, where we have shown that blood pressure fall occurring after the elimination of sympathetic vasoconstriction (by ganglionic blockade with pentolinium) is greater in SHR than in normotensive WKY rats. The significance of SNS in the development of genetic hypertension is well documented by the absence of high blood pressure in SHR subjected to neonatal sympathectomy (Lee et al., 1991; Korner et al., 1993). On the other hand, the crucial role of this system in the maintenance of blood pressure is underlined by the fact that basal blood pressure is proportional to the magnitude of its sympathetic (pentolinium-sensitive) component (Paulis et al., 2007; Hojna et al., 2007). The altered sympathetic function may arise from a variety of dysfunctions including increased sympathetic nerve firing rates (Grassi et al., 1998b), altered neuronal norepinephrine reuptake (Cabassi et al., 1998) and/or enhanced agonist sensitivity of vascular smooth muscles (Bhalla et al., 1987) based on altered postsynaptic mechanisms. These involve an imbalance in postsynaptic adrenoceptor function leading to the increase of intracellular concentration of calcium ions which are essential for the initiation of the contractile processes of vascular smooth muscle cells (Hilgers & Webb, 2005; Sonkusare et al., 2006). Indeed, increased vascular tone of SHR is often attributed to the elevated calcium entry into vascular smooth muscle cells through L-type of voltage-dependent calcium channels (L-VDCC) (Sonkusare et al., 2006; Cox, 2002). This was also confirmed by the results of the present study, since the dose-dependent blood pressure decreases elicited by acute cumulative injections of L-VDCC blocker, nifedipine, were significantly greater in SHR than in WKY. Moreover, we have also shown that the elimination of SNS by ganglionic blockade with pentolinium almost completely abolished hypotensive action of acute L-VDCC blockade by nifedipine in both normotensive and hypertensive rats. This led to the elimination of strain differences in blood pressure decreases induced by nifedipine. Although the blockade of L-VDCC prevented only a part of pentolinium-
induced blood pressure fall, nifedipine administration was also able to cancel the strain
difference in pentolinium-induced hypotensive responses (Table 2). The above mentioned
abolishing of the strain differences indicates that SHR are characterised by increased
contribution of calcium influx through L-VDCC to adrenergic vasoconstriction. However,
the ability of pentolinium to prevent pressor responses to L-VDCC inhibition is greater than
that of nifedipine to prevent blood pressure responses to ganglionic blockade. This can be
explained by the fact that the closure of L-VDCC by nifedipine affects only the tonic but
not the phasic vascular contraction (Paulis et al., 2007). Phasic contraction is mediated by
$\alpha_1$-adrenoceptor stimulation which is independent of L-VDCC calcium influx (van Zwieten
et al., 1983). All these results point out the strong relationship between nifedipine-induced
and pentolinium-induced blood pressure changes suggesting that calcium influx through L-
VDCC forms an important part of sympathetic vasoconstriction which is augmented in
hypertensive animals. These findings are in agreement with the earlier studies
demonstrating the increase in the open-state probability of L-VDCC by norepinephrine
(Benham & Tsien, 1988; Nelson et al., 1988) and greater contribution of these calcium
channels to agonist-induced responses in arteries from SHR as compared to WKY
(Matsuda et al., 1997).

5.2. Involvement of $G_i$ proteins and cAMP in the regulation of L-
VDCC activity

The activity of L-VDCC in the vascular smooth muscle cells is regulated primarily
by changes in membrane potential and/or by intracellular second-messenger systems,
including both cAMP/PKA and cGMP/PKG pathways (Xiong & Sperelakis, 1995). Indeed,
some studies have reported that vasoactive agents exert their action through the modulation
of L-VDCC activity. The elevation of cAMP has been shown to be implicated in the
vasorelaxation in response to the stimulation of $\beta$-adrenoceptors which are coupled to
stimulatory $G$ proteins (Orlov et al., 1996). On the other hand, the decrease of cAMP level
induced by the activation of inhibitory $G$ proteins ($G_i$) has been demonstrated to be
involved in $\alpha$-adrenoceptor- and arginine vasopressin-induced contractions of vascular
smooth muscles (Li & Triggle, 1993; Tabrizchi & Triggle, 1991; Liskova et al., 2007; Li &
Triggle, 1993). $G_i$ proteins have been shown to be involved particularly in alpha2-agonist-
induced contractions, which are strongly dependent on the availability of calcium ions from
extracellular source (Kazda et al., 1985). Thus, the changes of the adenylyl cyclase activity
in response to the altered function and/or amount of $G_i$ proteins in SHR (Anand-Srivastava, 1993) have been suggested to be one of the contributing factors in the pathogenesis of hypertension. Later reports confirmed the suggestion that altered $G_i$ protein-mediated pathway contributes to the development (Marcil et al., 1997; Li & Anand-Srivastava, 2002) as well as to the maintenance (Kost, Jr. et al., 1999) of high blood pressure in SHR. The results of the present work confirmed the important role of $G_i$ proteins in the maintenance of high blood pressure. The inactivation of $G_i$ proteins by in vivo PTX treatment led to a greater reduction of basal blood pressure in SHR than in WKY rats. Thus, the average arterial pressure of PTX-treated SHR reached similar values as in untreated normotensive WKY. These observations are in agreement with previous studies in conscious animals (Li & Anand-Srivastava, 2002; Kost, Jr. et al., 1999). Although the half-time of PTX-mediated ADP-ribosylation of $G_{ia}$ subunit and of PTX-induced decrease of the inhibition of adenylyl cyclase activity in cultured ventricular myocytes is about 200 minutes (Liang & Galper, 1988), the blood pressure reduction following in vivo PTX treatment is a matter of few days. Using radiotelemetry blood pressure measuring, Kost et al. (1999) have shown that blood pressure decrease induced by PTX treatment starts just after a single PTX injection and the maximal hypotensive effect of PTX treatment is evident after 48 hours. This effect was sustained for more than two weeks what is in agreement with our previous observations (unpublished data). Indeed, it has been demonstrated that ADP-ribosylation of $G_{ia}$ subunit and resulting attenuated inhibition of adenylyl cyclase activity is obvious even after four weeks after the treatment in hearts and mesenteric arteries from PTX-treated SHR (Li & Anand-Srivastava, 2002).

The present study has shown that the normalization of blood pressure level in SHR following PTX application was associated with a significant reduction of residual blood pressure (minimal blood pressure obtained after the full dilatation of resistance vessels by a direct NO donor - sodium nitroprusside) suggesting the alterations of structure and/or basal tone of resistance vessels. Since the decrease of residual blood pressure occurred within 48 hours after PTX application when rapid structural remodelling of hypertrophic resistance vessels can hardly occur, the acute normalization of blood pressure in SHR has to be achieved due to the normalization of basal tone of resistance vessels.

We have also demonstrated that PTX-induced blood pressure reduction is a result of pronounced attenuation of sympathetic vasoconstriction in PTX-treated rats, because $G_i$ protein inactivation by PTX caused the attenuation of blood pressure responses to ganglionic blocker, pentolinium, in both rat strains. This PTX effect was greater in SHR,
which are characterised by increased sympathetic vasoconstriction compared to WKY rats (Head, 1989). On the other hand, chronic inactivation of \( G_i \) proteins by PTX caused a substantial augmentation of otherwise moderate blood pressure responses to angiotensin converting enzyme inhibitor, captopril, in both rat strains. The augmented vasoconstrictor role of angiotensin II was compensating for a significantly attenuated role of SNS following PTX treatment. We suggest that this attenuation of sympathetic vasoconstriction was the result of a decreased pressor responsiveness to endogenous catecholamines, since PTX treatment also reduced blood pressure responses to exogenous norepinephrine.

The strong relationship between sympathetic vasoconstriction and calcium influx through L-VDCC in hypertension was also confirmed by our results showing that the attenuation of sympathetic vasoconstriction in PTX-treated SHR was accompanied by the decreased blood pressure sensitivity to the blockade of calcium entry through L-VDCC with nifedipine. Moreover, both chronic PTX treatment and acute nifedipine administration caused considerable rightward shift of norepinephrine dose-response curves in both rat strains. However, the combination of these two treatments had no additive effects. Thus, we can conclude that \( G_i \) proteins play an essential role in the signal transduction from \( \alpha \) -adrenoceptors to L-type of calcium channels suggesting the important role of cAMP in the modulation of L-VDCC activity (Fig. 19).

![Diagram of Molecular mechanisms of the control of membrane potential and calcium influx by cyclic nucleotides. AC adenylyl cyclase](image)

Figure 19. Molecular mechanisms of the control of membrane potential and calcium influx by cyclic nucleotides. AC adenylyl cyclase
5.3. Effect of G\textsubscript{i} protein inactivation on the balance of major vasodilator systems

Another interesting observation of this study was the fact that chronic inactivation of G\textsubscript{i} proteins by PTX caused a considerable attenuation of blood pressure responses to acute NO synthase blockade with L-NAME, the attenuation being more pronounced in WKY compared with SHR. This difference could be ascribed to a different sensitivity of L-VDCC to cyclic nucleotides in the two rat strains. The L-NAME-induced blood pressure responses were also largely prevented by acute nifedipine pretreatment (Pinterova et al., 2009). These findings support the observation of Lewis et al. (2005) that NO dilates resistance arteries by direct and/or indirect closure of L-VDCC. This is in agreement with studies demonstrating the ability of cGMP (Liu et al., 1997a; Taguchi et al., 1997; Ruiz-Velasco et al., 1998; Tewari & Simard, 1997) and cAMP (Ishikawa et al., 1993; Taguchi et al., 1997; Ruiz-Velasco et al., 1998; Ishikawa et al., 1993) to modulate calcium influx through this channel type. Although the involvement of cAMP in the control of L-VDCC activity seemed to be controversial, Ishikawa et al. (1993) have demonstrated that cAMP effects on L-VDCC channel activity are concentration-dependent. Under the physiological conditions a moderate rise of intracellular cAMP leads to a small enhancement of inward calcium current through L-VDCC, whereas higher levels of intracellular cAMP lead to its inhibition. In fact, one of the possible explanation of this could be that low cAMP concentrations are excitatory due to the stimulation of PKA, whereas higher cAMP levels lead to cross-over activation of PKG and hence to the closure of L-VDCC (Ruiz-Velasco et al., 1998). Thus, we can suggest that NO-dependent vasodilation and G\textsubscript{i}-protein-dependent vasoconstriction elicited by norepinephrine are two antagonistic actions operating via the control of calcium influx through L-VDCC (Liskova et al., 2007).

Besides NO-dependent vasodilation, PTX treatment altered another important vasodilator system, i.e. BK\textsubscript{Ca} channels (Jackson, 2005; Coleman et al., 2004; Peterssson et al., 1997). The activation of these potassium channels leads to the cell membrane hyperpolarization and closure of L-VDCC channels while BK\textsubscript{Ca} blockade with TEA causes membrane depolarization and hence L-VDCC opening (Ousterhout & Sperelakis, 1987; Sonkusare et al., 2006). The blockade of BK\textsubscript{Ca} channels with TEA elicited similar blood pressure rises in both strains of untreated rats and PTX treatment significantly augmented blood pressure responses to TEA in WKY and SHR, but this effect was considerably greater in hypertensive rats.
5.4. Involvement of BK\textsubscript{Ca} and K\textsubscript{V} channels in the cAMP action on L-VDCC

On the basis of the above described findings that PTX treatment attenuates blood pressure responses to L-NAME and increases blood pressure responses to BK\textsubscript{Ca} blockade with TEA, we suggested a hypothesis according to which the enhanced cAMP production (due to inactivation of G\textsubscript{i} protein pathway) can diminish L-type calcium influx through the activation of BK\textsubscript{Ca} channels. To evaluate this hypothesis we have performed the experiments in which cAMP level was increased by isoprenaline infusion (β-adrenoceptor agonist) or decreased by propranol administration (β-adrenoceptor antagonist). Then, our attention was focused on whether increased cAMP formation following isoprenaline infusion modulates the activity of L-VDCC through the activation of BK\textsubscript{Ca} channels.

Isoprenaline-induced overproduction of cAMP decreased the level of basal blood pressure in both rat strains. The depressor blood pressure responses elicited by isoprenaline infusion were accompanied by maximal heart rate increases due to the stimulation of cardiac β-adrenoceptors. Despite the fact of increased cardiac output, the isoprenaline infusion was followed by blood pressure lowering indicating that isoprenaline infusion caused large decrease of vascular resistance.

Likewise in animals with increased cAMP production following PTX treatment, the effect of isoprenaline on basal blood pressure was substantially greater in SHR compared to WKY rats. Thus, PTX action can be mimicked by isoprenaline infusion. Furthermore, there were no differences between blood pressure levels stabilized after PTX treatment or isoprenaline infusion. This was true in both SHR and WKY rats (data not shown). This indicates that the effect of increased cAMP levels on basal blood pressure is not dependent on the trigger of cAMP overproduction. The greater sensitivity of hypertensive SHR rats to vasodilator stimuli compared to normotensive WKY rats is in agreement with in vitro studies showing an enhanced β-adrenoceptor-mediated relaxation of isolated blood vessels from SHR (Carvalho et al., 1987; Deragon et al., 1978). However, other authors (Arribas et al., 1994) have observed that β-adrenoceptor-mediated relaxation of norepinephrine-precontracted arteries from SHR is decreased compared to those from WKY and they have ascribed this fact to impaired endothelial function. Since isoprenaline-elicted responses in their study were reduced by endothelial removal, they have suggested the existence of endothelial β-adrenoceptors which mediate vascular relaxation through NO release (Arribas et al., 1994). However, we have recently seen that vasodilation elicited by beraprost, a stable analogue of prostacyclin acting directly on vascular smooth muscle cells through
elevation of cAMP (Coleman et al., 1994; Narumiya et al., 1999; Norel, 2007), is enhanced in SHR compared to normotensive rats (M. Behuliak, unpublished data). Thus, our results question the idea of endothelial β-adrenoceptor-mediated vasorelaxation through the activation of NO pathway.

However, there arises an interesting question: “Why are hypertensive rats more sensitive to vasodilator stimuli than normotensive ones?” One of the possible explanations could be the increased expression of β-adrenoceptors in SHR compared to WKY. This increased β-adrenoceptor expression is, however, accompanied by the enormous increase in α-adrenoceptor expression (Oliver et al., 2009), leading to the imbalance between sensitivity of vascular smooth muscle to vasoconstrictor and vasodilator adrenergic stimuli. When arteries from hypertensive animals are precontracted by high doses of norepinephrine targeting both α- and β-adrenoceptors, the vasodilator effects of isoprenaline are counteracted by increased α-adrenergic vasoconstriction (Arribas et al., 1994). In contrast, in our in vivo study, where α-adrenoceptors were exposed only to physiological concentrations of endogenous norepinephrine, the increased effects of isoprenaline-induced β-adrenoceptor stimulation were not masked by the hyperactivity of α-adrenoceptors in SHR.

Likewise the elimination of G$_i$ proteins with PTX, isoprenaline infusion induced very similar effects on blood pressure responses to NO synthase blockade with L-NAME. Isoprenaline infusion prevented blood pressure rise elicited by L-NAME and abolished the already developed L-NAME-induced blood pressure elevation. This effect was mediated by the stimulation of β-adrenergic receptors, since it could be blocked by β-adrenoceptor antagonist, propranolol. However, unlike PTX treatment, the extent of isoprenaline-induced prevention of L-NAME-induced blood pressure rise was very similar in both WKY and SHR. This could be again explained by a smaller sensitivity of SHR to PTX doses used in our experiments or by increased β-adrenoceptor density in this strain compensating for increased α-adrenoceptor activity.

Our further attention was focused on the possible mechanism by which cAMP pathway could inhibit L-VDCC activity. Thus, we have tested the above mentioned hypothesis that increased cAMP formation (following isoprenaline infusion) inhibits L-VDCC through the activation of potassium channels. Since it has been reported that properties of both calcium-activated (BK$_{Ca}$) and voltage-dependent (K$_V$) potassium channels are altered in hypertension (Nelson & Quayle, 1995; Cox, 2002), we have evaluated the involvement of both potassium channel types in β-adrenoceptor-mediated
vasodilation in normotensive and hypertensive rats. The blockade of BK$_{\text{Ca}}$ or K$_V$ channels significantly diminished the isoprenaline-induced prevention of blood pressure rise following acute L-NAME injection in SHR rats suggesting that activation of both potassium channel types could be involved in the β-adrenoceptor-induced vasodilation (Fig. 19). This is in agreement with the in vitro studies performed in cultured vascular smooth muscle cells demonstrating that both BK$_{\text{Ca}}$ (Sadoshima et al., 1988; Scornik et al., 1993) and K$_V$ channels (Aiello et al., 1995) are regulated by cAMP-dependent pathway. However unlike in SHR, blockade of BK$_{\text{Ca}}$ or K$_V$ channels in WKY rats had no significant effect on isoprenaline-induced prevention of blood pressure responses to L-NAME. This difference could be explained by the increased expression of BK$_{\text{Ca}}$ channels and augmented K$^+$ current density in SHR (Liu et al., 1997b; Liu et al., 1995) suggesting the compensatory up-regulation of these potassium channels in hypertensive rats. However, the different strain effects of BK$_{\text{Ca}}$ or K$_V$ channel blockade failed to confirm the hypothesis on the primary lack of K$_V$ channel action demonstrated in the hypertensive animals (Cox & Rusch, 2002; Cox, 2002). However, the down-regulation of K$_V$ current was shown only in pulmonary vascular smooth muscle cells previously exposed to hypoxic conditions to produce pulmonary hypertension in rats (Platoshyn et al., 2001; Wang et al., 1997). On the contrary, Cox et al. (Cox et al., 2001) have shown the increased gene expression of prominent K$_V$ channel α and β subunits associated with a greater voltage-dependent K$^+$ current density in systemic arteries from SHR compared to WKY. These findings are in accordance with our recent results showing the greater blood pressure rises induced by infusion of K$_V$ channel blocker (4-aminopyridine) in hypertensive rats (data not shown, 21±4 mm Hg in WKY vs. 60±12 mm Hg in SHR).

To elucidate why the blockade of BK$_{\text{Ca}}$ or K$_V$ channels had no effect on isoprenaline-induced attenuation of blood pressure responses to L-NAME in WKY compared to SHR, we used the combined of BK$_{\text{Ca}}$ and K$_V$ channel blockade. The simultaneous infusion of both channel type inhibitors caused a restoration of blood pressure responses to L-NAME during isoprenaline infusion, which was not significantly different from the control L-NAME-induced blood pressure increases. Thus, we suggest that in normotensive WKY rats one class of K$^+$ channels can replace the missing effect of another K$^+$ channel family that was inhibited.
5.5. **Role of calcium sensitization in vascular contractility and high blood pressure maintenance**

In addition to fluctuation of intracellular calcium levels, it seems that the contractility of vascular smooth muscle is also regulated by alterations in the myofilament sensitivity to calcium mediated by the RhoA/Rho-kinase pathway (Somlyo & Somlyo, 2000). Using rabbit aorta, Asano et al. (1989) have shown that Rho kinase inhibition by fasudil (HA1077) produced a concentration-dependent rightward shift of the dose-response curves for various agonists including norepinephrine and angiotensin II. Since the impaired regulation of the vascular tone plays an important role in the pathophysiology of hypertension, it has been proposed that altered activity of RhoA/Rho-kinase pathway contributes to the increased peripheral vascular resistance in hypertension (Uehata *et al.*, 1997; Asano & Nomura, 2003; Mukai *et al.*, 2001). Indeed, Mukai et al. (2001) have demonstrated that mRNA expression of Rho kinase is significantly greater in blood vessels from SHR compared to those from WKY rats. Therefore, we aimed to evaluate the role of calcium sensitization in blood pressure maintenance and the effect of Rho kinase inhibition on the catecholamine-induced vasoconstriction. The inhibition of Rho kinase by cumulative administration of fasudil caused the dose-dependent blood pressure decreases in both rat strains. Although the effect of fasudil was more pronounced in SHR compared to WKY, the significant differences in fasudil-induced blood pressure changes between the two rat strains were seen only at the highest fasudil doses. This is in contrast with the study demonstrating significantly greater blood pressure decreases after oral administration of Y-27632 (another inhibitor of calcium sensitization) in SHR compared to normotensive Wistar rats (Uehata *et al.*, 1997).

Moreover, the comparison of fasudil-induced blood pressure responses with those induced by nifedipine has revealed that WKY rats are more sensitive to Rho kinase inhibition by fasudil than to L-VDCC blockade by nifedipine. On the contrary, hypertensive rats have shown the increased sensitivity to nifedipine compared to fasudil. To the best of our knowledge, the present study is the first one comparing the role of calcium influx with the role of calcium sensitization in normotensive and hypertensive animals.

To determine the contribution of Rho kinase signalling pathway to catecholamine-induced vasoconstriction and to uncover possible differences between hypertensive and normotensive rats, we focused our attention to the effects of Rho kinase blockade by either low or high fasudil doses on norepinephrine dose-response curves in these rat strains. Both fasudil doses shifted norepinephrine dose-response curves to the right in SHR as well as in
WKY rats. While low fasudil dose shifted the dose-response curve of SHR so that it was almost identical to that of control WKY, high fasudil dose caused an even more pronounced rightward shift of norepinephrine dose-response curves in both strains.

It should be mentioned that the effect of Rho kinase inhibition on norepinephrine-induced contraction was (at least after the high fasudil dose) greater in normotensive WKY compared to SHR. Contrary to our results, Mukai et al. (2001) have demonstrated that hydroxyfasudil inhibited the phenylephrine-induced contractile responses in several vascular beds significantly more in SHR than in WKY. Thus, they suggested that Rho kinase is substantially involved in the vascular hyperreactivity in hypertension. However, our present study did not confirm this suggestion. Moreover, in the above mentioned study (Mukai et al., 2001), the effect of Rho kinase inhibition on phenylephrine-induced contraction was greater in young prehypertensive SHR compared to adult hypertensive SHR, what corresponds with our results of greater fasudil effect in normotensive compared to hypertensive rats.

To reveal a possible additive effect of Rho kinase inhibition and blockade of calcium influx through L-VDCC, we also studied the effects of fasudil and nifedipine combination on the norepinephrine dose-response curves. Combination of both inhibitors caused the additional rightward shift of these curves, which was more pronounced in SHR. Thus, after this combined blockade the dose-response curves of both rat strains became identical, indicating different contribution of calcium influx and calcium sensitization in both rat strains. We suggest that norepinephrine-induced vasoconstriction is highly dependent on L-VDCC opening in SHR, whereas Rho kinase-mediated sensitization of contractile apparatus to calcium plays a greater role in normotensive rats.

It seems to be undeniable that Rho kinase pathway plays, together with calcium influx via L-type calcium channels, an important role in the vascular smooth muscle contractility. However, our results indicate that the dependence of blood pressure of normotensive rats on Rho kinase signalling is significantly greater than in hypertensive animals. Furthermore, the inhibition of Rho kinase had a more pronounced effect on norepinephrine dose-response curves again in WKY compared to hypertensive rats. Thus, we did not confirm the hypothesis that the increased activity of RhoA/Rho-kinase signalling pathway contributes to the maintenance of high blood pressure.
6. CONCLUSIONS

The present *in vivo* study was focused on better understanding of the pathological mechanisms contributing to high blood pressure maintenance in genetic hypertension represented by spontaneously hypertensive rats. Our results confirmed the well-known fact that SHR are characterised by elevated sympathetic vasoconstriction. Furthermore, we have demonstrated that:

- the main mechanism by which the augmented activity of sympathetic nervous system (SNS) contributes to the maintenance of high blood pressure is the increased nifedipine-sensitive calcium influx via L-type of voltage-dependent calcium channels (L-VDCC)

- the sympathetic control of L-VDCC is mediated by G\(_i\) protein-cAMP-coupled pathway, the activity of which is increased in hypertensive animals

One of the discussed mechanisms by which cAMP affects L-VDCC is the regulation of membrane potential by means of K\(^+\) current through certain potassium channels. The present results have showed that:

- cAMP overproduction induced by β-adrenoceptor stimulation can mimic hypotensive action induced by G\(_i\) protein inactivation by pertussis toxin (PTX) as well as the effect of PTX treatment on blood pressure responses to NO synthase blockade with L-NAME. These results confirm that cAMP pathway contributes to the control of calcium influx through L-VDCC

- the regulation of L-VDCC by increased endogenous cAMP levels is dependent on the activation of large-conductance Ca\(^{2+}\)-activated (BK\(_{Ca}\)) and voltage-dependent potassium (K\(_V\)) channels. While the inhibition of any of these channels led to the diminution of isoprenaline-induced blood pressure effects in hypertensive rats, we have demonstrated that the absence of one class of K\(^+\) channels in normotensive WKY rats could be compensated by the remaining K\(^+\) channel family
Recent studies indicate that besides the agonist-induced calcium influx, the contractility of vascular smooth muscle is also regulated by alterations in the myofilament sensitivity to calcium which is mediated by the RhoA/Rho-kinase pathway. It was suggested that the alterations of this pathway play very important role in the pathogenesis of hypertension. Our present results confirmed that:

- besides L-type calcium influx, Rho kinase pathway also plays an important role in blood pressure maintenance and in catecholamine-induced vascular contractility. However, the dependence of blood pressure maintenance and agonist-induced contractility on Rho kinase signalling was significantly greater in normotensive than in hypertensive rats disproving the hypothesis about the major involvement of RhoA/Rho-kinase pathway in the pathogenesis of genetic hypertension.

We hope that our results will contribute to the better understanding of altered vasoactive mechanisms involved in the maintenance of high blood pressure in genetic hypertension.
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8. LIST OF PUBLICATIONS

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