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## Altered Neural and Vascular Mechanisms in Hypertension

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### Summary

Essential hypertension is a multifactorial disorder which belongs to the main risk factors responsible for renal and cardiovascular complications. This review is focused on the experimental research of neural and vascular mechanisms involved in the high blood pressure control. The attention is paid to the abnormalities in the regulation of sympathetic nervous system activity and adrenoceptor alterations as well as the changes of membrane and intracellular processes in the vascular smooth muscle cells of spontaneously hypertensive rats. These abnormalities lead to increased vascular tone arising from altered regulation of calcium influx through L-VDCC channels, which has a crucial role for excitation-contraction coupling, as well as for so-called "calcium sensitization" mediated by the RhoA/Rho-kinase pathway. Regulation of both pathways is dependent on the complex interplay of various vasodilator and vasoconstrictor stimuli. Two major antagonistic players in the regulation of blood pressure, i.e. sympathetic nervous system (by stimulation of adrenoceptors coupled to stimulatory and inhibitory G proteins) and nitric oxide (by cGMP signaling pathway), elicit their actions *via* the control of calcium influx through L-VDCC. However, L-type calcium current can also be regulated by the changes in membrane potential elicited by the activation of potassium channels, the impaired function of which was detected in hypertensive animals. The dominant role of enhanced calcium influx in the pathogenesis of high blood pressure of genetically hypertensive animals is confirmed not only by therapeutic efficacy of calcium antagonists but especially by the absence of hypertension in animals in which L-type calcium current was diminished by pertussis toxin-induced inactivation of inhibitory G proteins. Although there is considerable information on the complex neural and vascular alterations in rats with established hypertension, the detailed description of their appearance during the induction of hypertension is still missing.

### Key words

Alpha- and beta-adrenoceptors • G proteins • L-type voltage-dependent calcium channels • Calcium influx • Potassium channels • RhoA/Rho-kinase pathway • Calcium sensitization • Spontaneously hypertensive rats • Sympathetic nervous system • Blood pressure

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### Introduction

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 (Antonaccio and Kerwin 1981, Grassi *et al.* 1998, K-Laflamme *et al.* 1997). Moreover, this can also be supported by the fact that blood pressure lowering of all widely used antihypertensive drugs (such as ACE inhibitors, AT<sub>1</sub> receptor antagonists and others) is

mediated by the attenuation of the SNS function (Antonaccio and Kerwin 1981, Clough *et al.* 1982, K-Laflamme *et al.* 1997). Since components of RAS are present not only in circulation but also in various tissues (such as blood vessel wall, heart, brain, kidney and others), 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 (Balt *et al.* 2003, De Jonge *et al.* 1982) and catecholamine release through AT<sub>1</sub> presynaptic mechanisms (Dendorfer *et al.* 1998), to decrease presynaptic reuptake (Raasch *et al.* 2004) as well as to affect postsynaptic  $\alpha$ -adrenoceptors (Eikenburg 1984, Richer *et al.* 1984). Therefore, it is not surprising that in patients with essential hypertension has been identified an increased sympathetic outflow attributing to essential hypertension a neurogenic character (Grassi *et al.* 1998).

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 review.

#### *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 (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 and Kuneš

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 and Farrell 1979, Tsuda and Masuyama 1991), which plays also a crucial role in the development and maintenance of hypertensive state in humans (Grassi *et al.* 1998, Grassi *et al.* 2008, Schlaich *et al.* 2004).

### **Neural mechanisms of blood pressure control**

#### *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 behavior (Allen 2002, Ross *et al.* 1984). 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).

#### *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* a glutamatergic

synapse (Hayward *et al.* 2002, Li and Pan 2010). 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 and Pan 2010, Sander and 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 adrenergic 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<sub>B</sub> (GABA,  $\gamma$ -aminobutyric acid) receptors, respectively (Guyenet 2006). The experiments of 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 transection, pharmacological blockade of  $\alpha$ - and  $\beta$ -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 (2002) has shown that inhibition of this region by the GABA<sub>A</sub> 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.

#### *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).

#### *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 terminal, the glutamate-gated channels and glutamate reuptake (Sander and 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 AT<sub>1</sub> 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 (Erdős *et al.* 2006).

#### ***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 (De Champlain 1990, Head 1989, 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).

#### ***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  $\alpha$ - and  $\beta$ -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  $\alpha_1$ - and  $\alpha_2$ -subtypes. Both  $\alpha_1$ - and  $\alpha_2$ -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  $\beta_1$ - and  $\beta_2$ -adrenoceptors. While  $\beta_1$ -adrenoceptors are present only in certain vascular smooth muscle cells (e.g. coronary),  $\beta_2$ -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).

#### ***Central $\alpha_2$ -adrenoceptors***

Central  $\alpha_2$ -adrenoceptor family participates in the regulation of the cardiovascular system at many levels. Although all three subtypes of  $\alpha_2$ -adrenoceptors ( $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -adrenoceptors) are expressed within the central cardiovascular control centres, the  $\alpha_{2A}$ -adrenoceptor is a predominant subtype (Kanagy 2005) exerting a sympathoinhibitory function (Makaritsis *et al.* 1999).

#### ***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  $\alpha_{2A}$ -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 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  $\alpha_{2A}$ -adrenoceptor-mediated inhibition of neurotransmitter release can lead to a hypertensive state (Tsuda and Masuyama 1991).

On the other hand, the activation of presynaptic  $\beta_2$ -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  $\beta$ -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 and 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  $\alpha$ -adrenoceptors (Gavras and Gavras 2001, Villalobos-Molina *et al.* 1999) and vasodilator  $\beta$ -adrenoceptors (Borkowski and 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 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 (Carvalho *et al.* 1987, Deragon *et al.* 1978). The latter data are supported by the results of 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.

## **Vascular mechanisms of blood pressure control**

### *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

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

	In situ (mV)		In vitro (mV)	
		Tetrodotoxin		Tetrodotoxin
WKY	-49	-48	-54	-54
SHR	-34	-45	-51	-52

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 and 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. 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 and 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 and Hermsmeyer 1981).

#### *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  $\alpha$ -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 transmembrane 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 and Hermsmeyer 1983) as well as by the strong  $\alpha$ -adrenergic input to small blood vessels in SHR which, as mentioned before, masks an endogenous hyperpolarizing  $\beta$ -adrenergic influence present in both WKY and SHR (Willems *et al.* 1982).

#### *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 5-7 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  $\text{Na}^+$  and  $\text{K}^+$  and thus causing an elevation of electrogenic  $\text{Na}^+$ - $\text{K}^+$  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 and Brayden 2010). TRPM4 channel is highly selective for monovalent cations (such as  $\text{Na}^+$ ) and it requires high levels ( $>1 \mu\text{M}$ ) of intracellular calcium for its activation. This channel is exclusively activated by calcium released from sarcoplasmic reticulum following  $\text{IP}_3$  receptor stimulation. TRPM4 activation enables a short-lasting  $\text{Na}^+$  influx leading to membrane depolarization (Gonzales *et al.* 2010). This explains why agonist-induced stimulation of  $\text{G}_{q/11}$  protein-coupled receptors causes a rapid nifedipine-insensitive phasic contraction (due to  $\text{Ca}^{2+}$  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). Moreover,  $\text{Na}^+$  influx can cause not only depolarization of plasma membrane, but also reversal of the  $\text{Na}^+$ - $\text{Ca}^{2+}$ -exchanger resulting in the calcium influx into the VSMC (Poburko *et al.* 2006).

#### *Interdependence of membrane potential and calcium ions*

One of the main determinants of vascular tone, the intracellular calcium concentration, is controlled by membrane potential. However, at the same time the intracellular calcium level influences a number of mechanisms affecting membrane potential. Thus calcium exerts a feedback control over membrane potential regulating calcium-dependent ion channels, exchangers and ATPases. Since calcium influx is voltage-dependent, the connections between intracellular calcium levels and membrane potential provide possibilities for both negative and positive feedback loops. However, the dominant action of calcium ions on membrane potential is depolarization. This positive feedback is balanced by induction of hyperpolarization through activation of potassium channels (Nilsson 1998) which will be further discussed in the chapter describing particular calcium-activated potassium channels.

#### ***Heterotrimeric G proteins***

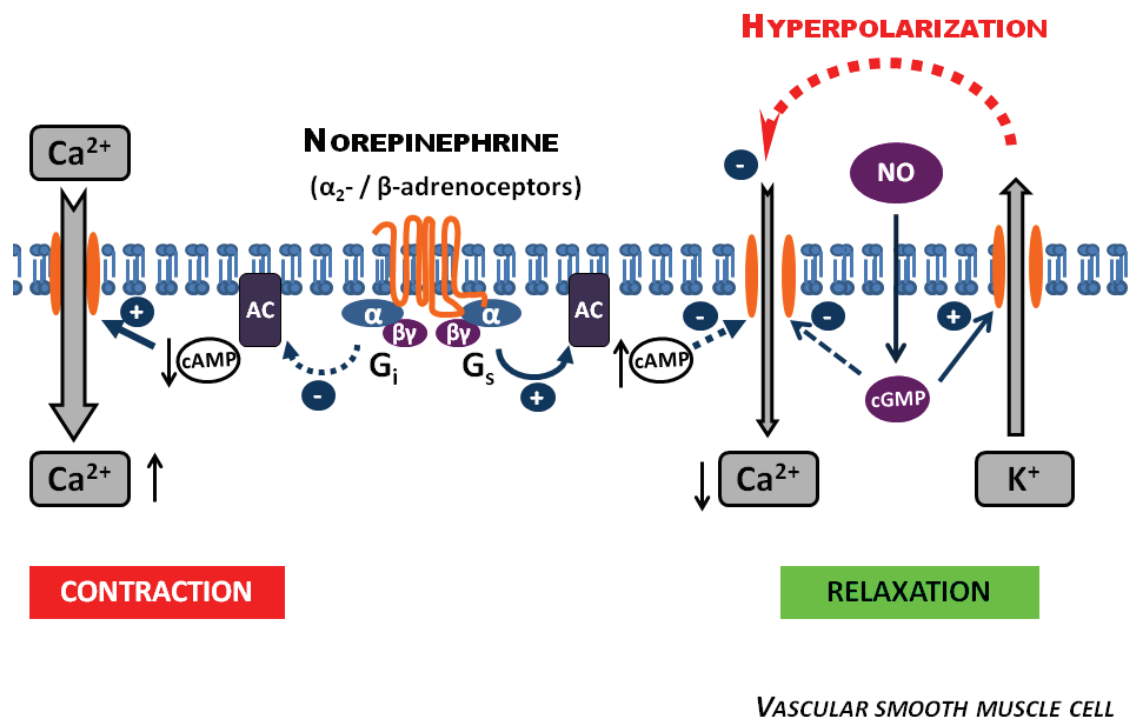
Heterotrimeric guanine nucleotide-binding proteins (G proteins) serve as ubiquitous signal transducers and regulators of cellular signaling in all eukaryotic cells. They are attached to the cell surface plasma membrane and transfer signals from membrane-bound G protein-coupled receptors to intracellular 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 signaling pathways and produce appropriate cellular responses.

G proteins consist of three subunits,  $\text{G}_\alpha$ ,  $\text{G}_\beta$  and  $\text{G}_\gamma$ . Under physiological conditions,  $\text{G}_\beta$  and  $\text{G}_\gamma$  subunits are tightly associated (non-covalently) and form  $\text{G}_{\beta\gamma}$  complex (Clapham and Neer 1993). Currently there are 20 known  $\text{G}_\alpha$ , 6  $\text{G}_\beta$  and 11  $\text{G}_\gamma$  subunits. The  $\text{G}_\alpha$  subunits are classified into subfamilies by its sequence homology and the downstream signal. These include four major families –  $\text{G}_{q/11}$ ,  $\text{G}_s$ ,  $\text{G}_{i/o}$  and  $\text{G}_{12/13}$  (Neves *et al.* 2002).

#### *Mechanism of G protein action*

G proteins become active on binding GTP but their intrinsic ability to hydrolyze GTP converts them to inactive GDP-binding form. Only  $\text{G}_\alpha$  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  $\text{G}_\alpha$  subunit and causes this subunit to dissociate from  $\text{G}_{\beta\gamma}$  complex and hence activate the downstream effectors. The activated state lasts until GTP is hydrolyzed to GDP by the intrinsic GTPase activity of  $\text{G}_\alpha$  subunit. Subsequent reassociation of  $\text{G}_\alpha$  with  $\text{G}_{\beta\gamma}$  complex turns off signal transduction and primes the system to respond to a new stimulus (Clapham and Neer 1993). Initially,  $\text{G}_{\beta\gamma}$  complex was considered to act as a binding partner for the  $\text{G}_\alpha$  subunit which suppresses spontaneous signaling and provides a membrane anchor for the  $\text{G}_\alpha$  subunit due to its hydrophobic character. However, recent evidences indicate that  $\text{G}_{\beta\gamma}$  complex also plays an important role in intracellular signaling (Milligan and Kostenis 2006).

In addition to the regulation of G protein activity by extracellular stimuli, the activity of  $\text{G}_\alpha$  subunit can also be modulated by extraneous factors such as bacterial toxins which modify the properties of G proteins. The  $\text{G}_{sa}$  subunit can be specifically ADP-ribosylated by cholera toxin (exotoxin of *Vibrio cholerae*) and thus irreversibly



**Fig. 1.** Molecular mechanisms of the control of membrane potential and calcium influx by cyclic nucleotides. AC – adenylyl cyclase

activates  $G_s$  proteins mediating the activation of adenylyl cyclase. On the other hand,  $G_{i\alpha}$  subunit can be ADP-ribosylated by pertussis toxin (exotoxin of *Bordetella pertussis*) at its COOH-terminal region preventing thus the interaction of  $G_i$  protein with the receptor. This results in the inhibition of adenylyl cyclase activity (Birnbauer *et al.* 1991). The  $G_{\beta\gamma}$  complex plays an important role in ADP-ribosylation of  $G_{i\alpha}$  subunit by pertussis toxin. It was revealed that this can happen only when  $G_{i\alpha}$  subunit is associated with  $G_{\beta\gamma}$  complex (Graf *et al.* 1992). Thus, cholera and pertussis toxins are invaluable tools for the study of the role of  $G_s$  and  $G_i$  proteins.

#### G protein pathways

As mentioned above, the  $G_\alpha$  subunits are divided into four subfamilies,  $G_{q/11}$ ,  $G_s$ ,  $G_{i/o}$  and  $G_{12/13}$ , 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  $G_{\beta\gamma}$  complex.

$G_s$  pathway stimulates adenylyl cyclase activity leading to accumulation of cAMP and to a subsequent activation of cAMP-dependent protein kinase (Milligan and Kostenis 2006). This pathway mediates vasodilator action of  $\beta$ -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  $G_s$  protein family,  $G_{i/o}$  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  $G_i$  proteins is also involved in the regulation of the  $\alpha_2$ -adrenoceptor-mediated arterial contraction (Li and Triggle 1993) leading to the opening of L-type of voltage-dependent calcium channels through cAMP-dependent mechanism (Pintérová *et al.* 2010).

$G_{q/11}$  pathway is activated by calcium-mobilizing hormones and stimulates PLC- $\beta$  to produce the intracellular messengers, inositol triphosphate ( $IP_3$ ) and diacylglycerol (DAG).  $IP_3$  triggers the release of calcium from intracellular stores, and DAG recruits protein kinase C to the membrane and activates it (Wickman and Clapham 1995).

Activation of  $G_{12}$  and/or  $G_{13}$  proteins is associated with stimulation of the low-molecular-weight G proteins Rho and their down-stream targets.  $G_{12}$  protein has been reported to directly interact with a GTPase-activating protein for Ras, RasGAP; while  $G_{13}$  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).

#### *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). It has been shown that the level of  $G_{sa}$  expression is unaltered, whereas the expression of  $G_{i\alpha-2}$  and  $G_{i\alpha-3}$  subunits is enhanced at protein as well as mRNA levels in SHR aorta (Anand-Srivastava 1992, Thibault and Anand-Srivastava 1992). 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 and 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 signaling 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 (Anand-Srivastava 1997, Lappas *et al.* 2005, Marcil and Anand-Srivastava 1995). 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.

Our recent studies with the long-term inactivation of  $G_i$  proteins by *in vivo* administration of pertussis toxin (Pintérová *et al.* 2009, Pintérová *et al.* 2010) demonstrated that chronic blood pressure decrease observed in pertussis toxin-treated rats was caused by the attenuation of sympathetic vasoconstriction due to a reduction of calcium influx through L-type voltage-dependent calcium channels. This was documented not only by diminished blood pressure fall after acute nifedipine injection but also by the absence of nifedipine-induced rightward shift of norepinephrine dose-response

curve in pertussis toxin-treated rats (Pintérová *et al.* 2010). Pertussis toxin-induced changes were more pronounced in SHR than in their normotensive controls, indicating a major role of  $G_i$  protein-mediated control of calcium influx in hypertension.

#### *Calcium channels in vascular smooth muscle cell*

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).

#### *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 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 (Cox and Rusch 2002, Sonkusare *et al.* 2006). 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).

#### *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). Signaling

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  $\alpha_{1C}$  subunits at the surface membrane (Keef *et al.* 2001). The function of  $\alpha_{1C-b}$  subunit also depends on the association with the  $\alpha_2\delta$  complex, where the  $\delta$  subunit is anchored in the membrane and the extracellular  $\alpha_2$  subunit interacts with the  $\alpha_{1C-b}$  subunit (Sanders 2001, Sonkusare *et al.* 2006).

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 and 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 and Worley 1989). Moreover, the inactivation of these calcium channels seems to be both voltage- and calcium-dependent (Sonkusare *et al.* 2006).

#### *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 and 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  $\alpha_2$ -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).

#### *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  $\alpha_{1C}$  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  $\alpha_{1C}$  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 (Sonkusare *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).

#### *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 signaling pathway (Tewari and Simard 1997), the stimulation of vessels by norepinephrine increases the open-state probability of these channels (Benham and Tsien 1988, Nelson *et al.* 1988) through G<sub>i</sub> protein-mediated mechanism resulting in the decrease of cAMP level (Li and Triggle 1993, Li and Anand-Srivastava 2002). While low doses of cAMP enhance L-type calcium channel current (Ruiz-Velasco *et al.* 1998, Taguchi *et al.* 1997), higher levels of intracellular cAMP lead to its inhibition (Ishikawa *et al.* 1993, Liu *et al.* 1997a). 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 of PKG 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<sup>2+</sup>-dependent potassium channels (BK<sub>Ca</sub>) (Sadoshima *et al.* 1988, Scornik *et al.* 1993) leading to a hyperpolarization of cell membrane (Ousterhout and Sperelakis 1987).

#### **Potassium channels in vascular smooth muscle cells**

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 endothelial cells (Nelson and 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.

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

#### *a) Large-conductance calcium-activated potassium channels (BK<sub>Ca</sub>)*

The dominant K<sub>Ca</sub> channels expressed by smooth muscle cells are large-conductance, BK<sub>Ca</sub> 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 and Quayle 1995). Under the low intracellular calcium, the BK<sub>Ca</sub> channels behave as a pure voltage-dependent potassium channels (Ledoux *et al.* 2006). BK<sub>Ca</sub> are composed of pore-forming Slo1 (or  $\alpha$ ) subunits and Slo $\beta$ <sub>1</sub> (or  $\beta$ <sub>1</sub>) subunits that modulate the calcium sensitivity of the channel. Slo $\beta$ <sub>1</sub> subunit, however, appears to be uniquely expressed in smooth muscle cells and its co-expression with Slo1 subunit results in BK<sub>Ca</sub> channels with increased calcium sensitivity (Amberg *et al.* 2003).

BK<sub>Ca</sub> 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 signaling complexes with L-VDCC channels, protein kinases (PKA, PKC, PKG), phosphatases and other signaling 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<sub>Ca</sub> 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 and Quayle 1995).

#### *b) Small conductance calcium-activated potassium channels (SK<sub>Ca</sub>)*

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

#### *c) Voltage-dependent potassium channels (K<sub>V</sub>)*

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-VGCC channels (Nelson and 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 signaling pathway such as adenosine or prostacyclin. Conversely, vasoconstrictors close  $K_V$  channels through signaling 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).

#### d) 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).

#### e) 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).

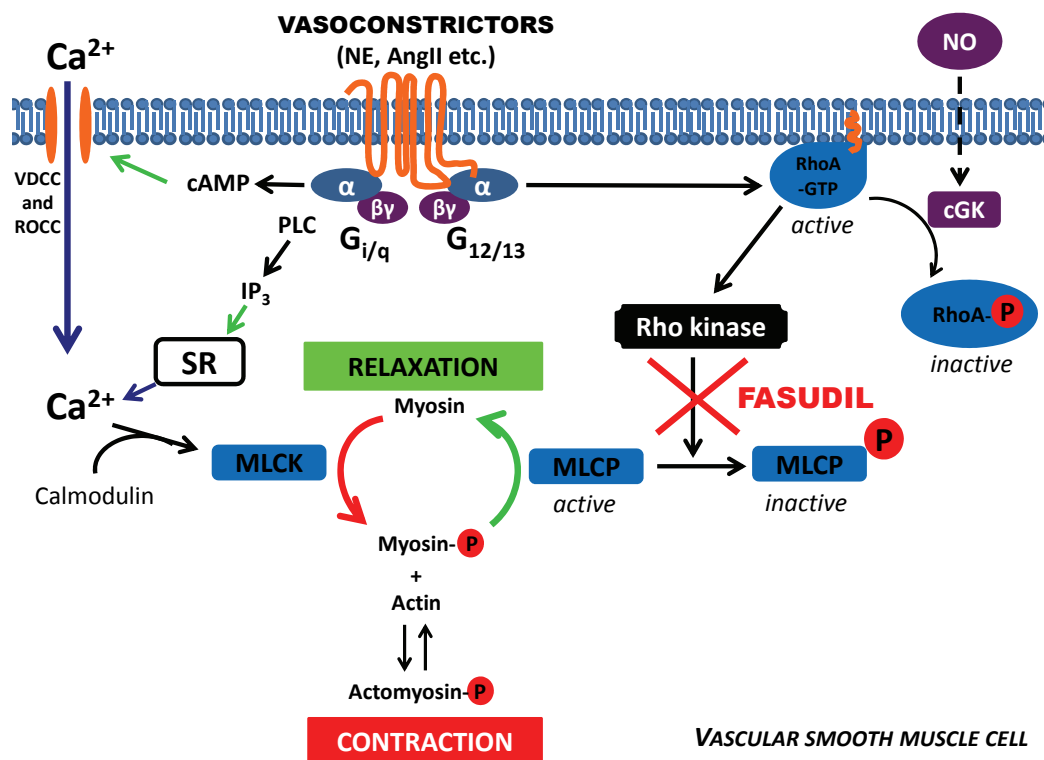
Properties and function of  $K_{ir}$  channels vary among vascular beds as well as with vessel size (Jackson 2005). The expression of  $K_{ir}$  channels is more abundant in the smooth muscle of autoregulatory vascular beds such as the coronary and cerebral circulations. In the

systemic circulation, the expression of the  $K_{ir}$  channel increases as the diameter of the artery decreases (Haddy *et al.* 2006). In coronary and cerebral microcirculation, smooth muscle  $K_{ir}$  channels serve as sensor for increases in extracellular  $K^+$  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).

#### Potassium channels in vascular endothelium

Although endothelial cells also express a similar complexity of potassium channels as vascular smooth muscle cells including  $K_{ATP}$ ,  $K_V$ ,  $K_{ir}$  and  $K_{Ca}$  channels, relatively little is known about these channels. They play an important role in endothelial cell signaling 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 ( $SK_{Ca}$ ) and intermediate conductance ( $IK_{Ca}$ ) channels. Both channel types have smaller single-channel conductance as compared with  $BK_{Ca}$  channels (10 pS for  $SK_{Ca}$  and 30-80 pS for  $IK_{Ca}$ ) (Jackson 2005). Distinct from  $BK_{Ca}$  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,  $SK_{Ca}$  channels can be blocked by apamin and tetrabutylammonium ions and  $IK_{Ca}$  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 (acetylcholine, bradykinin or histamine).



**Fig. 2.** Molecular mechanisms of agonist-induced vascular smooth muscle cell contraction and calcium sensitization mechanism involving RhoA/Rho-kinase signaling pathway. Ang II – angiotensin II, cGK – cGMP-dependent protein kinase, IP<sub>3</sub> – phosphatidylinositol 1,4,5-trisphosphate, MLCK – myosin light chain kinase, MLCP – myosin light chain phosphatase, NE – norepinephrine, PLC – phospholipase C, VDCC – voltage-dependent calcium channels, ROCC – receptor-operated calcium channels.

Physiological function of endothelial K<sub>V</sub> channels has not been established yet. They may participate in membrane potential oscillation and negative feedback regulation of membrane potential. Endothelial K<sub>ir</sub> 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).

#### 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 and 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<sub>Ca</sub> currents are larger and K<sub>V</sub> currents are smaller in VSMCs from hypertensive animals (Cox 2002). One of the mechanisms responsible for larger BK<sub>Ca</sub> current during hypertension appears to be increased expression of BK<sub>Ca</sub> 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<sub>Ca</sub> channel unit composition affecting the calcium sensitivity of BK<sub>Ca</sub> channels and hence leading to increased vascular tone (Amberg *et al.* 2003). Considering these results, it seems that regardless of the increased BK<sub>Ca</sub> currents reported during hypertension, the altered function of BK<sub>Ca</sub> channels is insufficient to oppose the effects of vasoconstrictors.

It seems that a decreased function of K<sub>V</sub> 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 K<sub>V</sub> 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 and Rusch 2002). Although K<sub>ATP</sub> channels appear to be also down-regulated in hypertension, in this regard there is lack of evidence (Jackson 2005).

#### 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<sub>3</sub>). IP<sub>3</sub> 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 (Kuneš *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).

#### *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 and 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 (Fig. 2). The role of MLCP will be discussed in the following section.

#### *Calcium sensitization and RhoA/Rho-kinase signaling 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 and Webb 2005).

Various vasoactive agonists activate specific heterotrimeric G protein-coupled receptors and lead not only to increases in intracellular calcium *via* G<sub>q/11</sub> or G<sub>i</sub> proteins, but also activate G<sub>12/13</sub> proteins that are coupled to Rho/Rho-kinase signaling pathway *via* guanine nucleotide exchange factors (RhoGEFs) (Wirth 2010) (Fig. 2). RhoA is a small monomeric G protein which is like 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 and 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).

#### *Crosstalk between nitric oxide and RhoA/Rho-kinase signaling*

RhoA/Rho-kinase pathway appears to have an impact on NO-signaling 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 signaling 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 (Lee *et al.* 1997, Wu *et al.* 1996) 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 signaling (Wirth 2010).

#### *Role of RhoA/Rho kinase pathway in hypertension*

Alterations of RhoA/Rho-kinase pathway promote the development of hypertension. Changes in RhoA/Rho-kinase signaling 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.

## **Conclusions**

The abnormal sympathetic vasoconstriction, arising from the altered central and peripheral control of SNS as well as from the altered membrane and intracellular transduction processes in VSMC, is one of the major factors contributing to hypertension. However, the final target of these alterations is myosin light chain, phosphorylation of which mediates the vascular smooth muscle contraction. Phosphorylation state of myosin light chain is regulated by two pathways containing phosphorylation-promoting calcium-dependent MLCK and counteracting dephosphorylation-mediating MLCP (also called calcium sensitization). Thus, the calcium influx through L-VDCC and calcium sensitization mediated by RhoA/Rho-kinase pathway are two major determinants of the VSMC contractile activity. Enhanced calcium current through L-VDCC during hypertension is elicited by vasoconstrictors (such as endogenous catecholamines) exerting their effects primarily through the modulation of cAMP/PKA pathway by inhibitory G proteins. On the contrary, cGMP/PKG pathway, which plays an important role in NO-induced vasodilatation, has an inhibitory effect on these calcium channels. However, these two major antagonistic players in the regulation of vascular tone (endogenous catecholamines and NO) as well as other vasoactive agonists also affect RhoA/Rho-kinase signaling pathway which modulates the phosphorylation level of myosin light chain by inactivation of MLCP. Thus, various vasoactive agents use both pathways to modulate myosin light chain phosphorylation and hence the vascular tone. Although the importance of enhanced calcium influx in the development and maintenance of hypertension has been well documented, it still remains to determine the exact role of RhoA/Rho-kinase pathway in hypertension.

## **Conflict of Interest**

There is no conflict of interest.

## **Acknowledgements**

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**Abbreviation list**

4-AP	- 4-aminopyridine	L-NAME	- nitro-L-arginine methyl ester
6-OHDA	- 6-hydroxydopamine	L-VDCC	- L-type voltage-dependent calcium channel
AT <sub>1</sub>	- angiotensin II-type-1 receptor	MAP	- mean arterial pressure
ATP	- adenosine triphosphate	MLCK	- myosin light chain kinase
AV3V	- third ventricle of the hypothalamus	MLCP	- myosin light chain phosphatase
BK <sub>Ca</sub>	- large-conductance calcium-activated potassium channel	MNDA	- <i>N</i> -methyl-D-aspartate
cAMP	- cyclic adenosine monophosphate	NAD(P)H	- nicotinamide adenosine dinucleotide phosphate
cGMP	- cyclic guanosine monophosphate	NO	- nitric oxide
CNS	- central nervous system	NTS	- nucleus tractus solitarii
CVLM	- caudal ventrolateral medulla	PVH	- paraventricular nucleus of the hypothalamus
DAG	- diacylglycerol	PTX	- pertussis toxin
EDHF	- endothelium-derived hyperpolarizing factor	RAS	- renin-angiotensin system
G protein	- heterotrimeric guanine nucleotide-binding protein	RVLM	- rostral ventrolateral medulla
GABA	- $\gamma$ -aminobutyric acid	SHR	- spontaneously hypertensive rat
GDP, GTP	- guanosine diphosphate, guanosine triphosphate	SK <sub>Ca</sub>	- small conductance calcium-activated potassium channels
IK <sub>Ca</sub>	- intermediate conductance calcium-activated potassium channel	SNS	- sympathetic nervous system
IP <sub>3</sub>	- inositol 1,4,5-triphosphate	TEA	- tetraethylammonium ions
K <sub>ATP</sub>	- ATP-sensitive potassium channel	TRPV, TRPC	- transient receptor potential vanilloid and transient receptor potential cation channels
K <sub>ir</sub>	- inward rectifier potassium channel	VSMC	- vascular smooth muscle cell
K <sub>v</sub>	- voltage-dependent potassium channel	WKY	- Wistar-Kyoto rat

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