Physiol. Res. 63 (Suppl. 1): S19-S27, 2014

REVIEW

The Interaction of Calcium Entry and Calcium Sensitization in the Control of Vascular Tone and Blood Pressure of Normotensive and Hypertensive Rats

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Received August 15, 2013
Accepted August 21, 2013

Summary

Increased systemic vascular resistance is responsible for blood pressure (BP) elevation in most forms of human or experimental hypertension. The enhanced contractility of structurally remodeled resistance arterioles is mediated by enhanced calcium entry (through L type voltage-dependent calcium channels – L-VDCC) and/or augmented calcium sensitization (mediated by RhoA/Rho kinase pathway). It is rather difficult to evaluate separately the role of these two pathways in BP control because BP response to the blockade of either pathway is always dependent on the concomitant activity of the complementary pathway. Moreover, vasoconstrictor systems enhance the activity of both pathways, while vasodilators attenuate them. The basal fasudil-sensitive calcium sensitization determined in rats deprived of endogenous renin-angiotensin system (RAS) and sympathetic nervous system (SNS) in which calcium entry was dose-dependently increased by L-VDCC opener BAY K8644, is smaller in spontaneously hypertensive rats (SHR) than in normotensive Wistar-Kyoto (WKY) rats. In contrast, if endogenous RAS and SNS were present in intact rats, fasudil caused a greater BP fall in SHR than WKY rats. Our *in vivo* experiments indicated that the endogenous pressor systems (RAS and SNS) augment calcium sensitization mediated by RhoA/Rho kinase pathway, whereas the endogenous vasodilator systems (such as nitric oxide) attenuate this pathway. However, the modulation of calcium entry and calcium sensitization by nitric oxide is strain-dependent because NO deficiency significantly augments low calcium entry in WKY and low calcium sensitization in SHR. Further *in vivo* and *in vitro* experiments should clarify the interrelationships between endogenous vasoactive systems and the contribution of calcium entry and/or calcium sensitization to BP maintenance in various forms of experimental hypertension.

Key words

Calcium sensitization • RhoA/Rho kinase • Fasudil • Ca^{2+} entry • Nifedipine • BAY K8644

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Introduction

Increased systemic vascular resistance (SVR) is responsible for blood pressure elevation in most forms of human or experimental hypertension. The enhanced contraction of structurally remodeled resistance arterioles is achieved by enhanced calcium entry (usually through L-type voltage-dependent calcium channels – L-VDCC) and/or augmented calcium sensitization (mediated by RhoA/Rho kinase pathway) which are characteristic hallmarks of hypertension (Kazda *et al.* 1986, Uehata *et al.* 1997, Pratt *et al.* 2002).

Figure 1 shows a simplified scheme of the above mentioned pathways responsible for arterial contraction stimulated by numerous vasoconstrictors (Nelson *et al.* 1988, Tsai and Jiang 2006) and attenuated by various vasodilators (Sauzeau *et al.* 2000, Somlyo and Somlyo 2000, Wirth 2010). It is generally assumed that the use of

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selective blockers of these pathways can reveal their participation in the control of vascular tone and blood pressure (BP) in normotensive or hypertensive animals. Nevertheless, this approach neglects the fact that the changes of vascular tone always reflect the existing activity of both pathways. It means that the magnitude of BP reduction elicited by either acute L-VDCC blockade (e.g. by nifedipine) or acute Rho kinase inhibition (e.g. by fasudil) is always dependent on the concomitant activity of the complementary pathway. Thus the increased BP response to fasudil might be not only due to the attenuation of a greater calcium sensitization at normal calcium entry but also due to the attenuation of a normal calcium sensitization at a greater calcium entry. The above mentioned facts might considerably complicate the evaluation of the role of these two pathways in BP control.

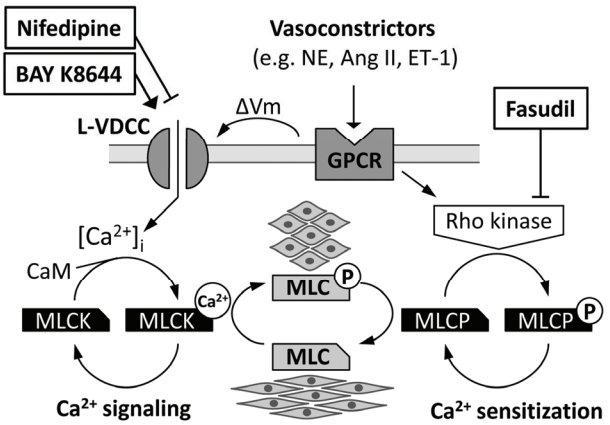

Fig. 1. The schematic representation of the principal pathways (Ca^{2+} entry signaling and Ca^{2+} sensitization) involved in the control of vascular smooth muscle tone. GPCR – G protein-coupled receptors, ΔVm – membrane potential change, L-VDCC – L type voltage-dependent calcium channels, MLC – myosin light chain, CaM – calmodulin, MLCK – myosin light chain kinase, MLCP – myosin light chain phosphatase, NE – norepinephrine, Ang II – angiotensin II, ET-1 – endothelin-1.

Recently we have developed a new approach for the evaluation of basal calcium sensitivity in conscious rats subjected to a combined blockade of renin-angiotensin system (RAS) and sympathetic nervous system (SNS) (Behuliak *et al.* 2013). Under these conditions we have demonstrated a considerably lower calcium sensitization in spontaneously hypertensive rats (SHR) which are characterized by enhanced calcium entry compared to their normotensive controls – Wistar-Kyoto (WKY) rats. Greater basal calcium sensitization of WKY rats was confirmed not only by a more pronounced fasudil-induced rightward shift of norepinephrine (NE) dose-response BP curve in conscious WKY than in SHR but also by a greater fasudil-induced attenuation of NE-induced contraction in WKY than SHR arteries (Behuliak *et al.* 2013).

Nevertheless, it remains to determine the relationship between *basal calcium sensitization* (determined in the absence of endogenous RAS and SNS) and *actual calcium sensitization* (seen in intact rats with functional vasoconstrictor systems) which might substantially differ in normotensive and hypertensive rats due to the differences in the activity of particular vasoactive systems.

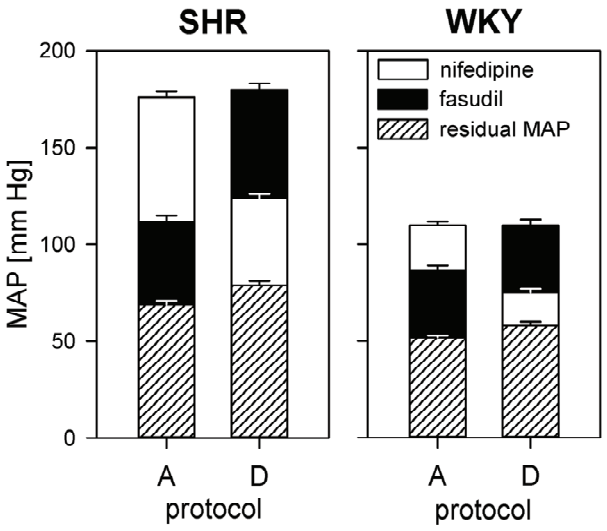

Fig. 2. MAP determined before and after the intravenous administration of nifedipine (0.75 mg/kg b.w.) and fasudil (10 mg/kg b.w.) in a different sequence (for details on protocols A and D see Table 1).

The cooperation of both pathways in blood pressure control in vivo

Figure 2 depicts mean arterial pressure (MAP) values found in SHR and WKY rats subjected to the acute inhibition of L-VDCC (by nifedipine, NIF) and Rho kinase (by fasudil, FAS) with a different sequence of inhibitor administration (Protocols A and D see Table 1). It is evident that in SHR the administration of the first inhibitor (nifedipine or fasudil) always elicited much greater MAP reduction (NIF: –64±3 mm Hg, FAS: –56±3 mm Hg) than the subsequent addition of the second reciprocal inhibitor (+FAS: –43±3 mm Hg, +NIF: –43±3 mm Hg). Thus the attenuation of either calcium entry or calcium sensitization in hypertensive animals diminishes BP effects exerted by the reciprocal pathway blockade. This can be explained by the fact that the second inhibitor always affects BP which has already been lowered by the

previous blockade. However, this is not the case in normotensive WKY rats in which MAP response to fasudil (applied as the first inhibitor: –35±3 mm Hg, applied as the additive inhibitor: –36±2 mm Hg) was always greater than that to nifedipine (–23±2 mm Hg and –17±2 mm Hg, respectively) irrespective of their order of administration (Fig. 2).

Since the magnitude of nifedipine- and fasudil-sensitive BP components depends on the mutual interaction of both pathways, we have also compared BP responses of adult male SHR and Wistar rats to these two inhibitors administered using different protocols (for details see Table 1). The magnitude of nifedipine- and fasudil-sensitive BP components in SHR was highly dependent on the sequence of inhibitor administration (Table 1, Fig. 3). If the full nifedipine dose was given initially (Protocol A) or before high BP was lowered substantially by preceding fasudil administration (Protocol C), nifedipine was a more potent BP-lowering drug than fasudil. However, similar major BP-lowering effect was also observed if fasudil was administered to SHR at high BP level (Protocols B and D). In Wistar rats fasudil was always a more efficient BP-lowering drug and even a partial fasudil pretreatment (Protocol C) further lowered the nifedipine-sensitive BP component (Table 1, Fig. 3).

Table 1. Mean arterial pressure (MAP) responses of SHR and Wistar rats to the different sequence of nifedipine and fasudil administration used in protocols A-D.

Protocol	Nifedipine	Fasudil	Nifedipine	Fasudil	Nifedipine
A	0.75 mg/kg	11 mg/kg			
B	0.15 mg/kg	11 mg/kg	0.6 mg/kg		
C		3 mg/kg	0.75 mg/kg	8 mg/kg	
D				11 mg/kg	0.75 mg/kg

Protocol	Strain	Total MAP change by nifedipine	Total MAP change by fasudil	Total MAP change by fasudil and nifedipine
A	*Wistar*	-23±2	-32±2	-55±3
B	*Wistar*	-14±4	-30±3	-44±1
C	*Wistar*	-11±2	-34±1	-45±3
D	*Wistar*	-16±1	-35±1	-51±3
A	*SHR*	-73±8	-43±4	-116±6
B	*SHR*	-43±5	-67±3	-110±4
C	*SHR*	-68±5	-46±6	-114±4
D	*SHR*	-46±4	-62±4	-108±5

Data (mm Hg) are means ± SEM, n=4-6 per group. Average initial MAP values were 111±2 mm Hg in Wistar rats and 185±3 mm Hg in SHR.

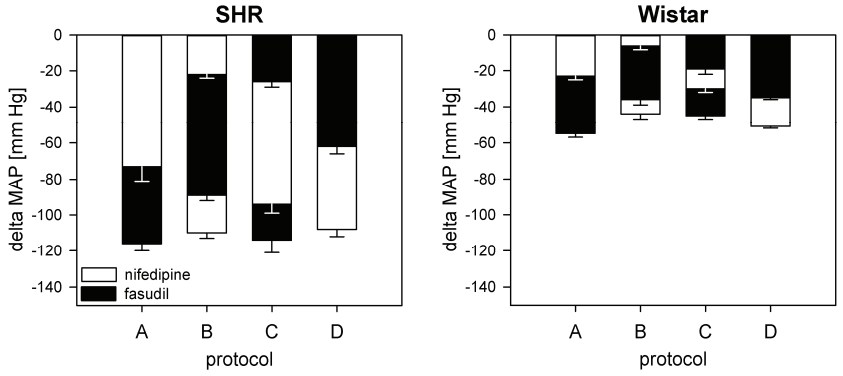

Fig. 3. MAP changes elicited in intact Wistar and SHR rats by intravenously administered fasudil or nifedipine using four different application protocols A-D (for details see Table 1). Data are mean ± SEM, n=4-6 per group.

The modification of both pathways by endogenous vasoactive systems

The *in vivo* determination of calcium entry or calcium sensitization in the absence of endogenous RAS and SNS activity is rather difficult because the elimination of endogenous pressor systems causes a pronounced hypotension in which both inhibitors cause only small MAP changes (Paulis *et al.* 2007, Behuliak *et al.* 2013).

We therefore recorded MAP responses to L-VDCC opening elicited by acute dose-dependent acute administration of BAY K8644 at two different levels of calcium sensitization, i.e. before and after Rho kinase blockade by fasudil (Fig. 4, left panel). The advantage of this method is that BAY K8644-induced calcium entry does not modify calcium sensitization mediated by RhoA/Rho kinase pathway (Alvarez *et al.* 2010). MAP response to BAY K8644 recorded in the presence of fasudil reflects BP effects of increasing calcium entry at major attenuation of calcium sensitization, whereas the difference between MAP changes recorded in the absence and in the presence of fasudil indicates the extent of basal calcium sensitization susceptible to Rho kinase inhibition. The right panel of Figure 4 shows that SHR are characterized by lower calcium sensitization but higher calcium entry compared to WKY rats (Behuliak *et al.* 2013).

When MAP changes obtained by the pharmacological inhibition of the two signaling pathways (L-VDCC and Rho kinase) in intact rats are compared with MAP changes achieved by the pharmacological opening of L-VDCC in animals deprived of endogenous pressor systems (RAS and SNS) (Table 2), there are some interesting relationships. If BP effects of calcium entry are measured after the reduction of calcium sensitivity by fasudil, both approaches indicated a greater magnitude of BP component dependent on calcium entry in SHR than in WKY. On the other hand, BP component corresponding to calcium sensitization was considerably smaller in intact WKY than SHR with preserved RAS and SNS, whereas this BP component was greater in WKY than in SHR deprived of endogenous RAS and SNS. Consequently, MAP reduction caused by a combined blockade of L-VDCC and Rho kinase in intact SHR was close to their maximal MAP rise elicited by BAY K8644-induced enhancement of calcium entry, whereas MAP response to combined blockade in intact WKY rats was equal to 50 % of BAY K8644-induced MAP rise in WKY animals subjected to the inhibition of endogenous RAS and SNS (Table 2).

Table 2. The contribution of calcium sensitization and calcium entry to blood pressure of SHR and WKY rats with and without endogenous RAS and SNS activity.

	Intact rats with preserved endogenous RAS and SNS	**Rats subjected to the blockade of endogenous RAS and SNS**
Total	***ΔMAP FAS + NIF***	***ΔMAP BAY K8644 prior to FAS***
SHR	-101±3	119±6
WKY	-52±2*	100±4*
Ca sensitization	***ΔMAP FAS prior to NIF***	***FAS-dependent ΔMAP BAY K8644***
SHR	-56±3	51±5
WKY	-35±2*	66±4*
Ca entry	***ΔMAP NIF after FAS***	***ΔMAP BAY K8644 after FAS***
SHR	-45±2	68±6
WKY	-17±2*	34±4*
Ca sensitization / Ca entry ratio		
SHR	1.24	0.75
WKY	2.06	1.94

The magnitude of MAP reduction elicited by fasudil (FAS) and/or nifedipine (NIF) in rats with intact endogenous RAS and SNS is compared to the magnitude of MAP rise induced by BAY K8644 (in the absence or presence of fasudil) in rats subjected to previous RAS and SNS blockade. Data (mm Hg) are mean ± SEM, n=6-8 per group. * Significantly different ($p<0.05$) from SHR.

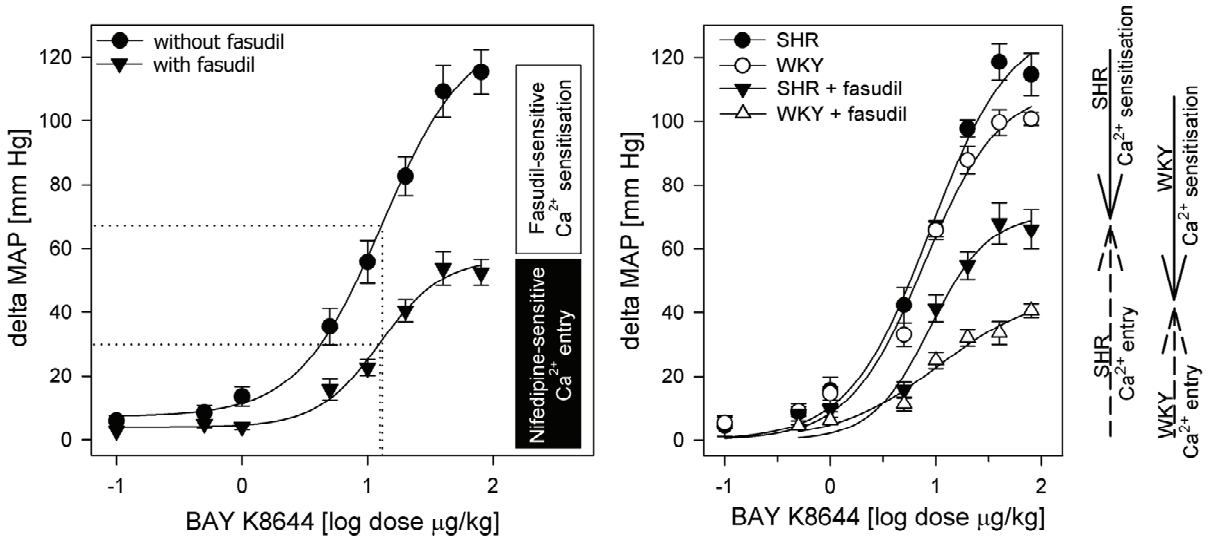

Fig. 4. Left panel. The determination of the complementary role of calcium sensitization and calcium entry in blood pressure control of conscious rats. Dose-dependent BP changes elicited by increasing cumulative doses of BAY K8644 (0.1, 0.5, 1, 5, 10, 20, 40 and 80 μg/kg b.w.) in the absence or presence of fasudil (10 mg/kg b.w.) in rats pretreated with captopril and pentolinium to eliminate the influence of endogenous RAS and SNS. **Right panel.** Dose-dependent MAP changes elicited by BAY K8644 in the absence (—o— WKY and —•— SHR) and in the presence (- - Δ - - WKY+FAS and - -▲- - SHR+FAS) of fasudil (10 mg/kg b.w.). Data are mean ± SEM, n=6 per group (modified from Behuliak *et al.* 2013).

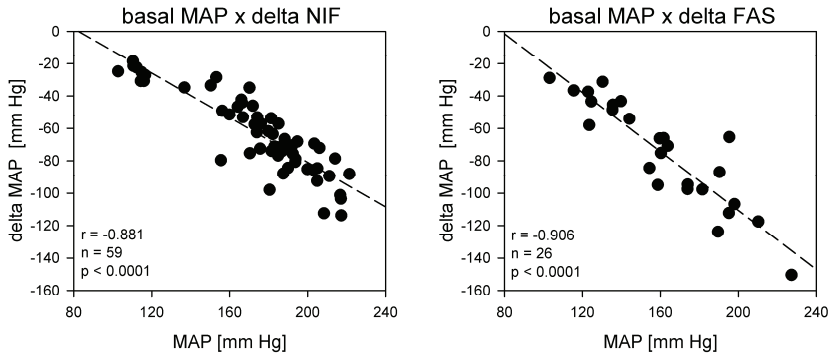

Fig. 5. Left panel. The correlation between basal MAP values and nifedipine-induced MAP changes in WKY and SHR (untreated and subjected to captopril or hydralazine antihypertensive treatment). **Right panel.** Correlation between basal MAP values and fasudil-induced MAP changes in WKY rats, some of them being treated with L-NAME for different periods of time.

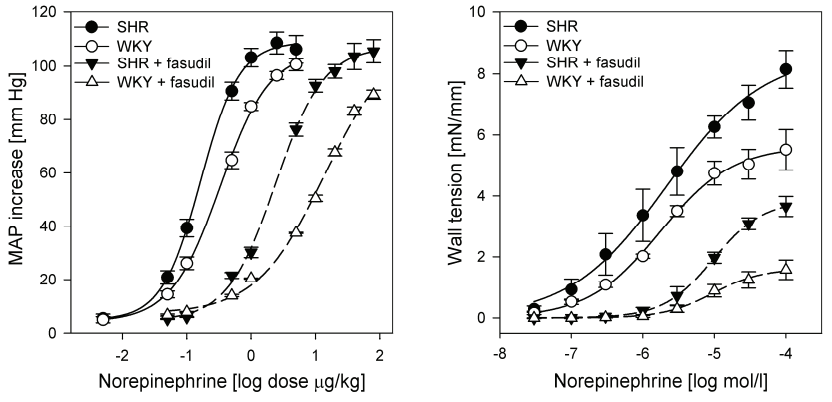

Fig. 6. Left panel. The *in vivo* effects of fasudil (10 mg/kg b.w.) on NE dose-response curves in WKY and SHR rats. The curves represent MAP in particular rat groups studied before (—o— WKY and —•— SHR) and after fasudil (- - Δ - - WKY+FAS and - -▲- - SHR+FAS) (n=6 per group). **Right panel.** The *in vitro* effects of fasudil (10-5 mol/l) on the development of NE-induced contractions of femoral arteries of WKY and SHR (untreated vessels: —o— WKY and —•— SHR; fasudil-pretreated vessels: - - Δ - - WKY+FAS and - -▲- - SHR+FAS). Data are mean ± SEM, n=5 per group (modified from Behuliak *et al.* 2013).

The interpretation of the above findings is not easy. However, the first surprising observation is the possibility to reach similar BP elevation in WKY by pharmacological opening of their L-VDCC as in SHR (Fig. 4, right panel). Thanks to the recording of BAY K8644-induced MAP changes also in the presence of Rho kinase inhibitor fasudil it is evident that BP rise in SHR was due to greater calcium entry even if their basal calcium sensitization was decreased. On the contrary, a similar BAY K8644-induced MAP rise in WKY rats was

achieved by a combination of higher calcium sensitization with lower calcium entry (Fig. 4, right panel) (Behuliak *et al.* 2013).

It should be pointed out that BP changes elicited by the acute administration of either nifedipine or fasudil to intact rats are always proportional to the basal BP level (Kuneš *et al.* 2004, Zicha *et al.*, unpublished data). This is documented by highly significant linear correlations between basal MAP values and MAP reductions induced by the respective inhibitors (Fig. 5). The similarity of both correlations seems to support the idea that BP changes resulting from the acute blockade of either pathway always reflect a product of the changes in existing calcium entry and calcium sensitization. This dependence on basal BP could explain why we observed a smaller BP response to nifedipine or fasudil in normotensive WKY rats unless we augmented their BP to hypertensive levels by L-VDCC opening.

Under the conditions of RAS and SNS blockade the resistance vessels of SHR are characterized by enhanced calcium entry and smaller calcium sensitization as compared to WKY rats (Behuliak *et al.* 2013). Nevertheless, it evident that under the normal conditions high BP of SHR is maintained by sympathetic hyperactivity (Head 1989, Paulis *et al.* 2007, Pintérová *et al.* 2009, 2010, 2011, Behuliak *et al.* 2013) which is known to augment not only calcium entry (Nelson *et al.* 1988) but also calcium sensitivity (Tsai and Jiang 2006). It is evident that there are major strain differences in the contribution of calcium sensitization and calcium entry to BP maintenance (Table 2). The ratio of BP changes induced by fasudil to those elicited by nifedipine was about twice as high in WKY than in SHR (intact rats: 2.06 vs. 1.24; RAS- and SNS-deprived rats: 1.94 vs. 0.75). Thus the endogenous pressor systems (RAS and/or SNS) do not augment calcium sensitization in intact WKY rats, but there is a considerable potentiation of calcium sensitization in intact SHR which might be a consequence of sympathetic hyperactivity.

The contribution of both pathways to vascular tone in vivo and in vitro

To evaluate the contribution of calcium sensitization and/or calcium entry to the control of vascular tone and blood pressure in WKY and SHR we have performed several *in vivo* and *in vitro* experiments. Figure 6 (left panel) indicates that the acute inhibition of Rho kinase by fasudil in conscious rats shifted norepinephrine (NE) dose-response MAP curve to the right in both rat strains but this change was more pronounced in WKY than in SHR (Behuliak *et al.* 2013). Fasudil also attenuated NE-induced contraction more in femoral arteries isolated from WKY than in those from SHR (Fig. 6, right panel). An even more pronounced strain difference in calcium sensitization was demonstrated using another Rho kinase inhibitor Y-27632 which attenuated phenylephrine (PE)-induced smooth muscle contraction more in WKY than in SHR endothelium-denuded femoral arteries (Fig. 7). Of course, the role of α_2- and/or β-adrenergic effects of norepinephrine (Pintérová *et al.* 2009) as well as the contribution of endothelial factors (Líšková *et al.* 2007) to calcium sensitization and/or calcium entry also deserve further investigations. Nevertheless, a comparison of the role of calcium sensitization in BAY K8644-induced BP elevation (Fig. 4, right panel) or PE-induced arterial contraction (Fig. 7) in the two rat strains clearly indicates that under the conditions of equal BP rise or equal arterial contraction calcium sensitization plays a greater role in WKY rats, whereas calcium entry is more important in SHR.

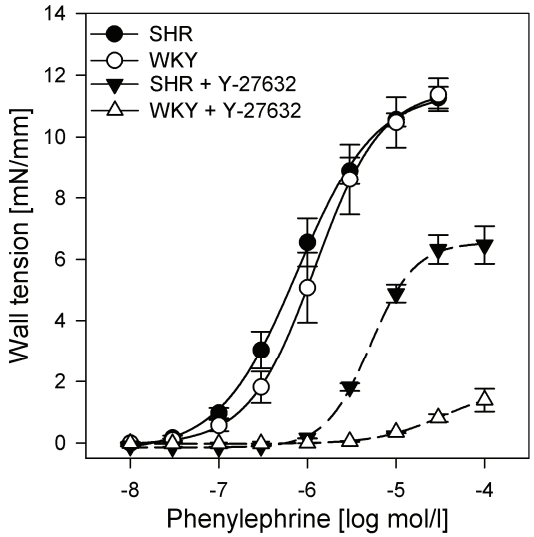

Fig. 7. The effects of Y-27632 (10^{-5} mol/l) on the development of phenylephrine-induced contractions of endothelium-denuded femoral arteries from WKY and SHR (untreated vessels: —o— WKY and —●— SHR; Y-27632-pretreated vessels: - - Δ - - WKY+ Y-27632 and - -▲- - SHR+ Y-27632). Data are mean ± SEM, n=5 per group.

Further series of *in vivo* and *in vitro* experiments was focused on the comparison of the effects of Rho kinase inhibition or calcium entry blockade on blood pressure and vascular contraction in WKY and SHR (Fig. 8). The *in vivo* experiments carried out in rats with

intact RAS and SNS indicated that increasing doses of nifedipine substantially attenuated the strain difference in BP, whereas fasudil had only a modest influence on this BP difference because it lowered BP in both rat strains (Fig. 8, upper panels). On the other hand, the *in vitro* experiments in NE-precontracted small femoral or mesenteric arteries with preserved endothelium did not indicate such considerable differences between vasorelaxing effects of fasudil and nifedipine (Fig. 8, middle and lower panels). These myographic experiments should be further extended to arteries of various diameter and location in order to find which vascular bed and/or experimental conditions correspond to the *in vivo* situation in intact rats with preserved endogenous vasoactive systems.

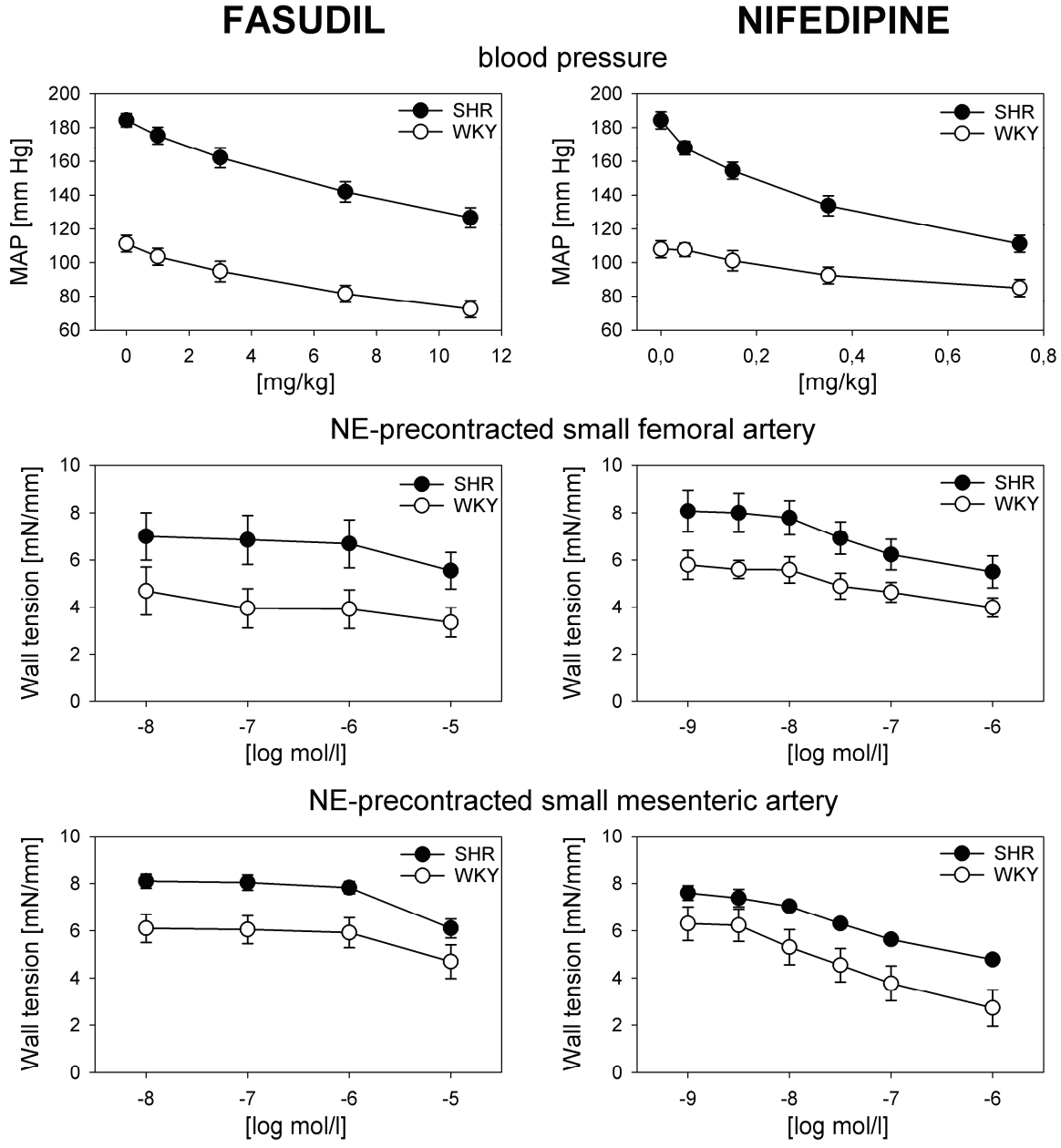

Fig. 8. Dose-dependent effects of intravenously administered fasudil (**left panels**) or nifedipine (**right panels**) on MAP of intact WKY (—o—) and SHR (—•—) rats (n=8 per group) (**upper panels**). Concentration-dependent effects of fasudil or nifedipine on the wall tension in small femoral arteries (diameter 250 µm) (**middle panels**) or mesenteric arteries (diameter 250 µm) (**lower panels**) of WKY and SHR precontracted with norepinephrine (NE, 10^{-5} mol/l). Data are mean ± SEM, n=4-5 per group.

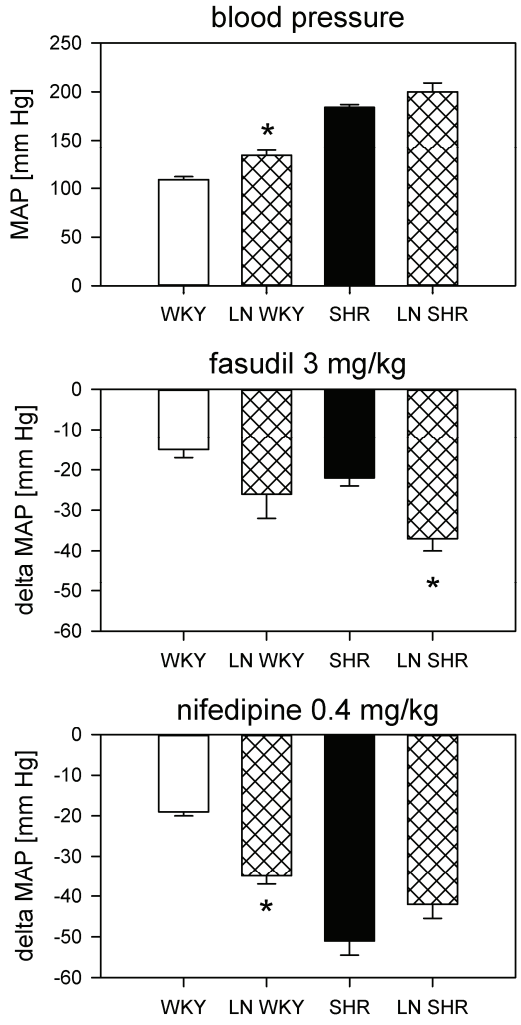

Fig. 9. MAP of intact (open bars) and L-NAME-pretreated (cross-hatched bars) WKY and SHR rats (**upper panel**) as well as their MAP response to acute intravenous administration of fasudil (3 mg/kg) (**middle panel**) or nifedipine (0.4 mg/kg) (**lower panel**). Data are mean ± SEM, n=4-8 per group. * P<0.05 vs. intact rats of the same genotype.

The role of nitric oxide in the modulation of calcium sensitization and/or calcium entry

Since NO was suggested to attenuate calcium sensitization mediated by RhoA/Rho kinase pathway (Sauzeau *et al.* 2000, Wirth 2010), we have focused our attention on the possible influence of endogenous NO on BP changes elicited by acute fasudil administration under the *in vivo* conditions. We have therefore compared dose-dependent BP reduction induced by fasudil in either intact rats or rats acutely pretreated with NO synthase inhibitor L-NAME (30 mg/kg b.w.). Figure 9 (upper panel) shows that the acute NOS inhibition elevated BP more in WKY than in SHR. The absence of endogenous NO augmented BP reduction induced by low doses of fasudil (up to 3 mg/kg b.w.) but this effect was significant in SHR only (Fig. 9, middle panel). On the other hand, acute NO deficiency almost doubled BP response to calcium entry blockade by nifedipine (0.4 mg/kg b.w.) in fasudil-pretreated WKY, whereas it had no effect on the already high nifedipine-sensitive BP component in fasudil-pretreated SHR (Fig. 9, lower panel) (Zicha *et al.*, unpublished data). These findings suggest that NO-dependent modulation of calcium entry and calcium sensitization is rather complex. Further *in vivo* and *in vitro* experiments should clarify the interrelationships between endogenous vasoactive systems and the contribution of calcium entry and/or calcium sensitization to BP maintenance in various forms of experimental hypertension.

In conclusions, our *in vivo* experiments indicated that the endogenous pressor systems (RAS and SNS) augment calcium sensitization mediated by RhoA/Rho kinase pathway, whereas the endogenous vasodilator systems (such as nitric oxide) attenuate this pathway.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by grants AV0Z 50110509, 305/09/0336 and 304/12/0259 (GA ČR).

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