

## Effect of Protein Kinase C and Protein Kinase A Inhibitors on Contraction of Isolated Femoral Arteries of SHR and Wistar Rats

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### Summary

Alterations of calcium handling and other second messenger cascades including protein kinase C (PKC) and A (PKA) were suggested to be responsible for abnormal vascular function in spontaneously hypertensive rats (SHR). However, the relative contribution of these pathways to vasoconstriction is still not completely understood. We investigated the effect of Ro 31-8220 (PKC inhibitor) and H89 (PKA inhibitor) on vasoconstriction induced by 120 mM KCl or by addition of 10  $\mu$ M noradrenaline (NA) in isolated femoral arteries of control Wistar rats and SHR. Moreover, we investigated these responses in the presence and absence of  $Ca^{2+}$  ions in the incubation medium in order to assess the role of calcium influx in these contractions. We observed that while the vasoconstriction in the presence of calcium was not different between Wistar and SHR, the difference between constriction elicited by NA addition in the absence and presence of external calcium was larger in SHR. The inhibition of PKC had no effect on constrictions in SHR, but diminished constrictions in Wistar rats. PKA inhibition slightly enhanced constrictions in Wistar rats, but reduced them in the presence of calcium in SHR. We conclude that vasoconstriction elicited by adrenergic stimulation is more dependent on extracellular calcium influx in SHR compared to Wistar rats. Moreover, the activation of PKA contributes to this calcium-dependent vasoconstriction in SHR but not in Wistar. On the other hand, PKC activation seems to play a less important role in vasoconstriction in SHR than in Wistar rats.

### Key words

Protein kinase C • Protein kinase A • Femoral artery • Calcium • Myograph

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### Introduction

Spontaneously hypertensive rats (SHR) represent an attractive model of essential hypertension. Augmented vasoconstrictor ability of arteries isolated from SHR was proposed to be involved in this type of hypertension. Several studies have reported abnormalities of vascular smooth muscle in SHR. These include disturbed cell  $Ca^{2+}$  handling (Hermsmeyer *et al.* 1989, Martens *et al.* 1998, Lompre 1999, Cox 2002) probably linked to abnormal properties of L-type voltage-dependent  $Ca^{2+}$  channels (VDCC) in SHR (Hermsmeyer *et al.* 1989, Matsuda *et al.* 1997), which display higher L-type  $Ca^{2+}$  current density due to a more frequent opening of the channels (Ohya *et al.* 1998). Available studies also suggest that there may be an increased number of L-type  $Ca^{2+}$  channel pores (Pratt *et al.* 2002) or increased  $Ca^{2+}$  stores (Goldberg *et al.* 1977, Miquel *et al.* 2005). Furthermore, the  $Ca^{2+}$ -sensitivity of vascular smooth muscle could be modulated by protein kinase C (Kitazawa *et al.* 2000) or protein kinase A (Wooldridge *et al.* 2004). PKC was proposed to be up-regulated (Kanashiro *et al.* 2001) and PKA less active in SHR (Alemany *et al.* 2006).

In vascular smooth muscle, PKC and PKA are

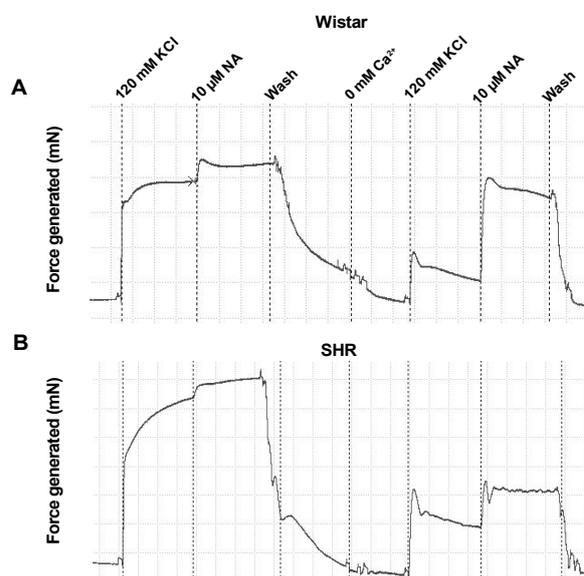
activated by noradrenaline through stimulation of  $G_q$  and  $G_s$  proteins, respectively (Nishizuka 1988, Somlyo *et al.* 1988) or to lesser extent by membrane depolarization (Takuwa and Rasmussen 1987, Ko *et al.* 2008). The role of PKC in the regulation of smooth muscle was suggested by the finding that tumor-promoting phorbol esters, which specifically activate PKC, induce sustained contractions in smooth muscles (Castagna *et al.* 1982, Rasmussen *et al.* 1987). The second messenger adenosine 3', 5'-cyclic monophosphate (cAMP) was shown to mediate its effects through PKA activation leading to relaxation of smooth muscle (Somlyo *et al.* 1994) mainly by  $Ca^{2+}$ -dependent or -independent effects (Somlyo *et al.* 2003, Nishimura *et al.* 1989).

The aim of our study was to evaluate the relative contribution of calcium influx to vascular constriction induced by KCl or adrenergic stimulation in SHR. Furthermore, we intended to elucidate the role of PKC and PKA recruitment in this vasoconstriction.

## Methods

Male 3-month-old SHR and Wistar rats were sacrificed by ether overdose. Femoral arteries were isolated, cleaned, and cut into segments of equal length. Attention was paid to preserve the endothelium of the arteries. The segments were subsequently mounted on standard Mulvany-wire myograph (610M; Danish Myo Technology, Aarhus, Denmark) filled with modified Krebs-Henseleit solution (KHS, pH 7.4) of the following composition (in mM): NaCl 120,  $NaHCO_3$  25, glucose 11.1,  $CaCl_2$  1.6, KCl 4.7,  $KH_2PO_4$  1.2,  $MgSO_4$  1.2, and oxygenated with 95 %  $O_2$ : 5 %  $CO_2$ . The rings were first equilibrated for 1 h at 37 °C and then constricted with 120 mM KCl followed by addition of 10  $\mu$ M noradrenaline (NA). After reaching a steady-state, the rings were washed with KHS containing nominally 0 mM  $CaCl_2$ , (KHS 0  $Ca^{2+}$ ) and once the resting tension was restored, the protocol was repeated in KHS 0  $Ca^{2+}$  solution (Fig. 1). In fresh arteries, the same protocol was implemented after a 30 min preincubation with PKC (Ro 31-8220) or PKA (H-89) inhibitor (both BIAFFIN, Germany). H-89 was dissolved as a stock solution of 1 mM in 100 % dimethyl sulfoxide, the final concentration of both inhibitors used was 1  $\mu$ M (Budzyn *et al.* 2006). All other drugs were dissolved and diluted in deionized water. During the experiment, the generated force was recorded and wall tension was automatically calculated by the myograph system. Data are expressed as mean  $\pm$  S.E.M.,  $P < 0.05$  (one-way, two-tailed,

unpaired Student's t-test) was considered significant. All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Physiology AS CR, and conform to the *European Convention on Animal Protection and Guidelines on Research Animal Use*.



**Fig. 1.** Original records of force generated by femoral artery of control (Wistar, upper panel) and spontaneously hypertensive rats (SHR, lower panel) stimulated with 120 mM KCl (6 min) followed by addition of 10  $\mu$ M noradrenaline (NA) in Krebs-Henseleit solution (KHS) containing 1.6 mM  $Ca^{2+}$  and in KHS nominally free of  $Ca^{2+}$ . Horizontal bar represents 5 min.

## Results

### *Basic parameters of the isolated arteries*

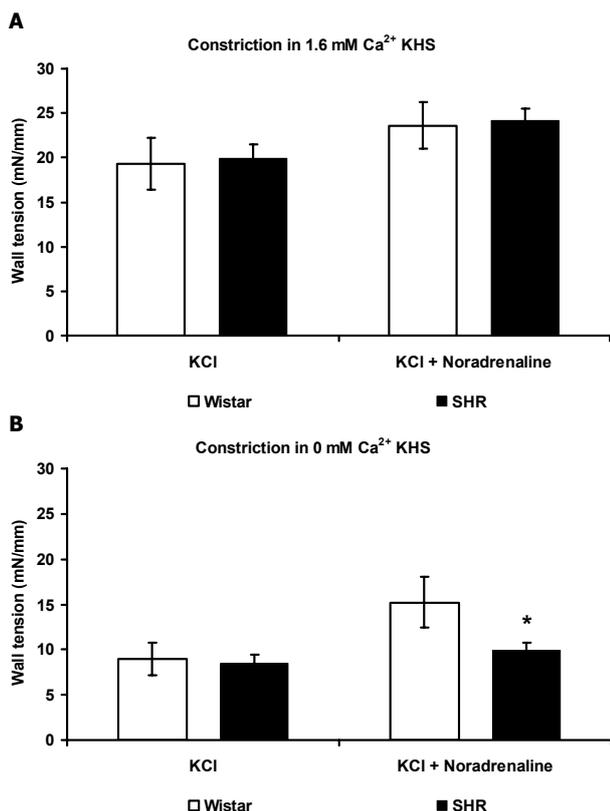
The normalized inner diameter under tension corresponding to pressure of 100 mm Hg was  $953.4 \pm 24.3$   $\mu$ m in Wistar controls vs.  $909.5 \pm 21.5$   $\mu$ m in SHR (non-significant). The segment length was not different between Wistar and SHR vessels ( $2.16 \pm 0.07$  mm in Wistar vs.  $2.19 \pm 0.06$  mm in SHR).

### *Constriction induced by KCl and KCl + noradrenaline*

The vasoconstriction elicited by KCl or KCl + NA in KHS 1.6  $Ca^{2+}$  or by KCl in KHS 0  $Ca^{2+}$  was not different between control rats and SHR. The vasoconstriction elicited by KCl + NA in KHS 0  $Ca^{2+}$  was lower in SHR compared to controls ( $P < 0.05$ ) (Fig. 2).

### *Effect of PKC inhibition on constriction induced by KCl or KCl + noradrenaline*

When PKC inhibitor was present in KHS



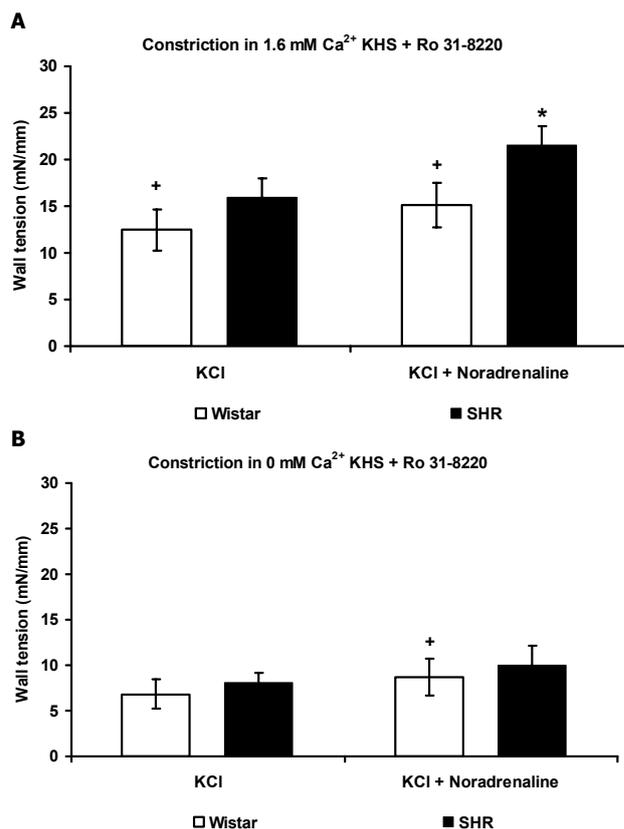
**Fig. 2.** Vasoconstriction obtained after stimulation of femoral artery of control (Wistar) and spontaneously hypertensive rats (SHR) stimulated with 120 mM KCl and KCl + 10  $\mu$ M noradrenaline (NA) in Krebs-Henseleit solution (KHS) containing 1.6 mM Ca<sup>2+</sup> (A) and in KHS nominally free of Ca<sup>2+</sup> (B). \* $P$ <0.05 vs. controls.

1.6 Ca<sup>2+</sup> medium, the constrictions of arteries isolated from control Wistar rats were decreased compared to constrictions seen in its absence ( $P$ <0.05). The incubation with PKC inhibitor did not affect constrictions of arteries from SHR and therefore the constriction elicited by KCl + NA in SHR became higher compared to Wistar controls ( $P$ <0.05) (Fig. 3A).

The presence of PKC inhibitor in KHS 0 Ca<sup>2+</sup> medium decreased the constrictions induced by KCl + NA in arteries isolated from Wistar rats compared to constrictions observed in its absence ( $P$ <0.05). The incubation with PKC inhibitor did not affect constrictions of arteries from SHR and therefore constriction elicited by KCl + NA in SHR was not different from respective controls (Fig. 3B).

#### *Effect of PKA inhibition on constriction induced by KCl or KCl + noradrenaline*

In the presence of PKA inhibitor in KHS 1.6 Ca<sup>2+</sup> medium the constrictions of arteries isolated from control Wistar rats were non-significantly higher



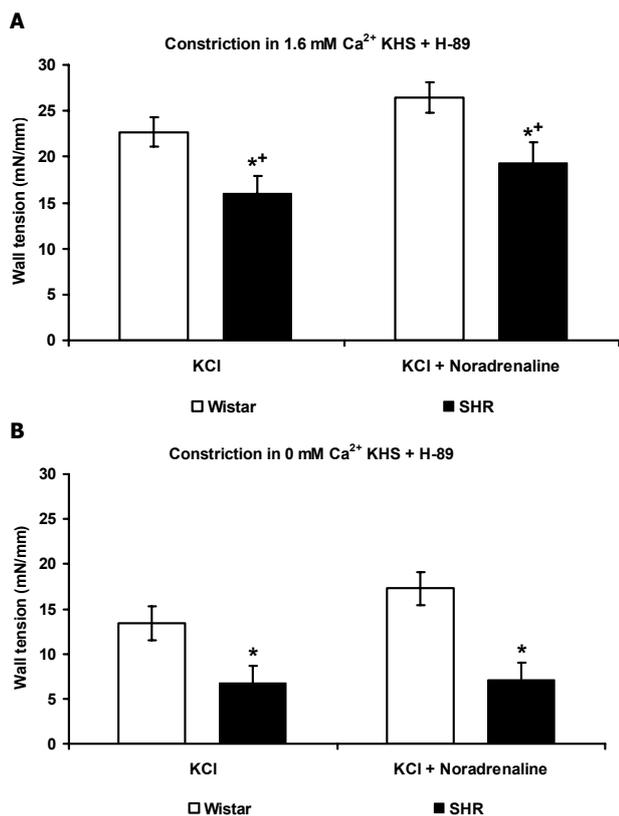
**Fig. 3.** Vasoconstriction in the presence of protein kinase C inhibitor Ro-31-8220 obtained after stimulation of femoral artery of control (Wistar) and spontaneously hypertensive rats (SHR) stimulated with 120 mM KCl and KCl + 10  $\mu$ M noradrenaline (NA) in Krebs-Henseleit solution (KHS) containing 1.6 mM Ca<sup>2+</sup> (A) and in KHS nominally free of Ca<sup>2+</sup> (B). \* $P$ <0.05 vs. controls, + $P$ <0.05 vs. respective group in the absence of the inhibitor.

compared to those recorded in its absence. The incubation with PKA inhibitor reduced constrictions of arteries from SHR ( $P$ <0.05) and therefore constriction in SHR became lower compared to controls ( $P$ <0.05) (Fig. 4A).

In the presence of PKA inhibitor in KHS 0 Ca<sup>2+</sup> medium the constrictions of arteries isolated from control rats were slightly but non-significantly higher compared to the conditions when PKA inhibitor was absent. The incubation with PKA inhibitor tended to reduce the constrictions of arteries from SHR, but the constriction in SHR was lower compared to Wistar controls ( $P$ <0.05) (Fig. 4A).

## Discussion

In our *in vitro* experiments on isolated femoral arteries we observed that the constriction elicited by KCl or KCl + noradrenaline (NA) in Krebs-Henseleit solution containing 1.6 mM Ca<sup>2+</sup> (KHS 1.6 Ca<sup>2+</sup>) or by KCl in the nominally calcium-free KHS (KHS 0 Ca<sup>2+</sup>) was not



**Fig. 4.** Vasoconstriction in the presence of protein kinase A inhibitor H-89 obtained after stimulation of femoral artery of control (Wistar) and spontaneously hypertensive rats (SHR) stimulated with 120 mM KCl and KCl + 10  $\mu$ M noradrenaline (NA) in Krebs-Henseleit solution (KHS) containing 1.6 mM Ca<sup>2+</sup> (**A**) and in KHS nominally free of Ca<sup>2+</sup> (**B**). \*  $P < 0.05$  vs. controls, +  $P < 0.05$  vs. respective group in the absence of the inhibitor.

different between control Wistar rats and SHR. However, the constriction elicited by KCl + NA in KHS 0 Ca<sup>2+</sup> was less pronounced in SHR. A larger inhibition of vasoconstriction of isolated mesenteric arteries after calcium chelation has already been reported by Kahonen *et al.* (1994) and is in agreement with our previous data reporting greater relaxation after nifedipine in SHR than in controls (Paulis *et al.* 2007). These results suggest that the activation of intracellular pathways and/or intracellular calcium stores is reduced in SHR. The relative contribution of extracellular calcium influx to vascular constriction is therefore increased in SHR, which have enhanced activity (Ohya *et al.* 1998) or higher number (Pratt *et al.* 2002) of voltage-dependent calcium channels (VDCC). Thus SHR seem to be more dependent on extracellular calcium influx and may require higher level of adrenergic stimulation.

The stimulation by noradrenaline involving the recruitment of G<sub>q</sub> and G<sub>s</sub> proteins leads to activation of protein kinase C (PKC) and protein kinase A (PKA), respectively (Nishizuka 1988, Somlyo *et al.* 1988). In

our experiments we aimed to investigate the significance of PKC and PKA in the recruitment of extracellular calcium influx and mobilization of intracellular calcium stores in SHR. Recent studies using inhibitors with improved selectivity for PKC have yielded conflicting results regarding its physiological importance in the vasculature (Chrissobolis *et al.* 2002, Shirao *et al.* 2002, McNair *et al.* 2004). In our experiment, the inhibition of PKC with Ro 31-8220 had no effect on constrictions in SHR, but diminished constrictions in control Wistar rats. Moreover, the constrictions in controls were attenuated in KHS 1.6 Ca<sup>2+</sup> as well as in KHS 0 Ca<sup>2+</sup> to similar extent. This fact indicates that in control rats PKC recruited either by membrane depolarization or by adrenergic stimulation enhances the smooth muscle tone independently on extracellular calcium influx either by modulation of sarcoplasmic reticulum calcium release (Bonev *et al.* 1997) or by direct action on myosine (Lamounier-Zepter *et al.* 2003). These pathways seem to be of less importance in SHR, despite the previously reported higher PKC expression in SHR (Kanashiro *et al.* 2001).

In contrast to the effect of PKC inhibition, the inhibition of PKA with H-89, a selective and potent inhibitor (Lochner *et al.* 2006), slightly enhanced constrictions in control rats in both KHS 1.6 Ca<sup>2+</sup> and KHS 0 Ca<sup>2+</sup> but reduced them in SHR in KHS 1.6 Ca<sup>2+</sup>. Apparently in control rats the PKA is involved in relaxation. The relaxing effect of PKA in Ca<sup>2+</sup>-free conditions is supposed to be mediated mainly by inhibition of myosin light chain kinase (Conti and Adelstein 1981). On the other hand, the reduced contraction after PKA inhibition in SHR, observed in our experiment, is more difficult to explain. The mild activation of PKA by lower cAMP concentrations was reported to increase VDCC current in smooth muscle cells and may lead to vasoconstriction (Taguchi *et al.* 1997, Ruiz-Velasco *et al.* 1998). In our experiments the effect of PKA on vasoconstriction in SHR was dependent on the presence of extracellular calcium supporting this hypothesis. Moreover, in rat aortic rings pre-incubated with the PKA inhibitor H-89 the albuterol-induced relaxation was attenuated (Ferro *et al.* 2004).

We conclude that vasoconstriction elicited by adrenergic stimulation is more dependent on extracellular calcium influx in SHR compared to controls. Moreover, the activation of PKA contributes to the vasoconstriction in SHR but not in Wistar. On the other hand, PKC activation seems to play a less

important role in vasoconstriction in SHR than in Wistar.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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