

## **Augmented agonist-induced Ca<sup>2+</sup>-sensitization of coronary artery contraction in genetically hypertensive rats. Evidence for altered signal transduction in the coronary smooth muscle cells.**

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*J Clin Invest.* 1994;**94**(4):1397-1403. <https://doi.org/10.1172/JCI117475>.

### Research Article

The Ca<sup>2+</sup> responsiveness of vascular smooth muscle myofilaments is not unique: it is increased during neuro-humoral activation and decreased during beta-adrenergic stimulation. In this study we tested whether an augmented Ca<sup>2+</sup> responsiveness of smooth muscle myofilaments may contribute to the increased coronary tone observed in hypertension using beta-escin-permeabilized coronary arteries from 3-mo-old stroke-prone spontaneously hypertensive rats (SHRSP) and their age matched normotensive reference strain (WKY rats). In intact coronary arteries, the response to 5-hydroxytryptamine (5-HT) but not to KCl was larger in SHRSP than in WKY rats. In beta-escin permeabilized coronary arteries in which the receptor effector coupling is still intact, 5-HT enhanced force at constant submaximal (Ca<sup>2+</sup>) (pCa 6.38) to a greater extent in SHRSP. The Ca<sup>2+</sup> sensitizing effect of 5-HT was mimicked by GTP gamma S (0.01-10 microM); again this effect was larger in SHRSP. In the absence of 5-HT or GTP gamma S the Ca<sup>2+</sup> force relation was similar in both groups. Forskolin induced relaxation at constant submaximal (Ca<sup>2+</sup>). This desensitizing effect was smaller in SHRSP than in WKY rats. In conclusion, this study shows that intracellular signalling pathways involved in modulating the Ca<sup>2+</sup> responsiveness of coronary smooth muscle myofilaments are altered in the genetically hypertensive animals favoring a hypercontractile state in the coronary circulation.

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# Augmented Agonist-induced $\text{Ca}^{2+}$ -Sensitization of Coronary Artery Contraction in Genetically Hypertensive Rats

## Evidence for Altered Signal Transduction in the Coronary Smooth Muscle Cells

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### Abstract

The  $\text{Ca}^{2+}$  responsiveness of vascular smooth muscle myofilaments is not unique: it is increased during neuro-humoral activation and decreased during  $\beta$ -adrenergic stimulation. In this study we tested whether an augmented  $\text{Ca}^{2+}$  responsiveness of smooth muscle myofilaments may contribute to the increased coronary tone observed in hypertension using  $\beta$ -escin-permeabilized coronary arteries from 3-mo-old stroke-prone spontaneously hypertensive rats (SHRSP) and their age matched normotensive reference strain (WKY rats). In intact coronary arteries, the response to 5-hydroxytryptamine (5-HT) but not to KCl was larger in SHRSP than in WKY rats. In  $\beta$ -escin permeabilized coronary arteries in which the receptor effector coupling is still intact, 5-HT enhanced force at constant submaximal ( $\text{Ca}^{2+}$ ) (pCa 6.38) to a greater extent in SHRSP. The  $\text{Ca}^{2+}$  sensitizing effect of 5-HT was mimicked by  $\text{GTP}\gamma\text{S}$  (0.01–10  $\mu\text{M}$ ); again this effect was larger in SHRSP. In the absence of 5-HT or  $\text{GTP}\gamma\text{S}$  the  $\text{Ca}^{2+}$  force relation was similar in both groups. Forskolin induced relaxation at constant submaximal ( $\text{Ca}^{2+}$ ). This desensitizing effect was smaller in SHRSP than in WKY rats. In conclusion, this study shows that intracellular signalling pathways involved in modulating the  $\text{Ca}^{2+}$  responsiveness of coronary smooth muscle myofilaments are altered in the genetically hypertensive animals favoring a hypercontractile state in the coronary circulation. (*J. Clin. Invest.* 1994. 94:1397–1403.) Key words: stroke-prone spontaneously hypertensive rat • permeabilized coronary arteries •  $\text{Ca}^{2+}$ -sensitivity • 5-hydroxytryptamine • forskolin

### Introduction

In the past, efforts to reduce the high coronary morbidity and mortality of patients with primary hypertension by antihyperten-

Portions of this work were published in abstract form in *Pflügers Arch. Eur. J. Physiol.* 420:R102, 1992.

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Received for publication 16 August 1994 and in revised form 2 June 1994.

*J. Clin. Invest.*

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0021-9738/94/10/1397/07 \$2.00

Volume 94, October 1994, 1397–1403

sive treatment have failed to reduce the coronary risk in this group of patients (1). This may reflect the fact, that the coronary disease process associated with primary hypertension is still poorly understood (2). Recent clinical (3, 4) and experimental reports using spontaneously hypertensive rats (5) have focused on the endothelial dysfunction as a possible cause of impaired coronary flow in hypertension. However, the vascular smooth muscle cells of the media may also contribute to a functionally altered regulation of coronary tone as suggested by a number of studies using isolated blood vessels obtained mainly from the mesenteric vascular bed and the aorta of genetically hypertensive rats (for reviews see references 6–8). It has been proposed that not only structural modifications of the blood vessels (6) but also alterations in the calcium homeostasis (7–9) of the smooth muscle cells may contribute to an increased responsiveness to a number of vasoconstrictor agents. The altered calcium homeostasis would ultimately lead to an increased availability of calcium for the myofilaments (7).

However, neurohumoral stimulation of vascular smooth muscle not only leads to an increase in the cytoplasmic  $\text{Ca}^{2+}$  concentration. It also increases the  $\text{Ca}^{2+}$  sensitivity of the myofilaments as has been demonstrated in a number of laboratories during the last decade (10–13). If the agonist induced  $\text{Ca}^{2+}$  sensitization of the myofilaments were augmented in hypertension this would then also contribute to an increased vasoconstrictor response. Such an augmented  $\text{Ca}^{2+}$  sensitization rather than alterations in the  $\text{Ca}^{2+}$  homeostasis of the smooth muscle cells may in fact underlie the higher sensitivity to extracellular  $\text{Ca}^{2+}$  of norepinephrine-induced contractions in intact mesenteric resistance arteries of spontaneously hypertensive rats (14). We therefore tested the hypothesis that the agonist induced  $\text{Ca}^{2+}$  sensitization of the smooth muscle myofilaments is augmented in coronary arteries from stroke-prone spontaneously hypertensive rats (SHRSP)<sup>1</sup> as compared with their normotensive reference strain, the Wistar Kyoto (WKY) rats (15). We chose 5-hydroxytryptamine (5-HT) as an agonist because in vivo it may be released from activated platelets (16) and it is capable of reducing coronary flow in perfused spontaneously hypertensive rat hearts (5).

Agonist-induced  $\text{Ca}^{2+}$  sensitization of smooth muscle myofilaments as well as the signalling cascade involved may be investigated in blood vessels permeabilized with  $\beta$ -escin (17). In these preparations, the receptor-effector coupling is functionally intact while the ionic composition, in particular ( $\text{Ca}^{2+}$ ),

1. Abbreviations used in this paper: 5-HT, 5-hydroxytryptamine; G protein, guanosine nucleotide binding protein; GTP, guanosine triphosphate;  $\text{GTP}\beta\text{S}$ , guanosine-5'-0-(3-thiodiphosphate);  $\text{GTP}\gamma\text{S}$ , guanosine-5'-0-(3-thiotriphosphate); PSS, physiological salt solution; SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar Kyoto.

may be clamped and small molecules such as nucleotides may be diffused into the smooth muscle cells. The agonist induced  $\text{Ca}^{2+}$  sensitization appears to be mediated by a G protein-coupled mechanism as it is mimicked by the nonhydrolyzable GTP analog,  $\text{GTP}\gamma\text{S}$ , which permanently activates G proteins (17–19). Downstream events may involve inhibition of myosin light chain phosphatase (20, 21) and activation of protein kinase C (PK-C, 22, 23).

In this study we show that in  $\beta$ -escin permeabilized coronary arteries from SHRSP and WKY rats, 5-HT increases the  $\text{Ca}^{2+}$  sensitivity of the myofilaments and this effect is more pronounced in the genetically hypertensive rats. The effect of 5-HT on  $\text{Ca}^{2+}$  sensitivity of force is mimicked by  $\text{GTP}\gamma\text{S}$ . Again, the effect of  $\text{GTP}\gamma\text{S}$  is larger in the coronary arteries from SHRSP. In contrast, in the absence of 5-HT or  $\text{GTP}\gamma\text{S}$ , the  $\text{Ca}^{2+}$  sensitivity of the myofilaments is not different between SHRSP and WKY rats (see reference 24). This suggests that the G protein effector coupling within the coronary artery smooth muscle cells involved in modulating  $\text{Ca}^{2+}$  sensitivity of force production may differ between genetically hypertensive and normotensive rats. This finding points to a novel mechanism contributing to the hyperresponsiveness of coronary arteries of SHRSP which is not related to altered  $\text{Ca}^{2+}$  homeostasis or endothelial dysfunction.

## Methods

**Animals.** 12–14-wk-old male rats were obtained from the colony at the Pharmacology Department of the University of Heidelberg (15). The body weight of the SHRSP rats was  $236 \pm 26$  g ( $n = 11$ ) and that of the WKY rats  $315 \pm 24$  g ( $n = 12$ ). Systolic blood pressure was measured before the experiments by tail plethysmography during light ether anesthesia. The values were (mm Hg):  $182 \pm 20$  in SHRSP and  $112 \pm 14$  in WKY rats ( $P < 0.05$ ).

**Force measurements.** Rats were anesthetized with ether and bled. First or second branches of the left coronary arteries (outer in situ diameter in  $\mu\text{m}$ ;  $135 \pm 19$  in SHRSP,  $144 \pm 21$  in WKY rats) were dissected in physiological salt solution (PSS) of the following composition (mM): NaCl 118, KCl 5,  $\text{Na}_2\text{HPO}_4$  1.1,  $\text{MgCl}_2$  1.2,  $\text{CaCl}_2$  1.6, Hepes 24 (pH 7.4 at 25°C) and glucose 10. PSS was gassed with 100%  $\text{O}_2$ . Ring segments were mounted on a myograph (AME 801 force transducer, Horten Norway) as described previously (25, 26). The mounting technique did not denude the vessels from the endothelium as judged by light microscopic examination of the vessels subjected to morphometry. The endothelium appeared to be functional in the intact vessels as precontracted vessels were relaxed by ADP. The ADP (10  $\mu\text{M}$ ) induced relaxation of vessels precontracted with 1  $\mu\text{M}$  5-HT amounted to  $82 \pm 11\%$  and  $46 \pm 15\%$  ( $n = 8$ ) in WKY and SHRSP rats, respectively. In  $\beta$ -escin-permeabilized preparations, the endothelium is not functional because of the treatment with detergent and the presence of dithioerythritol in the incubation solutions (27). The vessels were stretched to an optimum length at which the force induced by 125 mM KCl became maximum. On completion of the experiments in intact preparations, the vessels were permeabilized by treatment with  $\beta$ -escin according to the method of Kobayashi et al. (17). Briefly, the coronary arteries were incubated for 20 min in  $\text{Ca}^{2+}$ -free PSS followed by 10 min in relaxing solution. They were then incubated with 50  $\mu\text{g}/\text{ml}$   $\beta$ -escin for 30 min at 25°C in relaxing solution. The relaxing solution contained (in mM): imidazole 20, EGTA 4, ATP 7.5, potassium methanesulfonate 110, magnesium acetate 10,  $\text{NaN}_3$  1, creatine phosphate 10, creatine kinase 100 U/ml, at pH 7.0. The contracting solution contained in addition 4 mM  $\text{CaCl}_2$ . Alterations of the free  $\text{Ca}^{2+}$  concentration (calculated as in 26) were obtained by mixing contracting and relaxing solution in the appropriate ratio. All solutions contained in addition calmodulin (1  $\mu\text{M}$ ), leupeptin (1  $\mu\text{M}$ ), and dithioerythritol (DTE 2 mM). To deplete the sarcoplasmic reticulum of calcium and

maintain cytoplasmic ( $\text{Ca}^{2+}$ ) constant, every vessel was also treated with 10  $\mu\text{M}$  A23187 for 20 min in the relaxing solution.

**Morphometry.** The vessel dimensions were measured in (a) arteries that had been subjected to the mechanical experiments and (b) following retrograde perfusion fixation of the hearts in situ under constant pressure.

On completion of the mechanical experiments, the  $\beta$ -escin-permeabilized arteries were completely relaxed in relaxing solution (see above), fixed overnight at room temperature in phosphate-buffered (0.1 M, pH 7.4) glutaraldehyde (2.5%) and stored in the fixative at 4°C. The vessels were dehydrated with ethanol and propyleneoxide, and embedded in Epon. Cross-sections out of the middle of the segments (2  $\mu\text{m}$  thick) were mounted on glass slides and stained with Stevenel's Blue (28) and basic fuchsin. The slides were examined on a standard microscope (Polyvar 2, Leica, Germany). Morphological measurements were made on 7 segments out of 4 WKY rats and on 8 segments out of 4 SHRSP.

To measure the vessel dimensions in situ, four hearts of each group were perfused retrograde for 2 min with a modified Krebs-Henseleit solution containing (mM): NaCl 114.7, KCl 4.7,  $\text{MgSO}_4$  1.2,  $\text{KH}_2\text{PO}_4$  1.5,  $\text{NaHCO}_3$  25,  $\text{CaCl}_2$  2.5, glucose 11.1, and 10  $\mu\text{M}$  adenosine, gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , pH 7.4, at a constant pressure of 90 mm Hg at room temperature. This was followed by a 3–5 min perfusion at 90 mm Hg with 2.5% glutaraldehyde in 0.1 mM phosphate buffer, pH 7.4. A 3-mm-thick transverse slice was cut out of the medial third section of the heart (corresponding to the site where the vessels for the mechanical experiments were obtained) and was embedded with historesin<sup>TM</sup> (Cambridge Instruments, Cambridge, MA). The slice was cut into 2- $\mu\text{m}$ -thick cross sections, mounted on glass slides and stained with hematoxylin (hematoxylin solution Gill No. 3; Sigma Diagnostics, St. Louis, MO) and eosin. Morphological measurements were made on 2–4 vessels per heart.

Morphological analysis was performed at a magnification of 400 using a digitizing tablet interfaced to an IBM compatible personal computer running the program Sigma Scan (Jandel Scientific GmbH, Erkrath, Germany). The length of the internal elastic lamina and the cross-sectional area of the media were measured with the help of a drawing tube. The mean of three measurements of the limits of the vessels was used for further calculations. Using the method described by Furuyama (29) the thickness of the vessel wall (media thickness), the luminal diameter and the wall/lumen ratio were calculated for a standardized condition, in which the internal elastic membrane is smooth and circular. Because of oblique sections of coronary vessels in the cross section of the heart, the dimension of the in situ fixed vessel wall was determined as the ratio of surface area of the media to surface area of the vessel (= area of the lumen + area of the media) (30).

**Calculations and statistics.** To make comparisons between different vessel segments possible, forces (mN) were divided by the product of the longitudinal length ( $\mu\text{m}$ ) and the internal circumference ( $C$ , in  $\mu\text{m}$ ) to obtain mN per  $\text{mm}^2$  as described previously by Boels et al. (25). The internal circumference was calculated according to Hogestatt et al. (31):  $C = 2L + (\pi + 2)D$ , where  $D$  is the diameter of the wires and  $L$  the distance between them. All values were expressed as means  $\pm$  SEM, with  $n$  indicating the number of experiments. Difference of responsiveness among groups was tested by the one way analysis of variance (32), followed by paired or unpaired Student's  $t$  test as appropriate (one sided).  $P$  values  $< 0.05$  were considered to indicate significant differences.

**Drugs.** Forskolin and 5-hydroxytryptamine hydrochloride (5-HT) were from Sigma Chemical Co., St. Louis, MO). GTP, guanosine-5'-0-(3-thiotriphosphate) ( $\text{GTP}\gamma\text{S}$ ), and guanosine-5'-0-(2-thiodiphosphate) ( $\text{GDP}\beta\text{S}$ ) were obtained from Boehringer Mannheim, Germany. Forskolin was dissolved in methyl sulfoxide (DMSO); the final concentration of DMSO was less than 1%, which did not affect force.

## Results

**Morphological characteristics of the coronary arteries.** In  $\beta$ -escin-permeabilized coronary arteries, fixed completely re-

Table I. Light Microscopic Measurements of Coronary Arteries

	WKY	SHRSP
Permeabilized vessels		
Media diameter ( $\mu\text{m}$ )	14.2 $\pm$ 1.6	14.9 $\pm$ 1.0
Lumen diameter ( $\mu\text{m}$ )	134.4 $\pm$ 9	134.4 $\pm$ 9
Ratio media/lumen	0.1 $\pm$ 0.01	0.11 $\pm$ 0.01
Area of the media ( $\times 10^2 \mu\text{m}^2$ )	68.6 $\pm$ 10.4	70.8 $\pm$ 7.9
Area of the lumen ( $\times 10^2 \mu\text{m}^2$ )	68 $\pm$ 5	65 $\pm$ 6
Ratio area media/area vessel	0.48 $\pm$ 0.04	0.52 $\pm$ 0.02
In situ fixed intact vessels		
Lumen diameter ( $\mu\text{m}$ )	260 $\pm$ 50	232 $\pm$ 22
Area of the media ( $\times 10^2 \mu\text{m}^2$ )	270 $\pm$ 50	230 $\pm$ 30
Area of the lumen ( $\times 10^2 \mu\text{m}^2$ )	610 $\pm$ 130	470 $\pm$ 80
Ratio area media/area vessel	0.34 $\pm$ 0.03	0.36 $\pm$ 0.03

The permeabilized vessels were fixed in the myograph completely relaxed at the end of the mechanical experiment while the intact vessels were fixed in situ under retrograde constant pressure perfusion in the presence of adenosine. In permeabilized preparations 7 segments from 4 WKY rats and 8 segments from 4 SHRSP were investigated. In perfused hearts (four of each group) 2–4 vessels per animal were examined in a coronal section out of the medial third of the heart. The mean values of the vessel dimensions of the in situ fixed arteries are larger as vessels up to a diameter of 430  $\mu\text{m}$  were analyzed. Values are mean $\pm$ SEM, no statistically significant difference could be detected between SHRSP and WKY rats.

laxed in the myograph at the end of the experiment, no significant morphological difference between SHRSP and WKY rats could be detected (Table I). There was, however, some uncertainty in determining the media thickness in the permeabilized preparations as the cytoplasm was stained weakly. This made the identification of the boundary between media and adventitia somewhat ambiguous. For this reason we also analyzed coronary arteries fixed in situ under constant pressure perfusion and maximal vasodilation induced with adenosine (30). Again, there was no significant difference between SHRSP and WKY rats (Table I). The lumen diameters of the arteries measured in situ ranged from 80 to 430  $\mu\text{m}$ . The mean lumen diameters were higher than the mean lumen diameters of the permeabilized coronary arteries, because of large vessels in the coronal section of the heart. Again, there were no significant differences between SHRSP and WKY after subdividing the vessels into two size classes with lumen diameters  $> 250$  and  $\leq 250 \mu\text{m}$ .

**Mechanical experiments in intact coronary arteries.** Fig. 1 shows representative tension responses to stimulation with KCl (125 mM) and 5-HT ( $3 \mu\text{M}$ ). Tension response to KCl was biphasic with a rapid initial rise in force which was followed by a tonic component which was not significantly different between WKY and SHRSP rats (5.52 $\pm$ 0.68 mN/mm<sup>2</sup> in WKY rats and 6.12 $\pm$ 0.64 mN/mm<sup>2</sup> in SHRSP,  $n = 8$  each). 5-HT elicited monophasic tonic contractions in the presence of extracellular  $\text{Ca}^{2+}$  (Fig. 1). In the absence of extracellular  $\text{Ca}^{2+}$ , only small and transient contractions were induced by 5-HT (Fig. 1). Peak force elicited by  $3 \mu\text{M}$  5-HT was 5 $\pm$ 2% in WKY rats and 11 $\pm$ 4% in SHRSP ( $n = 8$ ) of that in the presence of  $\text{Ca}^{2+}$ ; the difference was not statistically significant.

Fig. 2 shows the dose-response relationship for 5-HT in the presence of extracellular  $\text{Ca}^{2+}$ . Coronary arteries from SHRSP rats showed a significantly higher efficacy of the response to 5-HT compared with those from WKY rats. This difference

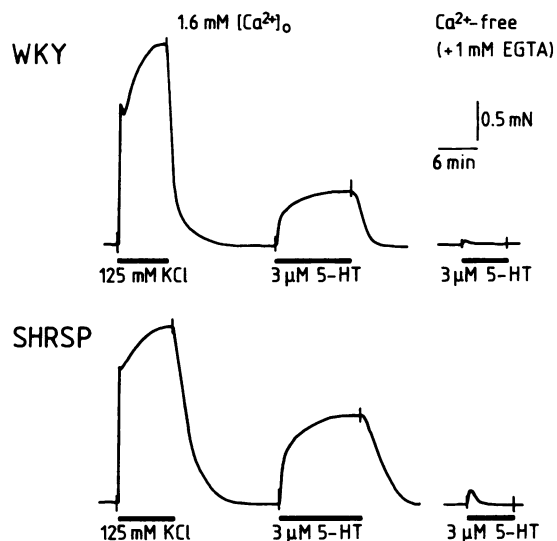


Figure 1. Tension responses in intact coronary arteries of SHRSP and WKY rats. Representative tension responses in intact coronary arteries to stimulation with KCl and 5-HT in the presence of extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o = 1.6 \text{ mM}$ , left two tracings), and in  $\text{Ca}^{2+}$ -free PSS with 1 mM EGTA (on the right side). Tension elicited by KCl was 5.52 $\pm$ 0.68 mN/mm<sup>2</sup> in WKY and 6.12 $\pm$ 0.64 mN/mm<sup>2</sup> in SHRSP (mean $\pm$ SEM,  $n = 8$ , not significantly different).

remained when force was expressed relative to the tension elicited by KCl (Fig. 2 B). However, the sensitivity of the response to 5-HT was not increased; the  $\text{EC}_{50}$  values in SHRSP and WKY rats were similar ( $\text{EC}_{50} = 0.16 \mu\text{M}$  in SHRSP and 0.18  $\mu\text{M}$  in WKY rats).

**Mechanical experiments in permeabilized coronary arteries.** In  $\beta$ -escin-permeabilized preparations, maximal force elic-

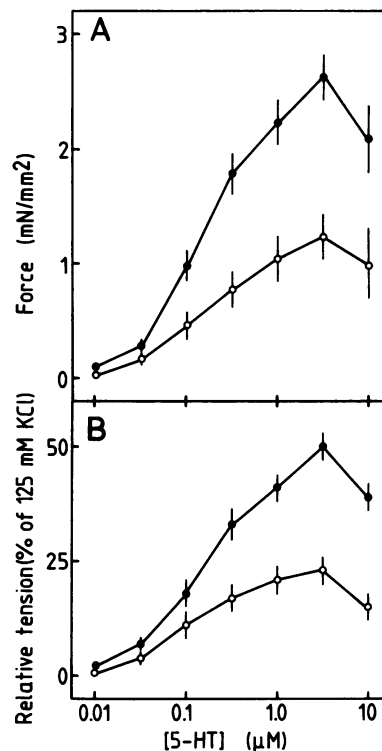
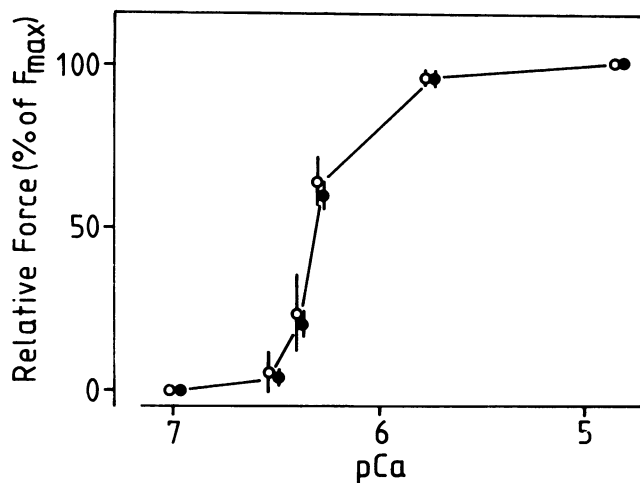


Figure 2. Dose-response curves to 5-HT in intact coronary arteries. The response to 5-HT is significantly larger in SHRSP ( $\bullet$ ) than in WKY rats ( $\circ$ ), both when expressed as absolute force (A) and when expressed as percent of the force elicited by KCl (B). The dose-response curves were obtained noncumulatively, i.e., the vessels were relaxed between each challenge of 5-HT. Each point is expressed as the mean $\pm$ SEM for eight observations.



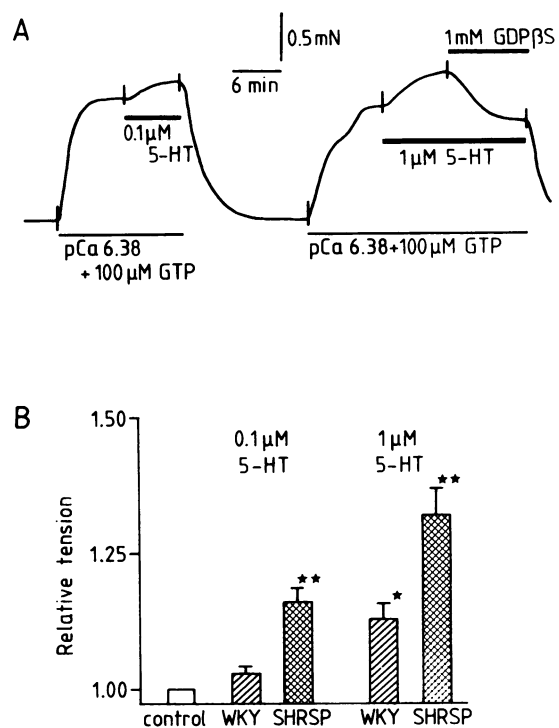
**Figure 3.** Force-pCa relations in  $\beta$ -escin-skinned coronary arteries from SHRSP ( $\bullet$ ) and WKY rats ( $\circ$ ). The vessels were contracted by cumulatively increasing concentrations of  $\text{Ca}^{2+}$  in the presence of  $1 \mu\text{M}$  calmodulin. Values were normalized to force at pCa 4.8. Each point is expressed as the mean  $\pm$  SEM for four observations.

ited by pCa 4.8 was not different between WKY and SHRSP and amounted respectively to  $125 \pm 10\%$  and  $134 \pm 12\%$  of the force developed in response to KCl in the intact preparation. There was also no difference in the  $\text{Ca}^{2+}$  sensitivity of tension development; pCa<sub>50</sub> was 6.31 in both groups (Fig. 3).

In a second series of experiments we tested whether 5-HT and GTP $\gamma$ S increased  $\text{Ca}^{2+}$  responsiveness and whether this effect was larger in SHRSP. A submaximal contraction was elicited by pCa 6.38 (in the presence of  $100 \mu\text{M}$  GTP, cf. Fig. 4 A) which amounted to  $30 \pm 7\%$  (WKY) and  $28 \pm 3\%$  (SHRSP) of the maximal response elicited by pCa 4.8 ( $n = 6$ ). 5-HT (in the continued presence of GTP) enhanced submaximal force concentration dependently at constant ( $\text{Ca}^{2+}$ ) (Fig. 4). The  $\text{Ca}^{2+}$ -sensitizing effect of 5-HT was reversible and was inhibited by GDP $\beta$ S (Fig. 4 A). The degree of force enhancement was significantly larger in SHRSP than in WKY rats and was shifted to lower concentrations (Fig. 4 B).

We then tested whether the  $\text{Ca}^{2+}$  sensitizing effect of 5-HT was mimicked by GTP $\gamma$ S. In these experiments, the blood vessels were activated with pCa 6.38 in the absence of GTP followed by incubation with cumulatively increasing concentrations of GTP $\gamma$ S. The  $\text{Ca}^{2+}$ -activated force in WKY rats and SHRSP was respectively  $28 \pm 5\%$  and  $25 \pm 5\%$  of maximal force. GTP $\gamma$ S modified neither resting force nor maximal force but enhanced submaximal  $\text{Ca}^{2+}$  activated force in both groups concentration dependently whereby the effect was larger in SHRSP (Fig. 5). The threshold concentration of GTP $\gamma$ S was also lower in SHRSP. Interestingly, in both groups the dose response relation was biphasic, i.e.,  $10 \mu\text{M}$  GTP $\gamma$ S was less effective than  $1 \mu\text{M}$  GTP $\gamma$ S (Fig. 5).

GTP $\gamma$ S nonspecifically activates all heterotrimeric G proteins, i.e., also G<sub>sa</sub> the stimulation of which leads to accumulation of cAMP through activation of adenylyl cyclase. Activation of the cAMP pathway in intact smooth muscle (33) and incubation of skinned coronary arteries with the catalytic subunit of the cAMP dependent protein kinase (34) leads to desensitization of the myofilaments to  $\text{Ca}^{2+}$ . We therefore tested whether direct activation of the adenylyl cyclase by forskolin affects force in  $\beta$ -escin-permeabilized coronary arteries. The blood vessels were



**Figure 4.** 5-HT increases force in  $\beta$ -escin-permeabilized coronary arteries at constant submaximal ( $\text{Ca}^{2+}$ ). (A) Original force tracing of a vessel from SHRSP. Following activation with pCa 6.38 in the presence of GTP, 5-HT was added which increased force concentration dependently. The effect was reversed by GDP $\beta$ S. B: Increase in  $\text{Ca}^{2+}$  responsiveness expressed relative to the force elicited by pCa 6.38 with GTP (control) in the presence of  $0.1 \mu\text{M}$  5-HT (left columns) and  $1 \mu\text{M}$  5-HT (right columns). Values are means  $\pm$  SEM for six observations; \*\*significant increase in  $\text{Ca}^{2+}$  responsiveness as well as significant difference between SHRSP and WKY rats ( $P < 0.05$ ). \*In WKY rats, there is only a significant increase in  $\text{Ca}^{2+}$  responsiveness in the presence of  $1 \mu\text{M}$  5-HT ( $P < 0.05$ ).

again submaximally activated (pCa 6.38). Once force had reached a plateau, forskolin ( $10 \mu\text{M}$ ) was added which induced a partial relaxation at constant  $\text{Ca}^{2+}$  (Fig. 6). In WKY rats the relaxing effect of forskolin on force was larger than in the SHRSP (Fig. 6).

## Discussion

The major finding of this study is, that 5-HT and GTP $\gamma$ S enhanced the  $\text{Ca}^{2+}$  responsiveness of force in  $\beta$ -escin-permeabilized coronary arteries from SHRSP to a greater extent than in their normotensive reference strain, the WKY rats. This augmented  $\text{Ca}^{2+}$  sensitization may underlie the increased responsiveness to stimulation with 5-HT observed in intact coronary arteries from the hypertensive animals. We also show that forskolin is less effective in decreasing force at constant ( $\text{Ca}^{2+}$ ) in  $\beta$ -escin-permeabilized coronary arteries from SHRSP. These findings indicate that pathways leading to an upregulation of  $\text{Ca}^{2+}$  responsiveness of the myofilaments are augmented while those leading to a downregulation are diminished in genetically hypertensive rats which would eventually favor a hypercontractile state in the coronary circulation.

*Responses in intact preparations.* Coronary arteries from SHRSP exhibited an increased responsiveness to stimulation

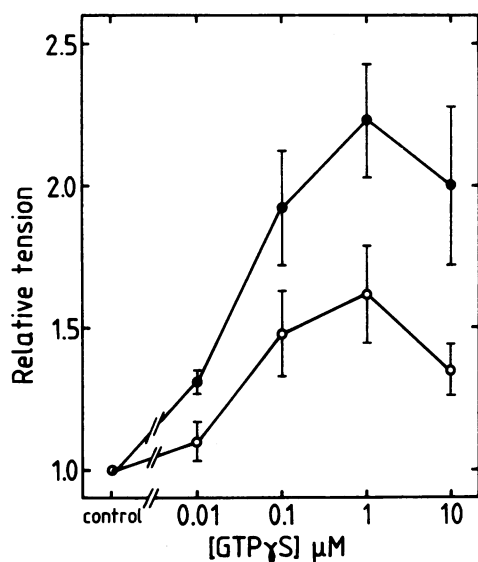


Figure 5. GTP $\gamma$ S increases Ca<sup>2+</sup> responsiveness in  $\beta$ -escin-permeabilized coronary arteries. After activation with pCa 6.38, GTP $\gamma$ S in the indicated concentrations was added cumulatively. Force is expressed relative to the tension obtained at pCa 6.38 (control); values are mean $\pm$ SEM for six observations, (●) SHRSP, (○) WKY rats.

with 5-HT but not to stimulation with KCl suggesting that the difference in the response to 5-HT was not due to a structural modification of the blood vessels. This was supported by morphometric analysis of the vessels subjected to the mechanical experiments and by an independent study of hearts fixed under retrograde constant pressure perfusion. It appears that coronary arteries from 12–14-wk-old SHRSP rats with a vessel size between 100 and 430  $\mu$ m have not yet developed morphologic changes. This is in accordance with findings in the superior mesenteric artery where the dimensions of the blood vessel were also not different in 10–12-wk-old SHR and WKY rats (35) whereas in peripheral mesenteric arteries structural alterations were already present in this age group (35, 36).

5-HT has a dual action on arterial tone: in the presence of an intact endothelium, it dilates precontracted blood vessels (16, 37); in the absence of an intact endothelium, it acts as a vasoconstrictor (16). Therefore it could be argued that the increased vasoconstrictor response of the intact coronary arteries of the SHRSP rats to stimulation with 5-HT might be due to a blunted endothelium-dependent vasodilatation. However, in contrast to other species, 5-HT does not release EDRF from endothelial cells of rat coronary arteries (38). Thus, the 5-HT responses in this study appears to reflect the responses of the medial smooth muscle cells themselves.

The difference in response to 5-HT and KCl suggests that the relative contribution of pharmacomechanical and electromechanical coupling to coronary artery tone differs between SHRSP and WKY rats (see Fig. 2 B). Pharmacomechanical coupling is associated with three events (39): the influx of Ca<sup>2+</sup> through specific channels in the plasma membrane, release of intracellularly stored Ca<sup>2+</sup> mediated by IP<sub>3</sub> and an increase in the Ca<sup>2+</sup> responsiveness of the myofilaments. Alterations related to Ca<sup>2+</sup> influx and sarcoplasmic reticulum function have been reported in hypertensive rats (7, 8). In addition, in SHRSP rats, alterations in the signalling pathways involved in modulating Ca<sup>2+</sup> sensitivity may contribute to the enhanced responsiveness to 5-HT as discussed below.

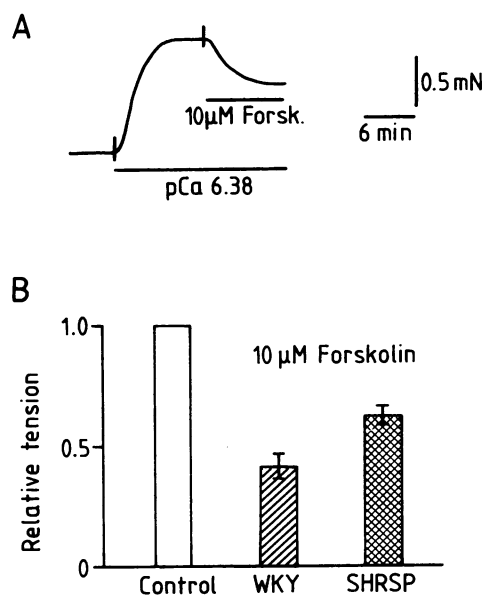


Figure 6. Forskolin relaxes submaximally activated coronary arteries at constant Ca<sup>2+</sup> (pCa 6.38). (A) Original tracing of a coronary artery from SHRSP. (B) 10  $\mu$ M forskolin has a significantly smaller effect ( $P < 0.05$ ) in SHRSP. Force with forskolin is expressed relative to force elicited by pCa 6.38 (control), values are mean $\pm$ SEM; six observations.

*Responses in  $\beta$ -escin permeabilized preparations.* As has been described previously in other types of vascular and non-vascular smooth muscles (17), the receptor-effector coupling in  $\beta$ -escin permeabilized rat coronary arteries is intact as indicated by the fact that they responded to stimulation with 5-HT with a reversible increase in force at constant submaximal (Ca<sup>2+</sup>). The 5-HT induced increase in Ca<sup>2+</sup> responsiveness was probably mediated by a G protein because it was blocked by GDP $\beta$ S which acts as a competitive inhibitor of GTP (40) and was mimicked by GTP $\gamma$ S. Both the response to 5-HT and to GTP $\gamma$ S was larger in the hypertensive rats. This suggests that the enhanced response of the intact tissue may, at least in part, be due to the augmented Ca<sup>2+</sup> sensitization of the smooth muscle myofilaments.

The enhanced Ca<sup>2+</sup> responsiveness observed in the hypertensive animals is probably not due to alterations of the receptors or the receptor G protein coupling as it is maintained when the G proteins were directly stimulated with GTP $\gamma$ S. Rather it may be due to a different G protein effector coupling whereby the alteration may be localized at the level of the G protein or downstream. However, it appears to be located upstream of the contractile proteins and their immediate regulatory proteins, myosin light chain kinase, and phosphatase, for the following reasons. (a) GTP $\gamma$ S had no direct effect on the contractile apparatus as the Ca<sup>2+</sup> sensitizing effect was absent in triton skinned microarteries (41) or after extensive permeabilization with saponin (20), and (b) the force-calcium relationship in the absence of an agonist or GTP $\gamma$ S was not different between SHRSP and WKY rats (Fig. 3). This finding is consistent with the previous reports showing that neither the pCa-force relationship nor Ca<sup>2+</sup>-induced myosin phosphorylation were altered in triton-skinned tail arteries from SHRSP (24). It is also consistent with the finding that the pCa-force relationship is not altered in saponin-skinned spastic coronary arteries of miniature pigs, which show hyperresponsiveness to histamine (42) or in

triton skinned aortas from rats with aldosterone-salt hypertension (43).

A complete picture of the signalling cascade mediating agonist induced  $\text{Ca}^{2+}$  sensitization has yet to emerge. It has been suggested that the G protein involved in  $\text{Ca}^{2+}$  sensitization of the myofilaments may not be identical to the one which leads to the  $\text{IP}_3$  mediated  $\text{Ca}^{2+}$  release (44). In fact, the agonist and  $\text{GTP}\gamma\text{S}$  induced increase in  $\text{Ca}^{2+}$  responsiveness may be mimicked by rhoA p21 (45) and H-ras p21 (46) which belong to the superfamily of monomeric, low molecular weight G proteins. It will therefore be interesting to see whether these monomeric G proteins or the proteins involved in regulating their activity are affected in hypertension. Ras p21 may increase  $\text{Ca}^{2+}$  sensitivity by at least two pathways. One may be an increased production of arachidonic acid (47). Arachidonic acid increases  $\text{Ca}^{2+}$  sensitivity by inhibiting myosin light chain phosphatase (48). It should be noted that the basal activity of myosin light chain phosphatase itself is presumably not decreased in SHRSP as suggested by the observation that the  $\text{Ca}^{2+}$  sensitivity of the myofilaments per se is not increased in SHRSP. The second pathway leading to  $\text{Ca}^{2+}$  sensitization may be the ras p21 induced activation of mitogen activated kinase (MAP kinase, 49) which in turn phosphorylates caldesmon in vitro (50) and presumably in vivo (51). In vitro phosphorylation of caldesmon by MAP kinase results in weakening of the binding of caldesmon to actin (50). Unphosphorylated caldesmon inhibits smooth muscle contraction (52, 53), and therefore phosphorylation of caldesmon may be a mechanism to increase  $\text{Ca}^{2+}$  sensitivity independent of an increase in myosin light chain phosphorylation. In this mechanism, a PK-C-dependent pathway may play an important role as it appears to make a large contribution to the activation of MAP kinase by ras p21 (49). As PK-C appears to be involved in the agonist induced increase in  $\text{Ca}^{2+}$  responsiveness (22, 23) it is interesting to note that blood vessels from spontaneously hypertensive rats exhibit a greater sensitivity to stimulation with phorbol esters (54, 55). Therefore, the augmented agonist induced  $\text{Ca}^{2+}$  responsiveness in SHRSP may be due to alterations in the PK-C pathway.

The dose response curve to  $\text{GTP}\gamma\text{S}$  is biphasic and declines at high concentrations of  $\text{GTP}\gamma\text{S}$ .  $\text{GTP}\gamma\text{S}$  nonspecifically activates all G proteins in the permeabilized arteries, i.e., the one(s) involved in calcium sensitization and  $\text{G}_{\text{sa}}$  the activation of which leads to an increase in the cyclic AMP levels which decreases the  $\text{Ca}^{2+}$  sensitivity of coronary arteries (34). Thus, the force response to  $\text{GTP}\gamma\text{S}$  is the sum of the activation of sensitizing and desensitizing pathways. It appears that at low  $\text{GTP}\gamma\text{S}$  concentrations the sensitizing pathways prevail while at high concentrations the desensitizing pathways become more important. It was therefore of interest to see, whether the desensitizing pathways are also affected in the hypertensive animals.

Forskolin increases cAMP levels by directly activating the adenylyl cyclase and acts therefore downstream of  $\text{G}_{\text{sa}}$ . Forskolin relaxed  $\beta$ -escin-permeabilized coronary arteries at constant submaximal ( $\text{Ca}^{2+}$ ). This effect was attenuated in the genetically hypertensive rats. Therefore, the attenuated desensitization to  $\text{Ca}^{2+}$  may in part explain the impaired  $\beta$ -adrenergic and forskolin-mediated relaxation of intact blood vessels from hypertensive animals (56). It is at present not clear whether the diminished response to forskolin is due to a decreased level of cAMP (57, 58). In some cases the impaired forskolin mediated relaxation appears not to involve activation mechanisms of cAMP dependent protein kinase (56) and may therefore involve events downstream of cAMP dependent protein kinase.

It will therefore be interesting to see whether the desensitizing action of the catalytic subunit of cAMP dependent protein kinase (34) is also impaired in SHRSP.

In conclusion we have shown that signalling pathways leading to the increase in  $\text{Ca}^{2+}$  sensitivity of smooth muscle myofilaments are augmented while those leading to a decrease are diminished in coronary arteries from SHRSP. It will be interesting to see whether these alterations also occur in vascular beds involved in blood pressure regulation such as mesenteric arteries. Due to the strategies required to obtain hypertensive and normotensive control strains, a multitude of genetic differences, many more than the few which confer the blood pressure phenotype, must be expected to be present (15). Future studies, therefore, have to show whether the alterations in the  $\text{Ca}^{2+}$  sensitivity modulation are related to the pathogenesis of hypertension in this animal model and/or whether they provide a link to the coronary dysfunction of the coronary circulation seen in experimental hypertension (e.g., 5). This novel mechanism may then act synergistically to those related to the altered  $\text{Ca}^{2+}$  homeostasis of the smooth muscle cells (7, 8) and to the endothelial dysfunction (5) in increasing coronary tone (5).

## Acknowledgments

The authors thank Professor J. C. Rüegg, University of Heidelberg, for critically reading the manuscript.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to G. Pfitzer (Pf 226/1-2, 226/4-1). S. Satoh was a recipient of a fellowship from the Alexander von Humboldt-Foundation.

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