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RESEARCH PAPER

Impairment of endothelial SK_{Ca} channels and of downstream hyperpolarizing pathways in mesenteric arteries from spontaneously hypertensive rats

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Background and purpose: Previous studies have shown that endothelium-dependent hyperpolarization of myocytes is reduced in resistance arteries from spontaneously hypertensive rats (SHRs). The aim of the present study was to determine whether this reflects down-regulation of endothelial K^+ channels or their associated pathways.
Experimental approach: Changes in vascular K^+ channel responses and expression were determined by a combination of membrane potential recordings and Western blotting.
Key results: Endothelium-dependent myocyte hyperpolarizations induced by acetylcholine, 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) (opens small- and intermediate-conductance calcium-sensitive K^+ channels, SK_{Ca} and IK_{Ca}, respectively) or cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (SK_{Ca} opener) were reduced in mesenteric arteries from SHRs. After blocking SK_{Ca} channels with apamin, hyperpolarizations to acetylcholine and NS309 in SHR arteries were similar to those of controls. Hyperpolarization to 5 mM KCl was reduced in SHR arteries due to loss of the Ba^{2+}-sensitive, inward-rectifier channel (K_{IR}) component; the contribution of ouabain-sensitive, Na^+/K^+-ATPases was unaffected. Protein expression of both SK_{Ca} and K_{IR} channels was reduced in SHR arteries; the caveolin-1 monomer/dimer ratio was increased.
Conclusions and implications: In SHRs, the distinct pathway that generates endothelium-dependent hyperpolarization in vascular myocyte by activation of IK_{Ca} channels and Na^+/K^+-ATPases remains intact. The second pathway, initiated by endothelial SK_{Ca} channel activation and amplified by K_{IR} opening on both endothelial cells and myocytes is compromised in SHRs due to down-regulation of both SK_{Ca} and K_{IR} and to changes in caveolin-1 oligomers. These impairments in the SK_{Ca}–K_{IR} pathway shed new light on vascular control mechanisms and on the underlying vascular changes in hypertension.
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Keywords: hypertension; EDHF; acetylcholine; apamin; TRAM-34; CyPPA; NS309; KCa3.1; KCa2.3; caveolin-1

Abbreviations: CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, native intermediate-conductance, calcium-sensitive K^+ channel; K_{IR}, inward-rectifier K^+ channel; L-NA, N^G-nitro-L-arginine; NS309, 6,7-dichloro-1H-indole-2,3-dione 3-oxime; SHR, spontaneously hypertensive rat; SK_{Ca}, native small-conductance, calcium-sensitive K^+ channel; TRAM-34, 1-[(2-chlorophenyl)-diphenyl-methyl]-1H-pyrazole; WKY, Wistar-Kyoto rat

Introduction

The endothelium plays an important role in the control of vascular tone and thus endothelial dysfunction can be a contributory factor in hypertension. Endothelium-derived nitric oxide and prostacyclin are major contributors to vasorelaxation but, in addition, myocyte hyperpolarization initiated by the opening of endothelial cell K^+ channels also induces vasodilatation (Félétou and Vanhoutte, 2007). This hyperpolarization was initially thought to be due to the release of an endothelium-derived hyperpolarizing factor (EDHF) and, for want of a better name, the term 'EDHF' is still employed to describe this phenomenon. Nevertheless, it is now universally accepted that, in most arteries, the *myocyte* hyperpolarization results *indirectly* from the opening of endothelial cell small- and intermediate-conductance, calcium-sensitive K^+ channels

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(SK_{Ca} and IK_{Ca}, respectively; ion channel nomenclature follows Alexander *et al.*, 2009). In some small arteries (such as the rat hepatic artery), the K^+ that leaves the endothelium via these channels induces activation of myocyte Na^+/K^+-ATPase (types 2 or 3) and partially relieves the rectifying block of inward-rectifier K^+ channels (K_{IR}), both of which actions result in smooth muscle hyperpolarization (Edwards *et al.*, 1998). In other vessels (such as guinea-pig internal carotid arteries), the hyperpolarization of the endothelium (due to the opening of the SK_{Ca} and IK_{Ca} channels) is transferred electrotonically to the myocytes via gap junctions (Edwards *et al.*, 1999a). In the rat mesenteric artery, which is commonly used to study the EDHF response, both of these pathways appear to play important roles (Edwards *et al.*, 1999a). In addition, it is now recognized that the sub-cellular distribution of the various components of the EDHF pathway is likely to be of relevance. Thus, the localization of SK_{Ca} channels to caveolae may be an important requisite for maximal activity (Absi *et al.*, 2007) and the clustering of IK_{Ca} channels together with Na^+/K^+-ATPases in endothelial cell projections may facilitate myo-endothelial cell coupling (Sandow *et al.*, 2006; Dora *et al.*, 2008; Harno *et al.*, 2008).

Several groups have reported that the EDHF response is impaired in various rat models of hypertension (see Fujii *et al.*, 1992; Sunano *et al.*, 1999; Alvarez de Sotomayor *et al.*, 2007; Hilgers and Webb, 2007; Dal-ros *et al.*, 2009) although the underlying cause has not been fully established. In view of our current understanding of the EDHF response, and the differential distribution of the two endothelial K^+ channels (Sandow *et al.*, 2006; Absi *et al.*, 2007; Dora *et al.*, 2008), the aim of the present study was to elucidate further the mechanisms underlying the reduced endothelium-dependent hyperpolarization in hypertensive rats. The study found no modification to endothelial cell IK_{Ca} channel signalling pathways. However, there was strong electrophysiological evidence of reduced SK_{Ca} and K_{IR} channel activity in the mesenteric arteries of spontaneously hypertensive rats (SHR). This was consistent with observed reductions in the corresponding α-subunit proteins, $K_{Ca}2.3$ and $K_{IR}2.1$. These changes, together with alterations in oligomeric forms of caveolin-1, show that an important endothelium-dependent vasodilator pathway is compromised in the mesenteric vessels of SHR. These are likely to lead to an increase in vascular tone *in vivo* and be underlying features responsible for the elevated blood pressure that is characteristic of the SHR strain.

Methods

Animals

All animal care and experimental procedures complied with the UK Animals (Scientific Procedures) Act, 1986. All animals were housed under a 12 h light–dark cycle with food and water available *ad libitum*. Systolic blood pressure was determined using tail-cuff plethysmography. Experiments were performed on second- and third-order mesenteric artery branches (approximately 150–250 mm diameter) dissected from 12- to 16-week-old male, SHR (mean body weight 298 ± 9 g, $n = 20$) and from strain- and age-matched normotensive Wistar-Kyoto (WKY) rats (mean body weight 295 ± 10 g, $n = 19$) killed by stunning and cervical dislocation.

Electrophysiology

Small segments of artery (second or third order; length 2–3 mm) were pinned to the Sylgard base of a thermostatically controlled bath and superfused (3 mL·min^{-1}) with a Krebs solution containing 10 μM indomethacin and 300 μM N^G-nitro-L-arginine (L-NA) and which was bubbled with 95% O_2/5% CO_2 (pH 7.5; 37°C). The composition of the Krebs solution was (mM): NaCl, 118; KCl, 3.4; $CaCl_2$, 1.0; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11. For membrane potential recordings, myocytes were impaled via the adventitial surface using microelectrodes filled with 3 M KCl (resistance 40–80 MΩ) as described previously (Edwards *et al.*, 1999b). Acetylcholine (ACh), cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA) and levcromakalim were each added as bolus injections directly into the bath in quantities calculated to obtain (transiently) the final concentrations indicated. Apamin, barium, ouabain and 1-[(2-chlorophenyl)-diphenyl-methyl]-1H-pyrazole (TRAM-34) were each added to the reservoir of Krebs solution superfusing the bath.

Western blotting

Western blotting was performed (as described previously; Gardener *et al.*, 2004) to determine any changes in the protein expression of the SK_{Ca}, IK_{Ca} and K_{IR} α-subunits ($K_{Ca}2.3$, $K_{Ca}3.1$ and $K_{IR}2.1$ respectively) and of caveolin-1. Briefly, endothelium-intact segments of rat mesenteric artery were homogenized in freshly prepared extraction buffer comprising 20 mM Tris, 250 mM sucrose, 5 mM EDTA, 10 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, 0.1% Triton X-100 and protease inhibitor cocktail (one vial per 100 mL of extraction buffer). Protein concentrations were determined using a Bradford reagent (Protein Assay, Bio-Rad, Hertfordshire, UK) and, for each lane, equal protein loading and transfer were visually assessed after staining for 5 min with 0.1% Ponceau S solution (Sigma-Aldrich, Dorset, UK). Consistency of protein loading was further confirmed by Western blot using β-actin as a loading control. Samples were mixed with Laemmli buffer (containing the detergent SDS; Laemmli, 1970), heated to 60°C ($K_{Ca}2.3$) or to 95°C for 5 min then separated on 8% ($K_{Ca}2.3$), 10% ($K_{IR}2.1$) or 12% (caveolin-1) polyacrylamide gels (as indicated) and transferred to nitrocellulose membranes.

The membranes were blocked with 2% bovine serum albumin (1 h at room temperature or overnight at 4°C) then incubated for 1 h at room temperature/overnight at 4°C with 1:500 rabbit anti-$K_{Ca}2.3$ antibody (anti-KCNN3, Abcam, Cambridge, UK), 1:200 rabbit anti-$K_{IR}2.1$ (Alomone, Jerusalem, Israel) or 1:5000 mouse anti-caveolin-1 antibody (BD Transduction Laboratories, Oxford, UK). Detection was achieved using horseradish peroxidase-conjugated goat anti-rabbit (1:5000, Promega, Madison, WI, USA) or goat anti-mouse secondary antibody (1:20 000; Jackson ImmunoResearch, Cambridge, UK) and ECL+ reagents (GE Healthcare, Bucks., UK). Following stripping with Western-Re-Probe Reagent (1:5,

Calbiochem, San Diego, CA, USA), membranes were re-probed by incubating for 1 h at room temperature/overnight at 4°C with 1:2000 anti-β-actin (mouse monoclonal; AC15; Abcam, Cambridge, UK) and immunoreactivity detected as above.

Statistics

Results were analysed by two-way ANOVA (with Bonferroni's *post hoc* test) or Student's *t*-test as appropriate and are expressed as means ± SEM. A *P*-value of 0.05 was considered significant.

Materials

CyPPA was generously provided by Dr H Draheim, Boehringer Ingelheim, Biberach, Germany. NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) was donated by Dr P Christophersen (Neurosearch A/S, Denmark). TRAM-34 was purchased from Toronto Research Chemicals, Toronto, Canada. Unless otherwise mentioned, all other drugs and chemicals were obtained from Sigma-Aldrich, Poole, UK.

Indomethacin was dissolved in absolute ethanol and barium in deionized water (10 mM stocks). CyPPA was prepared as a 100 mM stock and ACh, TRAM-34, NS309 and levcromakalim were prepared as 10 mM stock solutions in ethanol and subsequently diluted in Krebs solution. L-NA and ouabain were dissolved directly in Krebs solution.

Results

Systolic blood pressure was elevated in SHRs in comparison with WKYs (systolic, SHR: 235 ± 5 mmHg, $n = 20$; WKY: 174 ± 4 mmHg, $n = 16$; Student's *t*-test, $P < 0.0001$; diastolic, SHR: 157 ± 6 mmHg, $n = 20$, WKY: 112 ± 7 mmHg, $n = 16$, $P < 0.0001$) although the heart rate in the SHRs (414 ± 8 bpm, $n = 20$) was similar to that of WKYs (411 ± 13 bpm, $n = 16$) and the mean body weights did not differ (SHR, 293 ± 12 g, $n = 17$; WKY, 286 ± 11 g, $n = 15$).

Effects of ACh and NS309: evidence that SK_{Ca} channels are reduced in SHR vessels

In artery segments with an intact endothelium, the resting membrane potential of SHR mesenteric artery myocytes (–49.3 ± 0.4 mV, $n = 12$) was depolarized compared with that of the WKY controls (–53.6 ± 0.4 mV, $n = 12$; Student's *t*-test, $P < 0.0001$). ACh produced concentration-dependent myocyte hyperpolarizations that were significantly smaller in arteries from SHR animals than those in WKY arteries ($P = 0.001$, $n = 4$; two-way ANOVA; Figure 1). In contrast, NS309 (100 nM, an activator of both SK_{Ca} and IK_{Ca} channels) hyperpolarized SHR myocytes to a similar extent (two-way ANOVA with Bonferroni post-test, $P > 0.05$) in segments of artery from both SHR (by 13.5 ± 1.6 mV, $n = 4$) and WKY (by 16.0 ± 1.2 mV, $n = 4$) rats. To determine whether these results somehow reflected a change in the population of SK_{Ca} or IK_{Ca} channels in SHR myocytes, additional experiments were first conducted in the presence of apamin to block SK_{Ca} channels and thus to ensure that any changes subsequently mediated by either ACh or NS309 resulted from the opening of IK_{Ca} alone.

Apamin itself (100 nM; an SK_{Ca} blocker) had minimal effects on myocyte membrane potential in both SHR and WKY vessels. In SHR arteries, apamin also had no effect on responses to ACh or NS309 (two-way ANOVA) whereas the effects of each agent in WKY rats were reduced following SK_{Ca} blockade (Figure 1A,B; $P < 0.004$). One explanation for these findings was that the population of SK_{Ca} channels in hypertensive vessels was somehow reduced whereas the IK_{Ca}-mediated component of hyperpolarizations to ACh or NS309 (and revealed in the presence of apamin) was apparently unchanged (Figure 1A,B: Bonferroni post-test, $P > 0.05$).

To confirm that the residual hyperpolarizations observed in the presence of apamin did result from the opening of IK_{Ca} channels, TRAM-34 (10 μM; a blocker of IK_{Ca}) was subsequently added to the Krebs solution that already contained apamin. Under these conditions, the effects of NS309 were indeed abolished.

To provide some indication of the integrity of vessels at the end of these long impalements, arteries were finally challenged with 10 μM levcromakalim, an opener of myocyte ATP-sensitive K^+ channels. Levcromakalim hyperpolarized the membrane to a similar potential in the arteries from WKY and SHR rats (WKY, –77.7 ± 0.6 mV; SHR, –77.4 ± 1.2 mV, each $n = 4$; Figure 1C), an indication that these vessels were still in good condition.

Experiments with CyPPA: confirmation that SK_{Ca} channels are compromised in SHR vessels

The possible reduction in the SK_{Ca} component of the hyperpolarization to ACh could have been due to loss of muscarinic M_3 receptors, to modified receptor–channel coupling or to a loss of SK_{Ca} channels in SHR artery segments. In order to investigate this further, hyperpolarizations to CyPPA (a directly acting, selective opener of SK_{Ca} channels; Hougaard *et al.*, 2007) were elicited. This cyclohexylamine produced myocyte hyperpolarizations in endothelium-intact segments from both SHR and WKY rats and these changes were significantly smaller in arteries from hypertensive animals (CyPPA, 30 μM: SHR 8.8 ± 0.9 mV; WKY 19.4 ± 0.4 mV; both $n = 4$, $P < 0.0001$, two-way ANOVA). In the presence of the SK_{Ca} inhibitor, apamin (100 nM), the effects of CyPPA were always totally abolished, confirming that CyPPA-induced hyperpolarizations were indeed due solely to the opening of SK_{Ca} channels (Figure 2).

Collectively, these findings with ACh, NS309 and with CyPPA strongly indicate that either the number of functional SK_{Ca} channels and/or the pathways downstream of SK_{Ca} activation are compromised in SHR vessels.

Modification of SK_{Ca}-activated downstream pathways in SHR vessels

The activation of endothelial IK_{Ca} and SK_{Ca} channels generates an increase in the $[K^+]$ in the myo-endothelial space that we have called the 'K^+ cloud' (Edwards *et al.*, 1998; Edwards and Weston, 2004). Recent evidence suggests that the

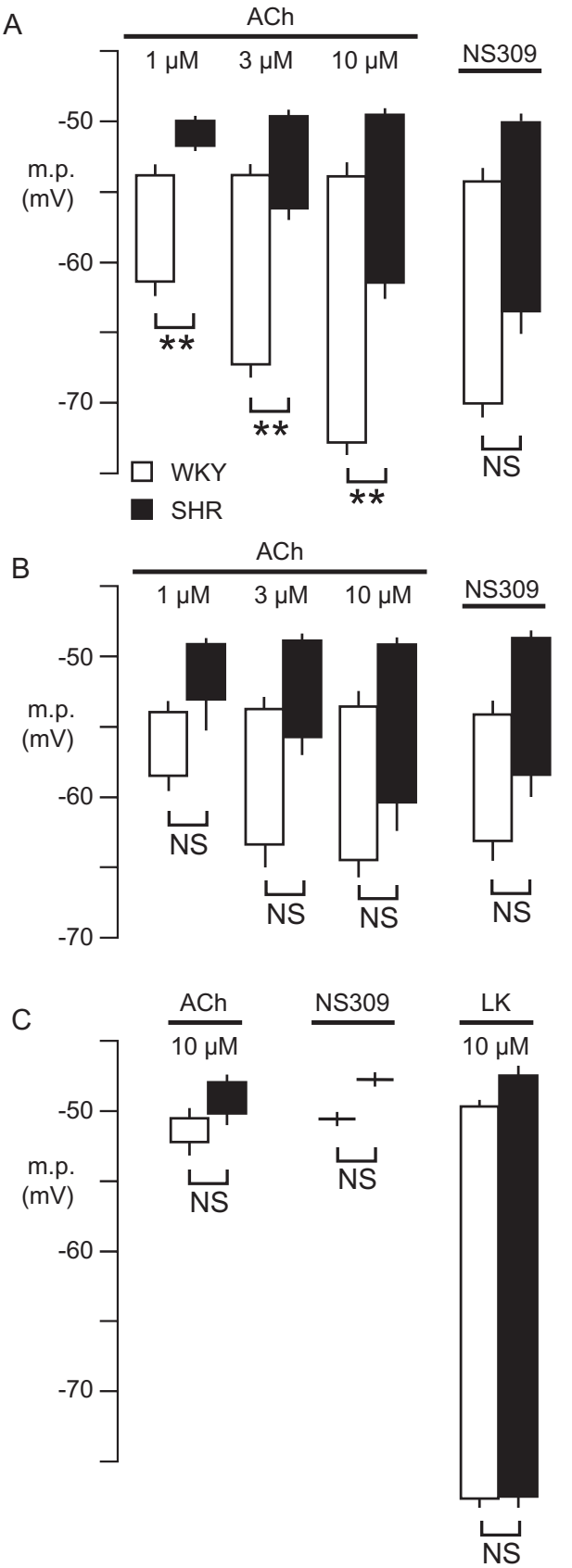

Figure 1 Comparison of hyperpolarizations induced by acetylcholine (ACh), 6,7-dichloro-1H-indole-2,3-dione 3-oxime (NS309) and levcromakalim (LK) in rat mesenteric artery segments from spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY) controls. (A) ACh induced a concentration-dependent myocyte hyperpolarization that was significantly greater in segments from WKY than in those from SHR ($P = 0.001$; two-way ANOVA). (B) In the presence of 0.1 μM apamin, the responses to ACh were reduced in artery segments from WKY but not from SHR. (C) In the additional presence of 10 μM 1-[(2-chlorophenyl)-diphenyl-methyl]-1H-pyrazole (TRAM-34), responses to ACh were almost abolished (the small, residual hyperpolarization is assumed to be due to activation of myocyte BK_{Ca} channels by nitric oxide). In the presence of TRAM-34 + apamin, there was no difference in the peak hyperpolarization induced by LK, suggesting that the myocyte E_K was similar in WKY and SHR vessels. Each bar represents mean membrane potential (m.p.) before, and peak response to, ACh or LK (±SEM, $n = 4$); asterisks indicate statistically significant differences between hyperpolarizations in the indicated groups (Bonferroni post-test; $^{**}P < 0.01$; NS, not significant).

apamin-sensitive component of the EDHF response (associated with a localized K^+ cloud that originates from K^+ efflux through SK_{Ca} opening) is coupled to the subsequent activation of Ba^{2+}-sensitive, inwardly rectifying K^+ channels (K_{IR}). The K^+ cloud, which originates from TRAM-34-sensitive, IK_{Ca} opening, predominantly then signals to the myocytes via activation of an isoform of Na^+/K^+-ATPase that is sensitive to low concentrations of ouabain (Dora *et al.*, 2008; Harno *et al.*, 2008).

Thus, to determine whether pathways downstream of SK_{Ca} activation were compromised in SHR vessels, hyperpolarizations generated following 5 mM elevation of extracellular [K^+] were used as a simple paradigm of the localized K^+-coupling process.

In the absence of the endothelium, the hyperpolarizations induced by 5 mM K^+ were smaller in SHR arteries (6.7 ± 0.3 mV, $n = 4$) than in WKY vessels (9.8 ± 0.6 mV, $n = 4$; Student's *t*-test, $P = 0.003$; Figure 3A). These presumably resulted from the activation of both Na^+/K^+-ATPases and K_{IR}; to reveal the contribution of K_{IR}, 500 nM ouabain was added to the Krebs solution to inhibit myocyte Na^+/K^+-ATPases. Under these conditions, the hyperpolarizations induced by 5 mM K^+ were smaller in SHR arteries (1.2 ± 0.3 mV, $n = 4$) than in WKY vessels (5.9 ± 0.8 mV, $n = 4$; Student's *t*-test, $P = 0.002$; Figure 3A) and were abolished by 30 μM Ba^{2+}, indicating that they were indeed generated by K_{IR} activation.

In the presence of the endothelium (Figure 3B), hyperpolarizations to exogenous (5 mM) K^+ were slightly (but significantly, $P < 0.05$, Student's *t*-test) greater (WKY, 13.2 ± 1.2 mV, $n = 4$; SHR, 10.1 ± 0.8 mV, $n = 4$) than in its absence (WKY, 9.8 ± 0.6 mV, $n = 4$; SHR, 6.7 ± 0.3 mV, $n = 4$). Under these conditions, addition of ouabain (500 nM) almost abolished the hyperpolarizing effect of K^+ but had no significant effect on the hyperpolarization to 5 mM K^+ in WKY arteries. In all endothelium-intact artery segments, addition of barium (30 μM) to the ouabain-Krebs abolished the residual response to K^+ (Figure 3B).

Taken together, these effects of increasing the bath K^+ concentration by 5 mM suggest that there is a reduction in functional K_{IR} channels in SHR arteries.

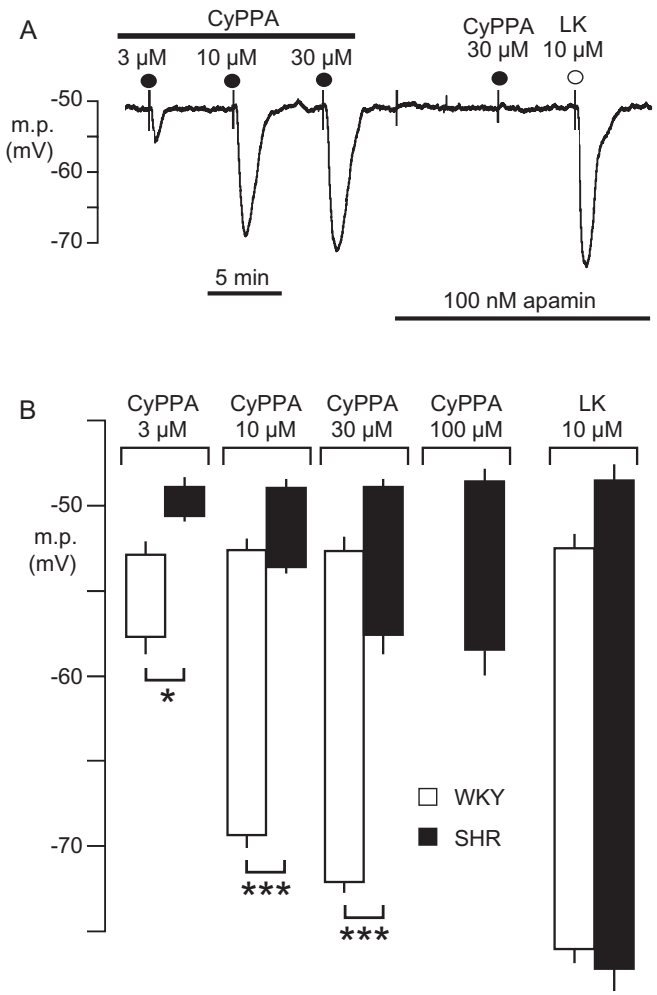

Figure 2 Comparison of hyperpolarizations induced by CyPPA (cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine) and levcromakalim (LK) in rat mesenteric artery segments from spontaneously hypertensive rat (SHR) and Wistar-Kyoto rat (WKY) controls. (A) Typical trace showing concentration-dependent smooth muscle hyperpolarization induced by CyPPA (a selective opener of SK_{Ca}, native small-conductance, calcium-sensitive K^+ channel) in a segment of artery from a WKY rat. (B) Mean results showing significantly greater hyperpolarization to CyPPA in segments from WKY than from SHR ($P = 0.001$; two-way ANOVA). In contrast, there was no difference in the membrane potential (m.p.) to which LK hyperpolarized the myocytes, suggesting that the myocyte equilibrium potential for K^+ was similar in WKY and SHR vessels. Each bar represents mean membrane potential (m.p.) before, and peak response to, acetylcholine or LK (±SEM, $n = 4$); asterisks indicate statistically significant differences between hyperpolarizations in the indicated groups (Bonferroni post-test; *$P < 0.05$; ***$P < 0.001$).

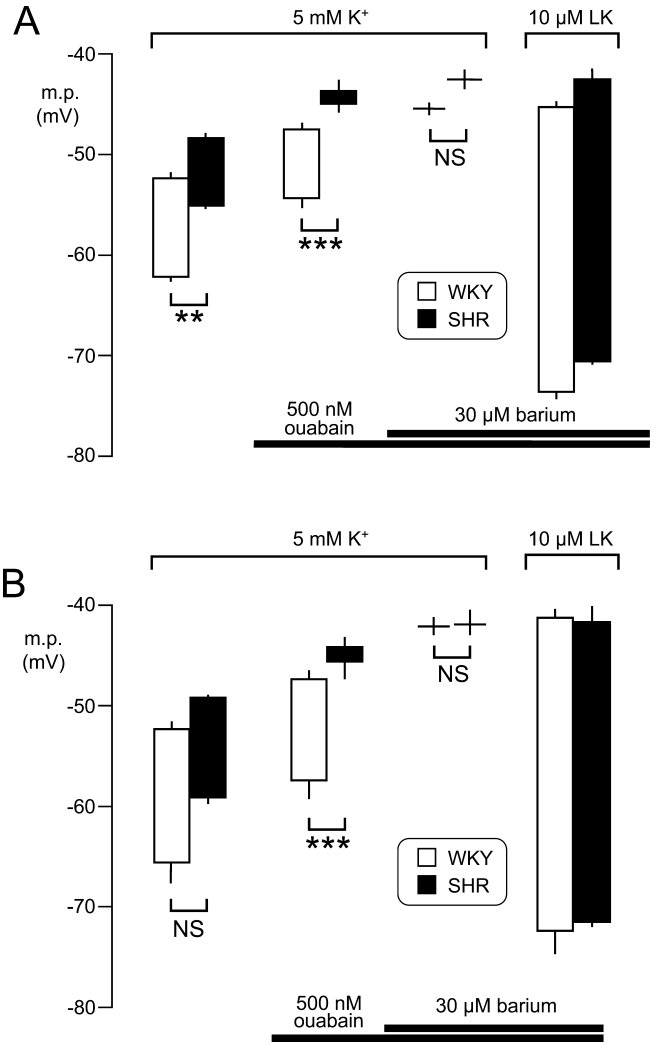

Figure 3 Effects of ouabain and barium on rat mesenteric artery myocyte hyperpolarizations to extracellular K^+. (A) In the absence of a functional endothelium, the resting membrane potential of the spontaneously hypertensive rat (SHR) artery myocytes was depolarized relative to that of the Wistar-Kyoto rat (WKY) myocytes ($P = 0.005$) and the hyperpolarizing effect of elevation of extracellular K^+ (by 5 mM) was reduced. The magnitude of the depolarization to ouabain was similar in SHR myocytes and WKY myocytes (each $n = 4$; $P = 0.47$). Elevation of extracellular K^+ by 5 mM produced a hyperpolarization that was partially inhibited by ouabain in segments of artery from SHR but not in those from WKY controls. (B) Hyperpolarizations to K^+ were increased by the presence of an intact endothelium but, under these conditions, significant inhibition by ouabain was only produced in the SHR artery segments. In the presence of both barium and ouabain, all responses to K^+ elevation were abolished. Each bar represents mean membrane potential (m.p.) before, and peak response to, acetylcholine or levcromakalim (LK) (±SEM, $n = 4$); asterisks indicate that differences between hyperpolarizations in the indicated groups were statistically significant (Student's *t*-test; **$P < 0.005$; ***$P < 0.001$; NS, not significant).

Western blotting

A polyclonal antibody was used for Western blot analysis of the $K_{Ca}2.3$ α-subunit expression in lysates of SHR and WKY mesenteric arteries. When SHR blot densities were adjusted according to their corresponding β-actin level and normalized relative to those from WKY samples, they showed a marked reduction in $K_{Ca}2.3$ immunoreactivity in SHR mesenteric arteries in comparison with those from WKY rats (Figure 4Ai,Bi). Using identical procedures, there was also a significant reduction in $K_{IR}2.1$ α-subunit protein expression in SHR arteries compared with vessels from the WKY controls (Figure 4Aii,Bii) whereas there was a marked increase in caveolin-1 (monomer) protein expression in vessels derived from the hypertensive rats (Figure 4Aiii,Biii). However, the concentration of the dimeric form was reduced (Figure 4Aiii,Biii), so the total relative concentration of caveolin-1 protein was essentially unchanged ($P > 0.05$; Student's *t*-test).

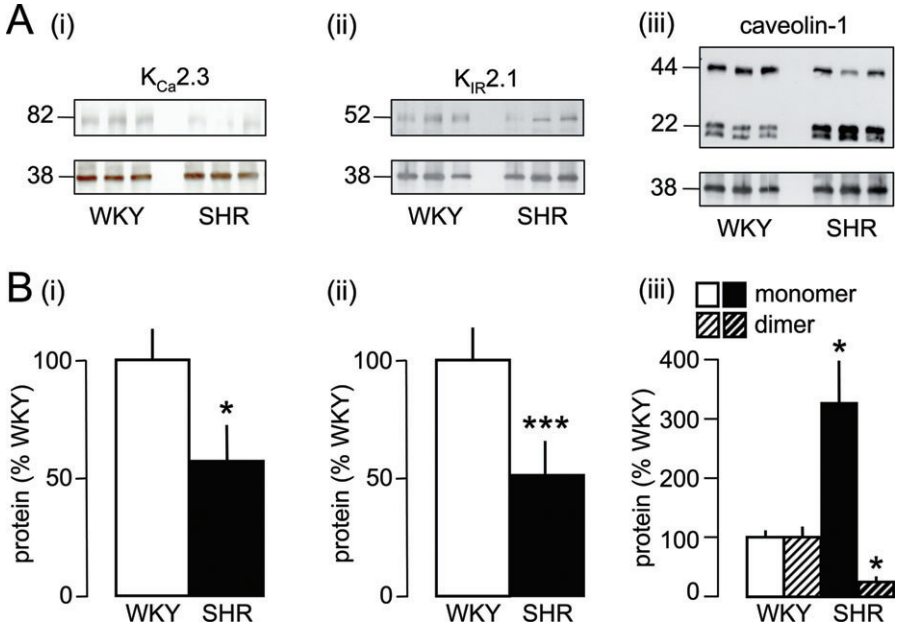

Figure 4 Changes in protein expression in mesenteric arteries from spontaneously hypertensive rat (SHR). Upper panel: typical Western blots for (i) $K_{Ca}2.3$ (ii) $K_{IR}2.1$ and (iii) caveolin-1 monomer and dimer, with size (kDa) of immunoreactive blot indicated. The three lanes on the left are from samples obtained from three different Wistar-Kyoto (WKY) rats and on the right are from three different SHRs. Lower panel: blots were stripped and re-probed for β-actin. (B) Semi-quantitative analysis of mean protein expression in SHR arteries relative to that of controls. Mean blot density (+SEM) for lysates from seven mesenteric beds showed significant reductions in (i) $SK_{Ca}2.3$ and (ii) $K_{IR}2.1$ protein and (iii) a large increase in caveolin-1 monomer and decrease in caveolin-1 dimer protein expression in SHR artery preparations relative to those from WKY (samples were adjusted for loading errors, using the β-actin blot density to standardize, prior to normalization relative to WKY values). Asterisks indicate the statistical significance of differences in relative protein concentrations in the SHR arteries compared with the corresponding WKY controls (Student's *t*-test; $*P < 0.05$; $***P < 0.0001$).

Discussion

Reduced hyperpolarization in SHR arteries reflects a modified SK_{Ca} channel pathway

The results from the present study clearly indicate that the ACh-induced myocyte hyperpolarization (the so-called EDHF response which in normal vessels follows from the activation of both endothelial SK_{Ca} and IK_{Ca} channels) is reduced in SHR arteries, an effect due largely to selective modification of pathways associated with activation of the SK_{Ca} component.

Thus, total ACh-mediated hyperpolarizations (activated by the interaction of ACh with the muscarinic M_3 receptor; Hammarström *et al.*, 1995; Wu *et al.*, 1997) in SHR vessels were halved in magnitude (compared with WKY controls), whereas the ACh-activated IK_{Ca} component (studied in the presence of apamin) was the same in both WKY and SHR arteries. This suggested that there had been a near abolition of the SK_{Ca} component in the SHR vessels. The unchanged IK_{Ca} component indicated that the endothelial M_3 receptor itself was unlikely to have been altered in the SHR vessels.

To test this directly, we made Western blot measurements of the expression of M_3 receptor protein using the Alomone AMR-006 anti-M_3 antibody. The results were, however, unclear, yielding multiple protein bands (data not presented) from which no firm conclusions could be drawn. Equivocal results were also obtained by measuring myocyte hyperpolarizations produced by NS309, the M_3 receptor-independent opener of IK_{Ca} and SK_{Ca} channels. These electrical changes were not significantly reduced in SHR arteries, perhaps reflecting the known selectivity of NS309 for IK_{Ca} (Strøbaek *et al.*, 2004). However, hyperpolarizations to CyPPA (a *selective* opener of SK_{Ca} channels; Hougaard *et al.*, 2007) were substantially reduced in SHR vessels. Furthermore, Western blot analysis showed that expression of the SK_{Ca} α-subunit protein ($K_{Ca}2.3$; Burnham *et al.*, 2002; Alexander *et al.*, 2009) was also markedly diminished in SHR vessels.

Collectively, these findings strongly support the conclusion that the SK_{Ca} pathway (and specifically the channel protein itself) is down-regulated in the hypertensive animals. A similar reduction in $K_{Ca}2.3$ mRNA and protein in mesenteric arteries from angiotensin II-induced hypertensive rats (Hilgers and Webb, 2007), together with the induction of hypertension in $K_{Ca}2.3$ gene-depleted mice (Taylor *et al.*, 2003), suggests that loss of SK_{Ca} may indeed be a general feature of a long-term increase in blood pressure.

Changes downstream of endothelial SK_{Ca} channel activation: additional down-regulation of K_{IR} in SHR vessels

Recent studies, using a combination of electrophysiological, ultrastructural and pharmacological techniques, have shown that activation of endothelial IK_{Ca} channels triggers myocyte hyperpolarization by mechanisms that are distinct from those that follow the opening of SK_{Ca} channels. Thus, endothelial cell IK_{Ca}-induced myocyte hyperpolarizations involve subsequent activation of types 2 and 3 Na^+/K^+-ATPases (Dora *et al.*, 2008; Harno *et al.*, 2008). In contrast, the dilator pathway

triggered by endothelial cell SK_{Ca} channel opening is associated with the subsequent activation of K_{IR} (Dora *et al.*, 2008; Harno *et al.*, 2008).

To determine whether these downstream pumps and channels (i.e. the Na^+/K^+-ATPases and /or K_{IR} channels) were, in addition to SK_{Ca} channels, functionally altered in SHR vessels, the simple technique of raising extracellular [K^+] (Edwards *et al.*, 1998; Weston *et al.*, 2002) was used to induce myocyte hyperpolarizations in the presence or absence of barium (to block K_{IR}) and ouabain (to block Na^+/K^+-ATPases). Increasing the bath concentration of K^+ by 5 mM hyperpolarized the membrane potential in both the presence and absence of the endothelium and the responses were similar in arteries from both the SHR and WKY controls. However, in contrast to WKY vessels, K^+-induced hyperpolarizations in SHR arteries were virtually abolished by low concentrations of ouabain. From this we conclude that these K^+-induced changes were almost totally dependent on Na^+/K^+-ATPase activation alone, an indication that K_{IR} channels were somehow compromised in SHR vessels. This was indeed confirmed in Western blot experiments that revealed a significant down-regulation in the expression of $K_{IR}2.1$ protein. This was a remarkable finding; it clearly shows that not only is the $K_{Ca}2.3$ protein reduced in SHR vessels but also that the K_{IR} channel 'downstream' of SK_{Ca} is also compromised in the hypertensive animals.

A functional loss of K_{IR} in mesenteric arteries from SHR was previously described by Goto *et al.* (2004). In their experiments, this was associated with a reduction in conducted vasodilatation (a poorly understood phenomenon in which vasodilatation initiated in one part of an artery is able to spread along the vessel; Welsh and Segal, 1998; Takano *et al.*, 2005). However, Goto *et al.* (2004) found no evidence of a decrease in K_{IR} mRNA and therefore proposed that the functional loss of K_{IR} might be a reflection of the more depolarized myocytes in SHR vessels and the associated reduction in current flow through K_{IR} channels.

In the present study, the myocytes from SHR vessels were also depolarized compared with their WKY controls. It therefore seems reasonable to conclude that the reduced K_{IR} involvement in SHR vessels results not only from the depolarized myocyte membrane potential but also from the observed significant down-regulation in $K_{IR}2.1$ protein expression.

Are caveolae modified in SHR vessels?

In an earlier study in mesenteric arteries, Absi *et al.* (2007) showed that SK_{Ca}- (but not IK_{Ca}-) mediated hyperpolarizations could be selectively inhibited by disruption of caveolae using methyl-β-cyclodextrin (Absi *et al.*, 2007). Furthermore, knockout of caveolin-1, a protein associated with caveolae, reduces EDHF-induced relaxant responses in mouse mesenteric arteries (Saliez *et al.*, 2008). We therefore wondered whether the compromised SK_{Ca}–K_{IR} pathway observed in SHR vessels in the present study might also extend to include some failure of the muscarinic M_3 receptor to signal specifically to SK_{Ca} channels located in endothelial cell caveolae (Absi *et al.*, 2007; Callera *et al.*, 2007; Grgic *et al.*, 2009). In favour of this possibility was the virtual abolition in our SHR vessels of ACh-induced hyperpolarizations mediated by SK_{Ca} opening, effects that were arguably too large to result solely from the observed (approximately 50%) reductions in $K_{Ca}2.3$ and $K_{IR}2.1$ proteins.

In the SHR vessels, there was no change in *total* caveolin-1 protein. However, the Western blot experiments showed a marked *increase* in the monomeric form, while caveolin-1 dimers were reduced. On the basis of earlier studies (Callera *et al.*, 2007; see also Grgic *et al.*, 2009) and of the present experiments, we speculate that possible changes within caveolae and/or to associated lipid rafts in SHR vessels affect the ability of activated M_3 receptors to induce the opening of SK_{Ca} channels. Such effects, when combined with additional reductions in $K_{Ca}2.3$ and $K_{IR}2.1$ proteins, account for the diminished ACh-mediated hyperpolarizations in SHR vessels and for their almost total dependence on IK_{Ca} channel opening.

In conclusion, the present study using mesenteric blood vessels from a rat model of hypertension has shown that there are specific changes in one of the two K_{Ca}-dependent dilator pathways associated with ACh-induced, endothelial-dependent hyperpolarization in myocytes. The pathway that involves activation of IK_{Ca} and subsequent stimulation of types 2 and 3 Na^+/K^+-ATPases remains intact. In contrast, both the SK_{Ca} and K_{IR} channels that are activated in a parallel signalling sequence are significantly down-regulated in mesenteric arteries from SHR. It is possible that changes in caveolae and/or lipid rafts also compromise the link between M_3 receptor activation and SK_{Ca} opening. This remarkable compromise of the SK_{Ca}–K_{IR} vasodilator pathway is likely to lead to an increase in vascular tone *in vivo* and thus be a factor that contributes to the elevated arterial blood pressure that is a feature of the SHR.

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Statement of conflicts of interest

None.

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