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RESEARCH PAPER

Activation of $K_{Ca}3.1$ by SKA-31 induces arteriolar dilatation and lowers blood pressure in normo- and hypertensive connexin40-deficient mice

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BACKGROUND AND PURPOSE

The calcium-activated potassium channel $K_{Ca}3.1$ is expressed in the vascular endothelium where its activation causes endothelial hyperpolarization and initiates endothelium-derived hyperpolarization (EDH)-dependent dilatation. Here, we investigated whether pharmacological activation of $K_{Ca}3.1$ dilates skeletal muscle arterioles and whether myoendothelial gap junctions formed by connexin40 (Cx40) are required for EDH-type dilatations and pressure depressor responses *in vivo*.

EXPERIMENTAL APPROACH

We performed intravital microscopy in the cremaster muscle microcirculation and blood pressure telemetry in Cx40-deficient mice.

KEY RESULTS

In wild-type mice, the $K_{Ca}3.1$-activator SKA-31 induced pronounced concentration-dependent arteriolar EDH-type dilatations, amounting to ~40% of maximal dilatation, and enhanced the effects of ACh. These responses were absent in mice devoid of $K_{Ca}3.1$ channels. In contrast, SKA-31-induced dilatations were not attenuated in mice with endothelial cells deficient in Cx40 ($Cx40^{fl/fl}$:Tie2-Cre). In isolated endothelial cell clusters, SKA-31 induced hyperpolarizations of similar magnitudes (by ~38 mV) in $Cx40^{fl/fl}$:Tie2-Cre, ubiquitous Cx40-deficient mice ($Cx40^{-/-}$) and controls ($Cx40^{fl/fl}$), which were reversed by the specific $K_{Ca}3.1$-blocker TRAM-34. In normotensive wild-type and $Cx40^{fl/fl}$:Tie2-Cre as well as in hypertensive $Cx40^{-/-}$ animals, i.p. injections of SKA-31 (30 and 100 mg·kg^{-1}) decreased arterial pressure by ~32 mmHg in all genotypes. The depressor response to 100 mg·kg^{-1} SKA-31 was associated with a decrease in heart rate.

CONCLUSIONS AND IMPLICATIONS

We conclude that endothelial hyperpolarization evoked by pharmacological activation of $K_{Ca}3.1$ channels induces EDH-type arteriolar dilatations that are independent of endothelial Cx40 and Cx40-containing myoendothelial gap junctions. As SKA-31 reduced blood pressure in hypertensive Cx40-deficient mice, $K_{Ca}3.1$ activators may be useful drugs for severe treatment-resistant hypertension.

Abbreviations

Cx40, connexin40; DP, diastolic pressure; EDH, endothelium-derived hyperpolarization; K_{Ca}, Ca^{2+}-dependent K^+-channel; L-NA, N^G-nitro-*L*-arginine; MAP, mean arterial pressure; SKA-31, naphtho[1,2-*d*]thiazol-2-ylamine; SP, systolic pressure; WT, wild-type

Introduction

The vascular endothelium controls the contractile state of the underlying smooth muscle and thereby regulates vascular diameter and blood pressure. This control is achieved by the release of endothelial autacoids, NO, prostaglandins and a third mechanism that induces hyperpolarization of the vascular smooth muscle and subsequent closure of voltage-gated calcium channels leading to a decrease in intracellular calcium and finally relaxation. Initially, it was assumed that this third mechanism acts similarly through the release of a diffusible endothelial factor (endothelium-derived hyperpolarizing factor). However, this view was later questioned by experiments demonstrating the involvement of gap junctions (Griffith *et al.*, 2002; Dora *et al.*, 2003; Chaytor *et al.*, 2005; Mather *et al.*, 2005; Sokoya *et al.*, 2006). These studies suggested that endothelial hyperpolarization is transferred from the endothelium to the smooth muscle by direct charge transfer through myoendothelial gap junctions (for in-depth reviews, see de Wit and Wölfle, 2007; Feletou and Vanhoutte, 2009; Grgic *et al.*, 2009; Heberlein *et al.*, 2009; Edwards *et al.*, 2010; de Wit and Griffith, 2010; Garland *et al.*, 2011). Whatever the exact mechanism, in multiple studies endothelial hyperpolarization has been demonstrated to have a crucial role in the endothelium-derived hyperpolarization (EDH)-type dilatations induced by agonists.

Endothelial hyperpolarization following activation of GPCRs like muscarinic (ACh) receptors requires Ca^{2+}-release from the endoplasmic reticulum. The resulting increase in intracellular Ca^{2+} activates Ca^{2+}/calmodulin-regulated K^+-channels (K_{Ca}) with intermediate ($K_{Ca}3.1$ or IK_{Ca}) and small conductance ($K_{Ca}2.3$ or SK_{Ca}). $K_{Ca}3.1$ is the predominant channel involved in ACh-induced EDH-type dilatation in many vessels (Si *et al.*, 2006), whereas $K_{Ca}2.3$ channels are possibly responsible for flow-induced dilatation and active hyperaemia (Brähler *et al.*, 2009; Milkau *et al.*, 2010). Importantly, both channels are voltage-independent and consequently do not inactivate during the ensuing hyperpolarization, which renders them an attractive target to induce a sustained, solid hyperpolarization towards the K^+-equilibrium potential. In mice deficient in the $K_{Ca}3.1$ gene, the ACh-induced EDH-type vasodilatation in large conduit arteries and in the cremaster microcirculation *in vivo* are attenuated compared to those responses in wild-type mice and – at the systemic level – this results in mild systolic hypertension (Si *et al.*, 2006). These results confirm a pivotal role for $K_{Ca}3.1$ channels. Intriguingly, pharmacological activation of this channel by the $K_{Ca}3.1$/$K_{Ca}2$ activator SKA-31, a compound with a 10-fold higher potency for $K_{Ca}3.1$ than $K_{Ca}2.3$, lowers blood pressure in normotensive mice in a $K_{Ca}3.1$-dependent fashion as well as in a murine model of short-term angiotensin II-induced hypertension (Sankaranarayanan *et al.*, 2009). Recently, an i.v. injection of SKA-31 has also been shown to transiently lower pressure in conscious dogs (Damkjaer *et al.*, 2012). As with NO, $K_{Ca}3.1$ (and $K_{Ca}2.3$) channels accordingly constitute promising novel pharmacological targets for lowering peripheral vascular resistance in hypertension or ischaemic heart disease.

However, it is not clear whether pharmacological activation of $K_{Ca}3.1$ produces vasodilatation of arterioles and thereby elicits a depressor response and whether myoendothelial gap junctions are involved in such dilatations, in analogy to the suggested role of gap junctions in EDH-type dilatations upon stimulation with ACh. Gap junctions are clusters of intercellular channels composed of connexin proteins. Six connexins oligomerize into a hemichannel in the plasma membrane, which docks to its counterpart in the adjacent cell to form an intercellular channel. Of the four connexins expressed in vascular cells, connexin40 (Cx40) connects endothelial cells homocellularly (de Wit, 2004) and is reportedly also an essential component in myoendothelial gap junctions (Isakson and Duling, 2005; Isakson *et al.*, 2006; 2008). Recently, we confirmed a role for Cx40-dependent myoendothelial coupling in ACh-induced EDH-type dilatations in an isometric experimental setting *in vitro*, whereas *in vivo* Cx40 did not appear to be necessary for EDH-type dilatations in small arteries (Boettcher and de Wit, 2011). Therefore, we hypothesized that activation of $K_{Ca}3.1$ induces dilatations in resistance-sized arterioles and lowers arterial pressure in the intact animal and that these effects are – similar to ACh-induced responses – independent of Cx40. To test this hypothesis, we studied vascular responses elicited by SKA-31 in mice ubiquitously deficient for Cx40 as well as in animals that lacked Cx40 only in endothelial cells. Global Cx40-deficient mice are hypertensive (de Wit *et al.*, 2000; 2003) due to an enhanced secretion of renin and activation of the renin-angiotensin-aldosterone system (Wagner *et al.*, 2007; 2010), while endothelial-specific Cx40-deficient mice are normotensive (Chadjichristos *et al.*, 2010; Wagner *et al.*, 2010). Thus, our approach also allowed us to study the effect of pharmacological $K_{Ca}3.1$ activation in a chronic renin-dependent, severe hypertension model.

Methods

Animals

Animal care and experiments were in accordance with the German Animal Welfare Act and were approved by Landwirtschafts- und Umweltministerium Schleswig-Holstein. The total number of mice used in the study was 63. Mice with endothelium-specific Cx40 deficiency ($Cx40^{fl/fl}$:Tie2-Cre) carrying a Cre recombinase under the control of the Tie2 promoter and homozygously the floxed Cx40 gene ($Cx40^{fl}$) were generated (Wagner *et al.*, 2010). Littermates without Cre-recombinase served as controls ($Cx40^{fl/fl}$). Ubiquitous Cx40-deficient ($Cx40^{-/-}$), $K_{Ca}3.1$-deficient mice ($K_{Ca}3.1^{-/-}$), and wild-type (WT) control littermates were derived from our breeding colonies.

The mice were kept at 20°C, max. 6 mice in a single cage, with a 12 h day/12 h night cycle and had free access to water and food (standard diet, Altromin). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Endothelial cell isolation and measurements of membrane potential

Endothelial cells were isolated from the carotid artery and measured as described (Brähler *et al.*, 2009). SKA-31 (1 $\mu mol \cdot L^{-1}$) was added to the bath solution followed by the

selective $K_{Ca}3.1$ blocker TRAM-34 (1 μmol·L^{-1}) (Wulff *et al.*, 2000) and the $K_{Ca}2.X$ blocker UCL1684 (1 μmol·L^{-1}) (Rosa *et al.*, 1998). Drugs did not modulate inter-endothelial electrical coupling as capacitance values remained unchanged. Electrical uncoupling was achieved by addition of 10 μmol·L^{-1} docosahexaenoic acid (Schmidt *et al.*, 2010).

Intravital microscopy of the microcirculation

Mice were anaesthetized with an i.p. injection of fentanyl (0.05 mg·kg^{-1}), midazolam (5 mg·kg^{-1}) and medetomidine (0.5 mg·kg^{-1}) followed by i.v. infusion. The mice were regularly pinched in the paw with forceps and also briefly blown on their whiskers to test for reactions and level of consciousness. The cremaster muscle was prepared as described previously (de Wit, 2010). Arteriolar diameters were measured before and during superfusion of SKA-31 (1–30 μmol·L^{-1}, dissolved at 20 mmol·L^{-1} in Cremophor EL). This protocol was repeated during superfusion of N^{G}-nitro-*L*-arginine (L-NA, 30 μmol·L^{-1}) and indomethacin (indo, 3 μmol·L^{-1}) to block NO synthase and COX. In a subset of experiments, dilatations induced by SKA-31 were studied before and after addition of the non-specific gap junction blocker carbenoxolone (30 μmol·L^{-1}) during L-NA and indomethacin. In a second protocol, SKA-31 (3, 30 μmol·L^{-1}) and ACh (0.03–10 μmol·L^{-1}) were superfused during L-NA and indomethacin either alone or together. The maximal diameter of the arterioles was determined by combined superfusion of ACh, adenosine and sodium nitroprusside (each 30 μmol·L^{-1}) before the animal was killed by an overdose of pentobarbital.

Blood pressure measurement

Mice were anaesthetized by inhalation of isoflurane (2%) and received fentanyl (0.07 mg·kg^{-1} i.p.) for implantation of telemetric pressure transducers (Data Sciences International, s'Hertogenbosch, Netherlands). SKA-31 was dissolved in peanut oil and administered i.p. Measurements were started 30 min before application and continued thereafter. Increasing concentrations of SKA-31 (1–100 mg·kg^{-1}) were studied in all animals non-recurring during consecutive nights.

Statistics and calculations

Data within groups were compared using paired *t*-tests and between different groups by ANOVA (one-way ANOVA) followed by the Bonferroni *post hoc* test. Time series measurements (pressure and HR) were analysed by univariate repeated measures ANOVA to test the hypothesis of a constant mean over time. The repeated measures ANOVA was also used to test whether curves differed between genotypes. Normal distribution of the residuals was validated using Q-Q plots. Differences were considered significant at a corrected error probability of $P < 0.05$. Data are given as mean ± SEM.

For further details on experimental methods, see supporting information.

Results

SKA-31-induced arteriolar dilatations require $K_{Ca}3.1$ and are additive to ACh dilatations

The necessity of $K_{Ca}3.1$ for SKA-31-induced arteriolar dilatation was studied by intravital microscopy in cremaster muscle arterioles in $K_{Ca}3.1$-deficient mice ($K_{Ca}3.1^{-/-}$) and WT littermates (n = 5 each genotype). The maximal diameter of the arterioles at the end of the experiment, induced by a combination of vasodilators, was not different between genotypes ($K_{Ca}3.1^{-/-}$: 36 ± 1 μm, n = 45; WT: 36 ± 2 μm, n = 42), but arteriolar resting tone was higher (i.e. resting diameter was lower) in $K_{Ca}3.1^{-/-}$ mice, as indicated by a lower ratio of resting to maximal diameter (0.46 ± 0.02 vs. 0.53 ± 0.03, $K_{Ca}3.1^{-/-}$ and WT, respectively, $P < 0.03$), as observed previously (Wölfle *et al.*, 2009). Local superfusion of SKA-31 over the cremaster muscle induced a concentration-dependent dilatation in WT arterioles that was significantly larger than the subtle diameter changes triggered by superfusing the solvent (Cremophor) alone (Figure 1A). Inhibition of NO and prostaglandin synthesis (L-NA, indomethacin) constricted the arterioles significantly (from 20 ± 2 to 16 ± 2 μm) but the SKA-31-induced dilatation was unchanged (Figure 1B). In marked contrast, arteriolar responses induced by SKA-31 in $K_{Ca}3.1^{-/-}$ mice were not different from the small responses induced by the solvent alone either in untreated preparations or after inhibition of NO and prostaglandins (Figure 1). However, $K_{Ca}3.1^{-/-}$ arterioles dilated considerably upon superfusion of adenosine (3 μmol·L^{-1}: 27 ± 5%; 10 μmol·L^{-1}: 74 ± 4%) or the exogenous NO-donor sodium-nitroprusside (10 μmol·L^{-1}: 39 ± 3%) excluding a general non-responsiveness. These responses were not attenuated after pretreatment with L-NA/indomethacin and were comparable to dilatations in WT mice. Together, these results demonstrate that arteriolar dilatations induced by SKA-31 critically depend on the presence of $K_{Ca}3.1$.

In a different series of WT animals, the effects of SKA-31 on EDH-type dilatations induced by ACh were investigated. In the presence of L-NA and indomethacin, ACh induced a concentration-dependent dilatation in arterioles with a maximal diameter of 34 ± 1 μm (81 arterioles in 10 mice). SKA-31 alone induced a dilatation of 8 ± 3% (3 μmol·L^{-1}) and 38 ± 5% (30 μmol·L^{-1}) in this series. The combined superfusion of ACh and SKA-31 (3 or 30 μmol·L^{-1}) induced dilatations that were significantly larger than dilatations induced by ACh alone, suggesting an additive effect of these dilators, except with the highest concentrations of ACh used (Supporting Information Figure S1).

SKA-31-induced arteriolar dilatations do not require endothelial Cx40

In the next series of experiments, we used mice lacking Cx40 in endothelial cells (Cx40$^{fl/fl}$:Tie2-Cre, n = 4) to investigate the importance of Cx40 for arteriolar dilatations induced by SKA-31. Animals carrying the floxed Cx40 gene without Cre-recombinase (Cx40$^{fl/fl}$, n = 5) served as controls. Resting and maximal diameter as well as tone of the arterioles under study did not differ between genotypes (resting: 16 ± 1 vs. 17 ± 1 μm; maximal: 34 ± 1 vs. 32 ± 1 μm; n = 45 and 36 arterioles, Cx40$^{fl/fl}$ and Cx40$^{fl/fl}$:Tie2-Cre respectively). Similar to WT arterioles (see above), SKA-31 induced concentration-dependent dilatation of arterioles in Cx40fl/fl controls (Figure 2A). This dilatation was not attenuated by pretreatment with L-NA and indomethacin (Figure 2B) despite the fact that this treatment reduced resting diameters (from 16 ± 1 to 12 ± 1 μm, $P < 0.05$). In Cx40$^{fl/fl}$:Tie2-Cre animals, SKA-31 likewise induced a concentration-dependent dilatation that

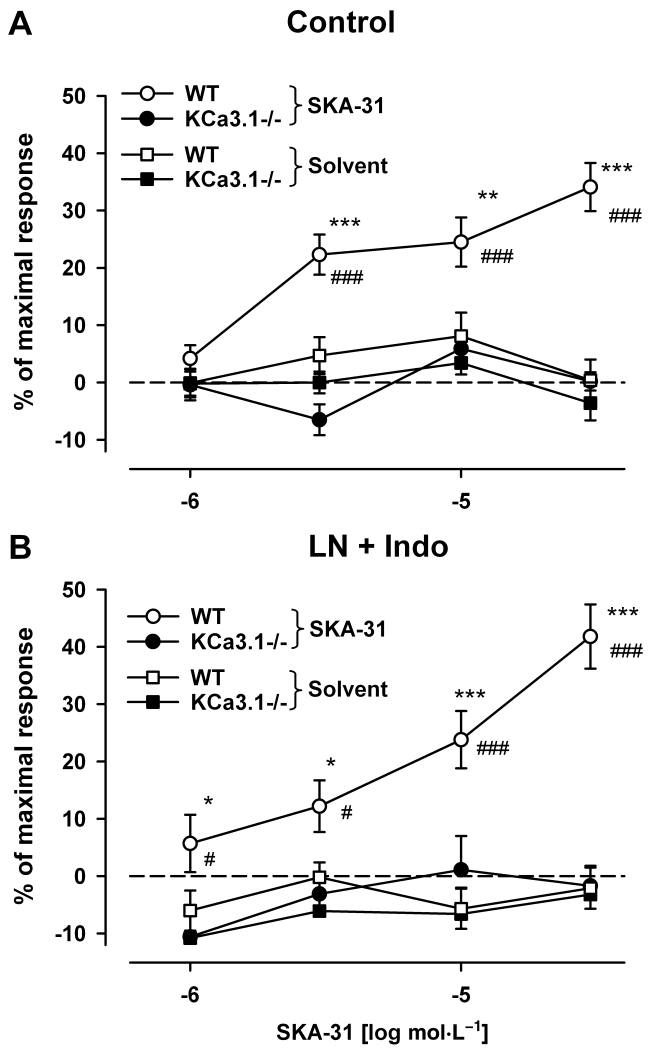

Figure 1

Concentration-dependent arteriolar dilatation upon superfusion of SKA-31 in WT mice before (control, A) and after L-NA and indomethacin treatment (3 and 30 μmol·L^{-1}, LN + Indo, B). In marked contrast, SKA-31 did not dilate arterioles in KCa3.1-deficient mice (Kca3.1-/-). The solvent (Cremophor) did not change the diameters significantly. WT: n = 42 arterioles in 5 animals, KCa3.1-/-: n = 45 arterioles in 5 animals; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus solvent, #: $P < 0.05$, ###: $P < 0.001$ versus KCa3.1-/-, paired and unpaired t-test respectively.

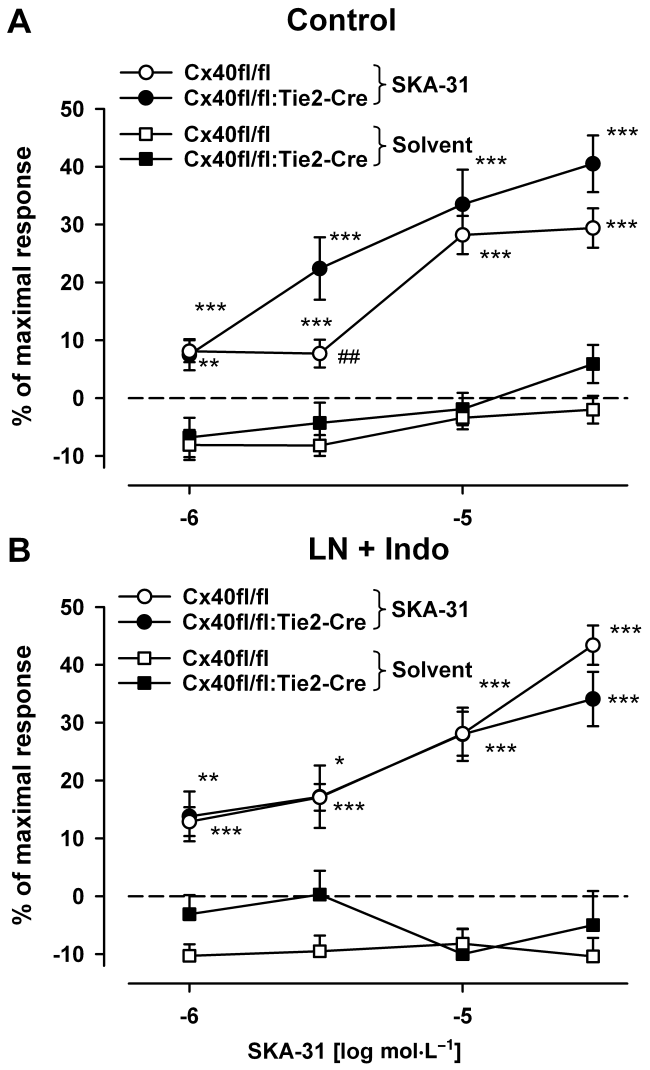

Figure 2

SKA-3-induced arteriolar dilatations in mice with endothelial cells deficient in Cx40 (Cx40$^{fl/fl}$:Tie2-Cre) without (control, A) and after blockade of NO synthase and COX (LN + Indo, B). These dilatations were not attenuated compared with controls (Cx40$^{fl/fl}$). Also, inhibition of NO synthase and COX did not attenuate the dilatations in either genotype. The solvent (Cremophor) did not induce a dilator effect. Cx40$^{fl/fl}$: n = 45 arterioles in 5 animals, Cx40$^{fl/fl}$:Tie2-Cre: n = 36 arterioles in 4 animals; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus solvent, ##: $P < 0.01$ versus Cx40$^{fl/fl}$:Tie2-Cre, paired and unpaired t-test respectively.

was not attenuated compared with Cx40$^{fl/fl}$ (Figure 2A). In fact, the dilatation was larger at one concentration of SKA-31 (3 μmol·L^{-1}). In Cx40fl/fl:Tie2-Cre, SKA-31 dilatations were also not attenuated by L-NA and indomethacin and responses were indistinguishable between Cx40fl/fl and Cx40fl/fl:Tie2-Cre (Figure 2B). The vascular effects of the solvent Cremophor were negligible at all conditions investigated (Figure 2). These results suggest that the dilatation induced by SKA-31 is not mediated by NO and prostaglandins and is independent of the expression of Cx40 in endothelial cells.

In a subgroup of WT mice (n = 40 arterioles in n = 5 mice), the effect of the non-specific gap junction blocker carbenoxolone (Cbx, 30 μmol·L^{-1}) on SKA-31-induced dilatation was studied in the presence of L-NA and indomethacin. Cbx itself caused dilatations and arterioles exhibiting very low tone in the presence of Cbx (ratio of resting to maximal diameter >0.8) were excluded from further analysis. The remaining arterioles (n = 16) dilated in response to Cbx from 12 ± 6 to 16 ± 7 μm ($P < 0.001$). However, SKA-31-induced dilatations remained unaffected (Supporting Information Fig. S2), indicating that these dilatations were independent of gap junctional coupling. Likewise, dilatations induced by ACh and sodium-nitroprusside were not attenuated (3 μmol·L^{-1}, not shown).

SKA-31 hyperpolarizes endothelial cell clusters

We next studied the hyperpolarizing efficacy of SKA-31 in isolated endothelial cell clusters (derived from the carotid artery to obtain feasible amounts of cell clusters) from mice either lacking Cx40 globally or only in endothelial cells. The initial membrane potential after seal rupture was similar in all genotypes ($Cx40^{fl/fl}$:–33 ± 6 mV; $Cx40^{fl/fl}$:Tie2-Cre:–30 ± 6 mV; $Cx40^{-/-}$:–45 ± 3 mV, n = 12–14 clusters isolated from three to four animals in each genotype, P = 0.15). From this level, 1 μmol·L^{-1} SKA-31 induced a rapid, sustained hyperpolarization to –71 ± 4 ($Cx40^{fl/fl}$), 69 ± 5 ($Cx40^{fl/fl}$:Tie2-Cre) and –76 ± 3 mV ($Cx40^{-/-}$) that was not different between genotypes. Amplitudes of the membrane potential change were likewise similar in all genotypes (Figure 3A,B). Addition of a specific $K_{Ca}3.1$ blocker (TRAM-34, 1 μmol·L^{-1}) reversed the SKA-31-induced hyperpolarization. A remaining small negative potential vanished after additional application of a specific $K_{Ca}2.3$ blocker (UCL1684, 1 μmol·L^{-1}). The membrane potential changes after addition of TRAM-34 and UCL1684 were similar in all three genotypes (Figure 3A,B). As expected for electrically coupled cells, membrane capacitance increased linearly with number of cells in clusters from $Cx40^{fl/fl}$ (P < 0.0001), but such a correlation was not found in $Cx40^{-/-}$ clusters (Figure 3C). While small clusters (≤6 cells) were electrically coupled, capacitance did not increase with cell number in larger clusters indicating weak coupling in $Cx40^{-/-}$ endothelial cells. After addition of docosahexaenoic acid (10 μmol·L^{-1}) to uncouple cells, capacitance decreased in both genotypes within 1 min to values ($Cx40^{fl/fl}$: 11 ± 1, $Cx40^{fl/fl}$: 10 ± 1 pF) corresponding to single murine endothelial cells (Schmidt *et al.*, 2010).

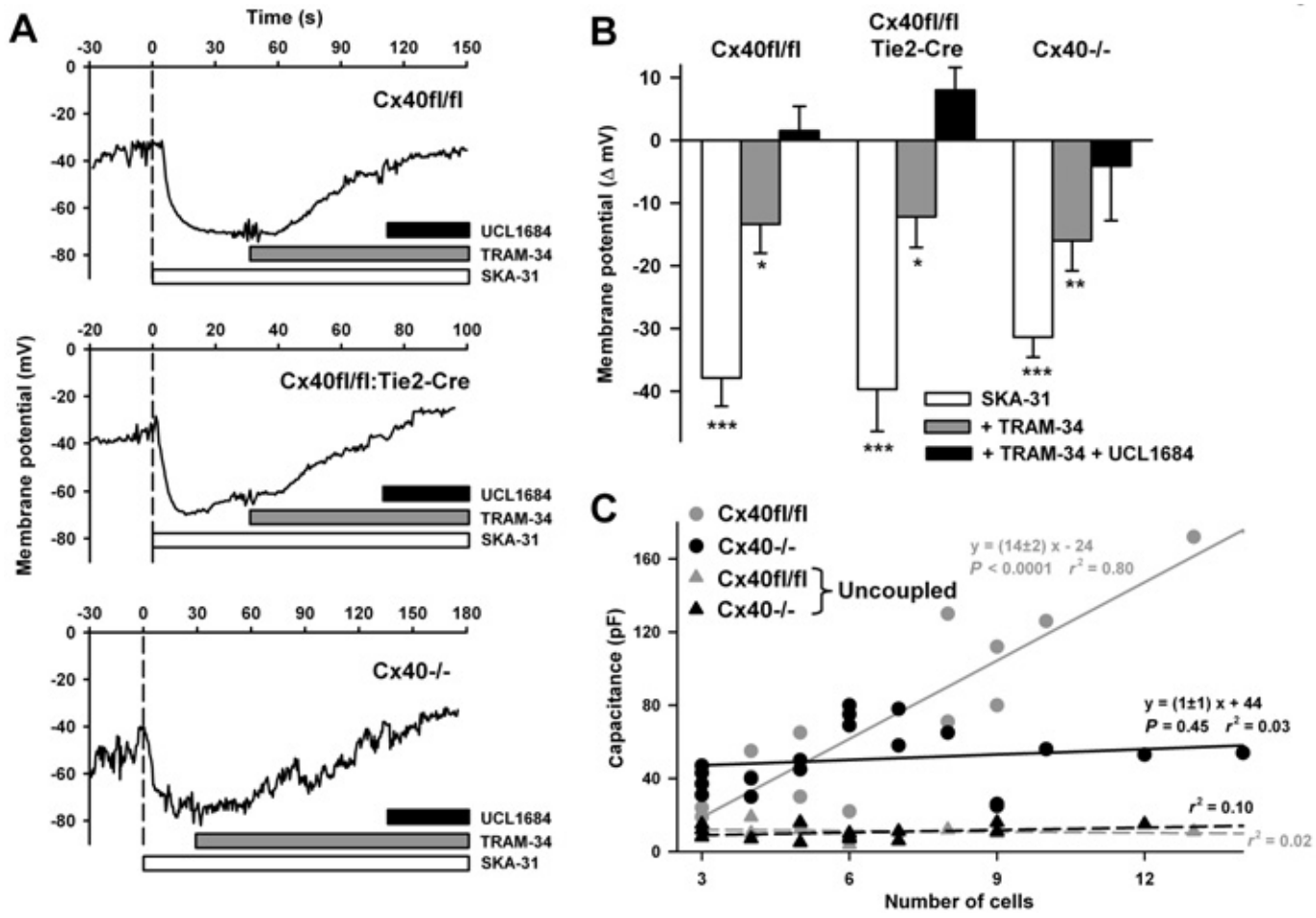

Figure 3

SKA-31-induced membrane hyperpolarization in endothelial cell clusters. SKA-31 (1 μmol·L^{-1}) hyperpolarized endothelial cells to a similar extent in $Cx40^{fl/fl}$, $Cx40^{fl/fl}$:Tie2-Cre and $Cx40^{-/-}$. This membrane potential change was largely reversed by the specific blocker of $K_{Ca}3.1$ (TRAM-34, 1 μmol·L^{-1}) and completely inhibited after additional block of $K_{Ca}2.3$ (UCL1684, 1 μmol·L^{-1}) as shown in representative traces (A) and summary data (B). (C) Capacitance is depicted as a function of cell number in the clusters showing that only in $Cx40^{fl/fl}$ (correlation coefficient, r^2 = 0.80), but not in $Cx40^{-/-}$ (r^2 = 0.03) capacitance correlated linearly with cell number in untreated cells (circles). After pharmacological uncoupling by docosahexaenoic acid (10 μmol·L^{-1}, triangles) capacitance values dropped, corresponding to a single cell and were thus independent of cell number in either genotype. The relationship was modelled by linear regression and is depicted for both genotypes in untreated cells and after uncoupling. n = 12–14 endothelial cell clusters derived from carotid arteries of three animals of each genotype; *: P < 0.05, **: P < 0.01, ***: P < 0.001 versus initial value.

Effect of SKA-31 on arterial pressure and HR in conscious Cx40-deficient mice

Five days after transmitter implantation, arterial pressure and HR was measured in conscious mice starting 30 min before and continuing for 2 h after i.p. application of SKA-31. Wild-type mice (n = 8) exhibited a systolic pressure (SP) of 128.3 ± 5.5 and a diastolic pressure (DP) of 96.4 ± 5.6 mmHg [mean arterial pressure (MAP): 109.8 ± 4.3 mmHg] at a HR of 601 ± 11 beats min^{-1}. Similar values were found in Cx40$^{fl/fl}$:Tie2-Cre mice (n = 6; SP: 125.4 ± 5.7, DP: 96.6 ± 4.4, MAP: 109.4 ± 4.3 mmHg, HR: 600 ± 23 beats min^{-1}). In marked contrast, Cx40$^{-/-}$ mice (n = 6) were hypertensive (SP: 170.3 ± 5.3; DP: 122.9 ± 2.9; MAP: 145.5 ± 3.8 mmHg; all P < 0.01 vs. other genotypes) at a similar HR of 612 ± 16 beats min^{-1}. Injection of SKA-31, i.p., at all dosages as well as injection of the vehicle induced a slight increase in pressure (Figure 4) and a transient increase in HR in all genotypes (Figures 5,6B) that most likely reflects excitement and sympathetic activation upon animal handling. At 1 and 3 mg·kg^{-1}, SKA-31 did not produce a significant change from MAP baseline in any genotype (Figure 4). SKA-31 was also without significant effect at 10 mg·kg^{-1} in WT or Cx40$^{fl/fl}$:Tie2-Cre but significantly reduced MAP in Cx40$^{-/-}$ mice (Figure 4B). At the higher dosage of 30 mg·kg^{-1}, SKA-31 lowered MAP in all genotypes starting at 20 min after injection and lasting for about 35 min (Figure 4). Absolute changes in MAP were similar in all genotypes (Figure 6A) and pressure dropped by maximally 21 ± 9 in WT, by 13 ± 4 in Cx40$^{fl/fl}$:Tie2-Cre, and by 30 ± 11 mmHg in Cx40$^{-/-}$ animals (P = 0.45 between genotypes) as assessed from individual pressure curves. At 100 mg·kg^{-1}, SKA-31 induced a more prolonged pressure drop that was similar in all genotypes and lasted for up to 120 min after injection (Figures 4,6A). The maximal pressure drop amounted to 42 ± 8 (WT), 32 ± 8 (Cx40$^{fl/fl}$:Tie2-Cre) and 32 ± 10 mmHg (Cx40$^{-/-}$, P = 0.66 between genotypes). These values were not different from the response to 30 mg·kg^{-1} except for that in Cx40$^{fl/fl}$:Tie2-Cre mice (P < 0.05 vs. 30 mg·kg^{-1}). HR decreased significantly from ~600 to 350 beats min^{-1} in all genotypes after 100 mg·kg^{-1} but remained mostly unaltered after 30 mg·kg^{-1} SKA-31 (Figures 5,6B).

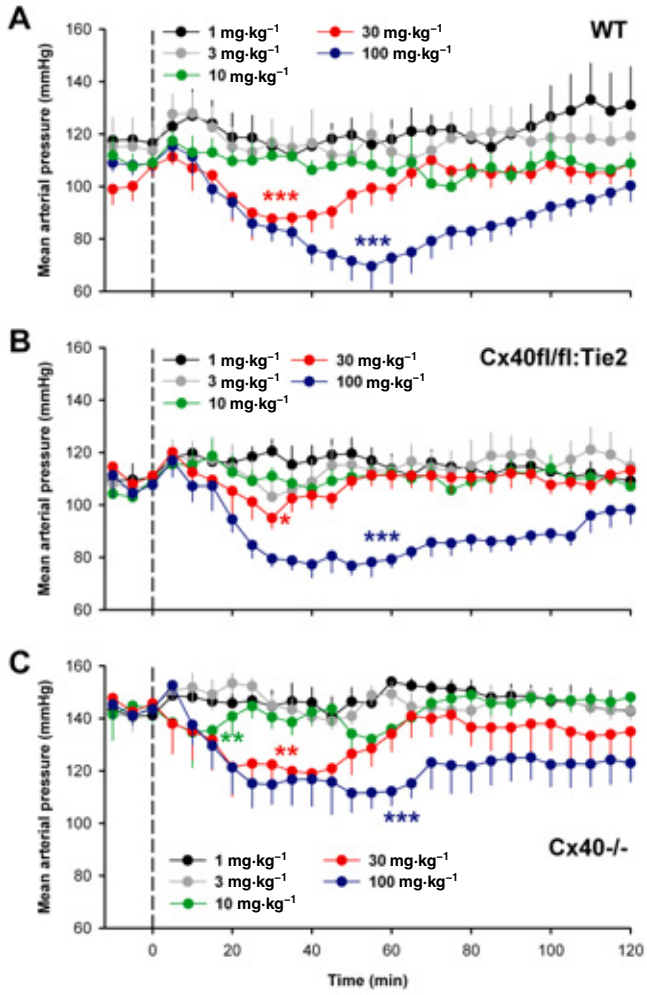

Figure 4

Mean arterial pressure measured telemetrically in conscious mice before and after i.p. application of SKA-31 (at 0 min) in WT (A), Cx40$^{fl/fl}$:Tie2-Cre (B) and Cx40$^{-/-}$ mice (C), the latter being significantly hypertensive. At low SKA-31 dosages (1–10 mg·kg^{-1}) pressure remained unchanged in all genotypes except for a significant depressor effect in Cx40$^{-/-}$ mice. SKA-31 (30 and 100 mg·kg^{-1}) lowered pressure in WT, normotensive Cx40$^{fl/fl}$:Tie2-Cre and hypertensive Cx40$^{-/-}$ mice in a similar fashion in all genotypes with a more sustained pressure drop at 100 mg·kg^{-1}. n = 6–8 mice each genotype; *P < 0.05, **P < 0.01, ***P < 0.001: indicates a significant difference from constant mean at applied dosage (univariate repeated measures ANOVA, the first two time points were excluded from analysis).

Discussion

This study demonstrated *in vivo* that: (i) the $K_{Ca}3.1/K_{Ca}2$ activator SKA-31 induces arteriolar dilatations that require the presence of $K_{Ca}3.1$ channels but not Cx40; (ii) concomitant stimulation by ACh and SKA-31 produces additive dilatory effects; (iii) SKA-31 hyperpolarizes endothelial cells and the SKA-31-induced dilatation resembles an EDH-type dilatation that is independent of Cx40; (iv) SKA-31 lowers MAP in WT mice but also in normotensive mice lacking Cx40 specifically in endothelial cells as well as in ubiquitous Cx40-deficient mice exhibiting a renin-dependent hypertension; and (v) a high dosage of SKA-31 (100 mg·kg^{-1} i.p.) lowers HR in mice. Together, these data substantiate that SKA-31 is a potent dilator of arterial resistance vessels in its own right and is capable of acutely lowering pressure in normotensive as well as chronically hypertensive animals. Because SKA-31 induces hyperpolarization in endothelial cells and dilates arterioles in a $K_{Ca}3.1$-dependent manner, we suggest that endothelial hyperpolarization *per se* is able to initiate EDH-type dilatations. The finding that arteriolar dilatations and pressure-lowering effects following local or systemic SKA-31 application were fully intact in animals lacking Cx40 suggests that dilatations initiated through endothelial $K_{Ca}3.1$ channels *in vivo* do not require myoendothelial gap junctions formed by Cx40. As a deficiency in Cx40 also concomitantly impairs the presence of Cx37 in endothelial cell membranes (Simon and McWhorter, 2003; de Wit, 2010; Jobs *et al.*, 2012), it is questionable whether EDH-type dilatations *in vivo* do indeed

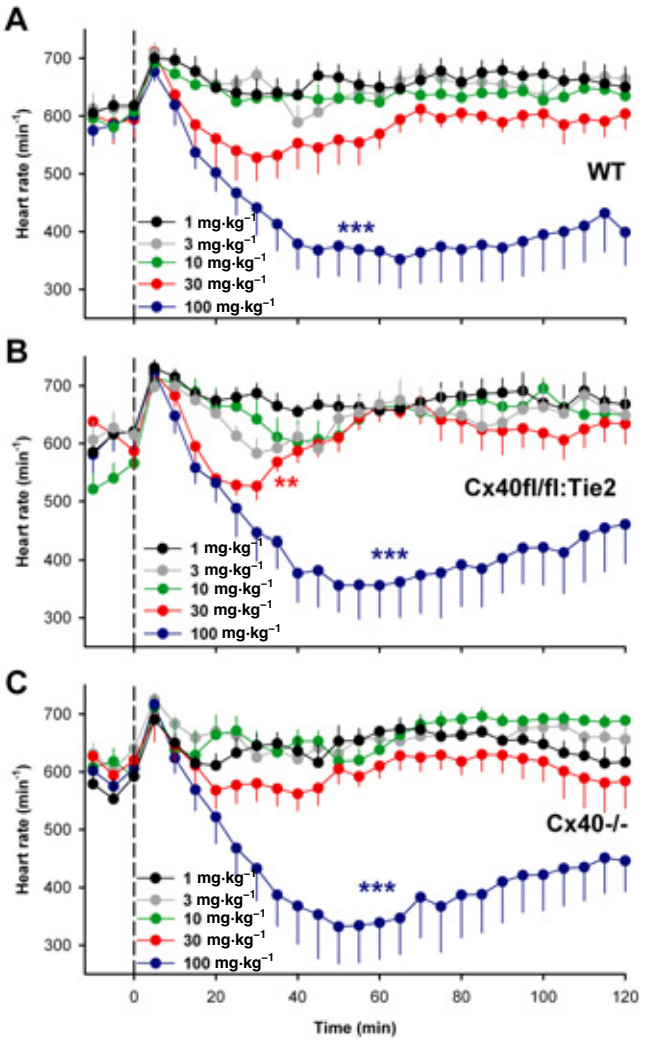

Figure 5

HR measured in conscious mice before and after i.p. application of SKA-31 (at 0 min) in WT (A), $Cx40^{fl/fl}$:Tie2-Cre (B) and $Cx40^{-/-}$ mice (C). In all genotypes at every dosage of SKA-31, a transient increase in HR was evident, most likely due to animal handling. Thus, the first two data points were excluded from statistical analysis. Low dosages of SKA-31 (1–10 mg·kg^{-1}) did not lower HR. Likewise, HR remained unchanged in WT and Cx40-/- during the measurement period after 30 mg·kg^{-1} SKA-31. However, in $Cx40^{fl/fl}$:Tie2-Cre animals a significant change was evident (a decrease followed by an increase). The highest dosage of SKA-31 (100 mg·kg^{-1}) depressed HR in all genotypes similarly. n = 6–8 mice for each genotype; $^{**}P < 0.01$, $^{***}P < 0.001$: indicates a significant difference from constant mean at applied dosage (univariate repeated measures ANOVA). Evidence for non-parallel curves between genotypes in response to a single dosage was not found (repeated measures ANOVA, at least $P = 0.21$).

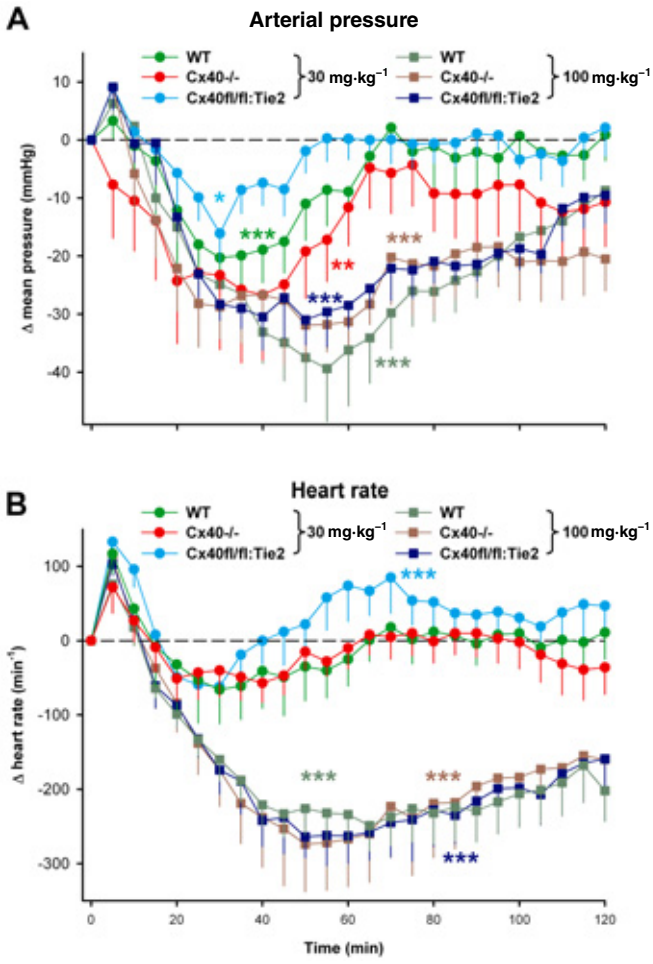

Figure 6

Amplitudes of changes in mean arterial pressure (A) and heart rate (B) by SKA-31 (30 and 100 mg·kg^{-1}) in WT, $Cx40^{fl/fl}$:Tie2-Cre and $Cx40^{-/-}$ mice. The amplitudes of pressure changes were similar in different genotypes for each SKA-31 dosage. Note that heart rate increased during the first 10 min (most likely caused by animal handling) and returned to baseline at the dosage of 30 mg·kg^{-1}. SKA-31 at 100 mg·kg^{-1} decreased heart rate significantly in all genotypes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: indicates a significant difference from constant mean at the applied dosage (univariate repeated measures ANOVA, the first two time points were excluded from analysis). Evidence for non-parallel curves between genotypes was not found (repeated measures ANOVA, at least $P = 0.41$).

require myoendothelial gap junctions, as suggested from results of *in vitro* studies (Mather *et al.*, 2005).

SKA-31 activates $K_{Ca}3.1$ channels with an EC_{50} value of 0.26 µmol·L^{-1} and $K_{Ca}2.3$ channels with a 10-fold lower potency (2.9 µmol·L^{-1}). In the murine vasculature, both channels are selectively expressed in endothelial cells (Brähler *et al.*, 2009; Potocnik *et al.*, 2009). Up to now, *in vitro* studies have shown that SKA-31 enhances ACh-induced dilatation of murine carotid arteries and of canine mesenteric arteries in the presence of a vasospasm agent (Sankaranarayanan *et al.*, 2009; Damkjaer *et al.*, 2012). However, in these vessels SKA-31 was not a vasodilator in its own right. Herein, we provide the first evidence that SKA-31 *per se* is capable of dilating resistance vessels in skeletal muscle. This arteriolar dilatation is strictly dependent on $K_{Ca}3.1$ because it was absent in $K_{Ca}3.1^{-/-}$ mice. The SKA-31-mediated dilatation was potent, amounting to about 40% of the vessel's maximal diameter, but did not reach the maximal levels achieved on stimulation with ACh (range of 75%) (Koeppen *et al.*, 2004; Wölfle and de Wit, 2005). This lower efficacy of SKA-31 may

be due to its limited access to the endothelial cells when it is applied with the superfusion solution onto the cremaster muscle and this may also explain the need for higher concentrations compared with the reported EC_{50} values of 0.26 μmol·L^{-1}, which were determined by measuring channel activity in transfected HEK293 cells or isolated endothelial cells. The SKA-31-induced dilatation is of a similar magnitude to those reported for the less selective activator of K_{Ca} DCEBIO, which also induces dilatations in a K_{Ca}3.1-dependent manner (Wölfle *et al.*, 2009). The present experiments demonstrate that the SKA-31-initiated response does not require NO or prostaglandins and that it therefore can be deemed a pure EDH-type dilatation (Figure 1). Nonetheless, it is possible that SKA-31 does increase NO production, as reported for rat arteries (Sheng *et al.*, 2009; Stankevicius *et al.*, 2011). However, if this is so, similar to the responses to ACh, this NO does not seem to contribute to the SKA-31-induced dilatation in murine arterioles in the *in vivo* experiments presented here (Koeppen *et al.*, 2004).

The combined application of SKA-31 and ACh induced additive effects evident at lower but not at higher concentrations at which a solid hyperpolarization to near the K^+ equilibrium limits the dilator response. Despite the effects being mainly additive, SKA-31 may still restore endothelial function in situations where the dilatation induced by ACh is impaired (Grgic *et al.*, 2009; Kohler *et al.*, 2010) or when the K_{Ca}3.1-mediated pathway is up-regulated (Simonet *et al.*, 2012), because SKA-31 has been shown to potentiate ACh-induced dilatations in other species and vascular beds without exhibiting a dilator effect in itself (Sankaranarayanan *et al.*, 2009; Damkjaer *et al.*, 2012). From a more methodological perspective, SKA-31's efficacy at producing arteriolar dilatation without the need to stimulate GPCRs may render it a novel pharmacological tool to investigate pure EDH-type dilator responses and related electrical mechanisms of dilatation such as direct charge transfer through myoendothelial coupling involving Cx40, as reported for rat small mesenteric arteries (Mather *et al.*, 2005).

A major aim of the present study was to determine whether Cx40 is required for K_{Ca}3.1-mediated arteriolar dilatations. Our results clearly show that arteriolar dilatations induced by SKA-31 were not attenuated in mice lacking Cx40 in endothelial cells. We therefore conclude that K_{Ca}3.1-mediated endothelial hyperpolarization *per se* is able to induce dilatation even in the absence of endothelial Cx40, thus excluding the possibility that Cx40-dependent myoendothelial gap junctions are a prerequisite for K_{Ca}3.1-mediated (resembling EDH-type) dilatations in murine skeletal muscle arterioles, as was reported for rat mesenteric vessels (Mather *et al.*, 2005). We further suggest that EDH-type dilatations may be even completely independent of myoendothelial gap junctions because Cx40-deficient endothelial cells also exhibit a significantly reduced expression of Cx37 (Simon and McWhorter, 2003; de Wit, 2010; Jobs *et al.*, 2012). In fact, we demonstrate herein for the first time a substantial impairment of electrical coupling in endothelial cell clusters derived from Cx40$^{-/-}$ carotid arteries (Figure 3C). The fact that the non-specific gap junction blocker carbenoxolone did not affect SKA-31-induced dilatation further supports the hypothesis that these dilatations do not require gap junctional coupling. This conclusion is also corroborated by our previous observations in Cx40-deficient animals, which exhibited intact ACh-induced EDH-type dilatations in the *in vivo* setting (de Wit *et al.*, 2003; Boettcher and de Wit, 2011). We therefore suggest that mechanisms other than the mere transmission of an endothelial hyperpolarization towards the vascular smooth muscle need to be re-evaluated to explain EDH-type dilator responses, at least in murine skeletal arterioles.

I.p. injections of SKA-31 have been reported to lower MAP, after 24 h, by approximately 5 mmHg in normotensive mice and by about 12 mmHg in angiotensin-II-infused hypertensive mice, which requires the presence of K_{Ca}3.1 (Sankaranarayanan *et al.*, 2009), while i.v. SKA-31 produced an immediate and strong but transient depressor response in conscious dogs (Damkjaer *et al.*, 2012). In the present study, we provide further insights into the time course of SKA-31's systemic cardiovascular actions. Administration of 30 and 100 mg·kg^{-1} SKA-31 lowered pressure by 20–32 mmHg within 2 h after i.p. injection in normo- and hypertensive genotypes. Lower concentrations of 1 and 3 mg·kg^{-1} had no effect while 10 mg·kg^{-1} only produced a depressor response in hypertensive Cx40-deficient mice. It is most likely that these depressor responses were elicited by a decrease in peripheral resistance due to dilatation of arterioles, as observed in the microcirculation. The time course of the SKA-31 effects is compatible with reported plasma concentrations of SKA-31, which peak 2 h after a single i.p. injection (Sankaranarayanan *et al.*, 2009). The pressure drop was not accompanied by significant changes in HR in WT mice at 30 mg·kg^{-1} SKA-31, suggesting that neither the pressure drop was due to a change in HR nor that the return to baseline was driven predominantly by a reflex increase in HR. In fact, unlike dogs (Damkjaer *et al.*, 2012), mice seem to lack reflex tachycardia to SKA-31-induced depressor responses. However, other slow counterbalancing regulatory effects may have possibly offset a more prolonged reduction in pressure, for example, an increase in stroke volume or other humoral mechanisms such as increased catecholamine secretion or activation of the renin-angiotensin-aldosterone system.

In normotensive endothelial-cell-specific Cx40-deficient animals, SKA-31 induced a comparable decrease in arterial pressure starting at a dosage of 30 mg·kg^{-1} without a drop in HR. In fact, HR was moderately enhanced in this group. Hypertensive ubiquitous Cx40-deficient mice responded in a similar fashion to 30 mg·kg^{-1} and even to 10 mg·kg^{-1} without changes in HR. Thus, SKA-31 decreases blood pressure independently of endothelial Cx40 and, most interestingly, is also effective in a model of chronic hypertension related to a renin excess and chronic activation of the renin-angiotensin-aldosterone system (de Wit *et al.*, 2003; Wagner *et al.*, 2007; Schweda *et al.*, 2009).

Higher concentrations of SKA-31 (100 mg·kg^{-1}) exerted a strong and long-lasting depressor response but this was accompanied by a significant slowing of HR. To our knowledge, KCa3.1 channels are not expressed in sinus node cells or pacemaker tissue and are not implicated in pacemaker functions. However, mRNA encoding for K_{Ca}2 channels has been identified in cardiac atrial and ventricular myocytes (Tuteja *et al.*, 2010). Accordingly, cardiac overexpression of K_{Ca}2.2 channels shortened the action potential in pacemaker tissue and enhanced firing rate and, conversely, their ablation

decreased spontaneous firing (Zhang *et al.*, 2008), suggesting that modulation of $K_{Ca}2$ channels may affect electrical behaviour in pacemaker tissue. However, the consequent effects on pacemaker tissue induced by pharmacological stimulation of $K_{Ca}2$ channels may differ from those resulting from genetic overexpression of these channels, because overexpressed $K_{Ca}2$ channels still respond to physiological stimuli. Although speculative, SKA-31 could potentiate the activity of $K_{Ca}2$ channels, which would keep the pacemaker tissue in a hyperpolarized state that leaves pacemaker cells further away from the threshold to fire an action potential and, ultimately, decrease HR. Additionally, a central sedative effect with a resulting decrease in sympathetic drive may add to the slowing of the HR, as $K_{Ca}2$ channels induce an afterhyperpolarization in neurons. As SKA-31 is able to penetrate the brain, activation of these channels may induce inhibition of neural activation. In fact, SKA-31 exerts a sedative effect at higher dosages, which is independent of $K_{Ca}3.1$ because it was also observed in animals deficient in $K_{Ca}3.1$ (Lambertsen *et al.*, 2012). Thus, we suggest that the bradykardia observed at the highest SKA-31 dosage in mice is probably due to activation of $K_{Ca}2$ channels through an effect on pacemaker tissue in conjunction with a potential sedative effect. However, this view cannot be confirmed from the telemetric pulse wave recordings obtained in the present study, but our findings do demonstrate that specific activation of $K_{Ca}3.1$ channels is desirable to induce dilatation and a pressure decrease in mice *in vivo*.

In conclusion, the endothelial $K_{Ca}3.1$ channel provides an attractive pharmacological target to initiate EDH-type dilatations. Upon activation, endothelial cells hyperpolarize and induce an arteriolar dilatation that is independent of NO and prostaglandins. *In vivo*, this dilatation surprisingly does not require the presence of Cx40 in endothelial cells, suggesting that in arterioles myoendothelial gap junctions either do not transfer the hyperpolarization from the endothelium to the underlying smooth muscle or that they do so without the need for Cx40. The finding that $K_{Ca}3.1$ activation can still induce EDH-type dilatations and lower pressure in Cx40-deficient mice, which exhibit severe chronic renin-dependent hypertension, suggests that $K_{Ca}3.1$ activators like SKA-31 should be evaluated as novel treatment options for severe renal hypertension.

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 ACh induced a concentration-dependent dilatation during inhibition of NO synthase and COX (LN and Indo, 3 and 30 μmol·L^{-1}) in arterioles of wild-type mice. SKA-31 applied alone dilated these arterioles by 8 ± 3% (3 μmol·L^{-1}) and 38 ± 5% (30 μmol·L^{-1}). Combined application of ACh and SKA-31 (A: 3 μmol·L^{-1}; B: 30 μmol·L^{-1}) induced a significantly stronger dilatation that was comparable to the sum of the dilatations initiated by each substance alone (expected dilatation, dashed line) with the exception of high ACh concentrations. A: n = 57–65 arterioles in 7 mice, B: n = 24 arterioles in 3 mice; *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus control.

Figure S2 SKA-31 induced a concentration dependent dilatation during inhibition of NO synthase and COX (LN and Indo, 3 and 30 μmol·L^{-1}) in arterioles of wild-type mice that was unaffected in the presence of the non-specific gap junction blocker carbenoxolone (Cbx, 30 μmol·L^{-1}). n = 16 arterioles in 5 mice, arterioles that exhibited a very low resting tone (i.e. ratio of resting to maximal diameter > 0.8, n = 24) in the presence of Cbx were excluded from analysis.