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

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Running title: Cx40-independent dilation upon $K_{Ca}3.1$ activation

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Abbreviations:

ACh	Acetylcholine
K_{Ca}	Ca^{2+}-dependent K^+-channel
EDH	Endothelium-derived hyperpolarization
Cx40	Connexin40
EDHF	Endothelium-derived hyperpolarizing factor
L-NA	N^G-nitro-*L*-arginine
SP	Systolic pressure
MAP	Mean arterial pressure
DP	Diastolic pressure
HR	Heart rate
wt	wild-type

Keywords: Myoendothelial coupling, SKA-31 (Naphtho[1,2-*d*]thiazol-2-ylamine), Ca^{2+}-activated K^+ channel, hypertension, gap junction, microcirculation, endothelium-derived hyperpolarizing factor

Summary

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 dilations. We here 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 dilations 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 naphtho[1,2-*d*]thiazol-2-ylamine (SKA-31) induced pronounced concentration-dependent arteriolar EDH-type dilations amounting to ~40% of maximal dilation and enhanced acetylcholine effects. These responses were absent in mice devoid of $K_{Ca}3.1$ channels. In contrast, SKA-31-induced dilations were not attenuated in mice deficient for Cx40 in endothelial cells ($Cx40^{fl/fl}$:Tie2-Cre). In isolated endothelial cell clusters, SKA-31 induced hyperpolarizations of similar magnitudes (by ~38mV) 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, intraperitoneal injection of SKA-31 (30 and 100 mg/kg) decreased arterial pressure by ~32 mmHg in all genotypes. The depressor response was associated with a decrease in heart rate at 100 mg/kg SKA-31.

Conclusions and Implications. We conclude that an endothelial hyperpolarization through pharmacological activation of $K_{Ca}3.1$ channels induces EDH-type arteriolar dilations that are independent of endothelial Cx40 and Cx40-containing myoendothelial gap junctions. Since SKA-31 reduces blood pressure in hypertensive Cx40-deficient mice, $K_{Ca}3.1$ activators may be useful in severe treatment-resistant hypertension.

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, nitric oxide (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. Although it was initially assumed that this mechanism acts likewise through the release of a diffusible endothelial factor (endothelium-derived hyperpolarizing factor, EDHF) this view was later questioned by experiments demonstrating the involvement of gap junctions (Griffith *et al.*, 2002; Dora *et al.*, 2003; Mather *et al.*, 2005; Chaytor *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; Grgic *et al.*, 2009; Feletou and Vanhoutte, 2009; Heberlein *et al.*, 2009; Edwards *et al.*, 2010; de Wit and Griffith, 2010; Garland *et al.*, 2011)]. Whatever the exact mechanism is, endothelial hyperpolarization has been demonstrated to be crucial for the capability of agonists to induce EDH-type dilations in multiple studies.

Endothelial hyperpolarization following activation of GPCRs like muscarinergic (acetylcholine, ACh) receptors requires Ca^{2+}-release from the ER. The resulting increase of intracellular Ca^{2+} produces activation of 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 dilation in many vessels (Si *et al.*, 2006), while $K_{Ca}2.3$ channels are possibly responsible for flow-induced dilation and active hyperemia (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. Confirming a pivotal role of $K_{Ca}3.1$ channels, genetic deficiency of $K_{Ca}3.1$ in mice abrogates ACh-induced EDH-type vasodilation in large conduit arteries and in the cremaster microcirculation *in vivo* and - at the systemic level - results in mild systolic hypertension (Si *et al.*, 2006). Intriguingly, pharmacological activation of this channel by the $K_{Ca}3.1$/$K_{Ca}2$ activator naphtho[1,2-*d*]-2-ylamine (SKA-31), a compound with a ten-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, intravenous injection of SKA-31 has also been shown to transiently lower pressure in conscious dogs (Damkjaer *et al.*, 2012). In addition to nitric oxide, $K_{Ca}3.1$ (and $K_{Ca}2.3$) channels accordingly constitute promising novel pharmacological targets for lowering peripheral vascular resistance in hypertension or ischemic heart disease.

However, it is not clear whether pharmacological activation of $K_{Ca}3.1$ produces vasodilation of arterioles and thereby elicits a depressor response and whether myoendothelial gap junctions are involved in such dilations, in analogy to the suggested role of gap junctions in EDH-type dilations 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; Isakson *et al.*, 2008). Recently, we confirmed a role for Cx40-dependent myoendothelial coupling in ACh-induced EDH-dilations in an isometric experimental setting *in vitro*, whereas *in vivo* Cx40 seemed dispensable for EDH-type dilations in small arteries (Boettcher and de Wit,

2011). Therefore, we hypothesized, that activation of $K_{Ca}3.1$ induces dilations 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; de Wit *et al.*, 2003) due to enhanced secretion of renin and activation of the renin-angiotensin-aldosterone system (Wagner *et al.*, 2007; Wagner *et al.*, 2010), while endothelial-specific Cx40-deficient mice are normotensive (Wagner *et al.*, 2010; Chadjichristos *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 approved by local authorities. Mice 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 control littermates were derived from our breeding colonies.

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 µmol/L) was added to the bath solution followed by adding the selective $K_{Ca}3.1$ blocker TRAM-34 (1 µmol/L) (Wulff *et al.*, 2000) and the $K_{Ca}2.X$ blocker UCL1684 (1 µmol/L) (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 docosahexaenoic acid (Schmidt *et al.*, 2010).

Intravital microscopy of the microcirculation:

Mice were anesthetized with intraperitoneal injection of fentanyl, midazolam, and medetomidin followed by intravenous infusion. The cremaster muscle was prepared as described (de Wit, 2010). Arteriolar diameters were measured before and during superfusion of SKA-31 (1–30 µmol/L, dissolved at 20 mmol/L in CremophorEL). This protocol was repeated during superfusion of N^G-nitro-*L*-arginine (L-NA, 30 µmol/L) and indomethacin (indo, 3 µmol/L) to block NO-synthase and cyclooxygenase. In a subset of experiments, dilations induced by SKA-31 were studied before and after addition of the nonspecific gap

junction blocker carbenoxolone (30 µmol/L) during L-NA and indomethacin. In a second protocol, SKA-31 (3, 30 µmol/L) and ACh (0.03-10 µmol/L) 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) before sacrificing the animal by pentobarbital.

Blood pressure measurement:

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

Statistics and calculations:

Data within groups were compared using paired t-tests and between different groups by analysis of variance (one-way ANOVA) followed by the Bonferroni post hoc test. Time series measurements (pressure, heart rate) 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 supplement.

Results

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

The necessity of K_{Ca}3.1 for SKA-31 induced arteriolar dilation was studied by intravital microscopy in cremaster muscle arterioles in K_{Ca}3.1-deficient mice ($K_{ca}3.1^{-/-}$) and wild-type (wt) littermates (n=5 each genotype). The maximal diameter of the arterioles at the end of the experiment by combining 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 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 dilation in wt arterioles that was significantly larger than the subtle diameter changes triggered by superfusing the solvent (cremophor) alone (Fig.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 dilation was unchanged (Fig.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 neither in untreated preparations nor after inhibition of NO and prostaglandins (Fig.1). However, $K_{Ca}3.1^{-/-}$ arterioles dilated considerably upon superfusion of adenosine (3 µmol/L: 27±5%; 10 µmol/L: 74±4%) or the exogenous NO-donor sodium-nitroprusside (10 µmol/L: 39±3%) excluding a general non-responsiveness. These responses were not attenuated after pretreatment with L-NA/indomethacin and were comparable to dilations in wt mice. Together, these results demonstrate that arteriolar dilations induced by SKA-31 critically depend on the presence of K_{Ca}3.1.

In a different series of wild-type animals the effects of SKA-31 on EDH-type dilations induced by ACh were investigated. In the presence of L-NA and indomethacin, ACh induced

a concentration-dependent dilation in arterioles with a maximal diameter of 34±1 µm (81 arterioles in 10 mice). SKA-31 alone induced a dilation of 8±3% (3 µmol/L) and 38±5% (30 µmol/L) in this series. Combined superfusion of ACh and SKA-31 (3 or 30 µmol/L) induced dilations that were significantly stronger than dilations induced by ACh alone suggesting an additive effect of these dilators except for the highest concentrations of ACh used (Fig.S1).

SKA-31 induced arteriolar dilations 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 dilations 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 dilated arterioles in $Cx40^{fl/fl}$ controls concentration-dependently (Fig.2A). This dilation was not attenuated by pretreatment with L-NA and indomethacin (Fig.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 dilation that was not attenuated compared to $Cx40^{fl/fl}$ (Fig.2A). In fact, the dilation was stronger at one concentration of SKA-31 (3 µmol/L). In $Cx40^{fl/fl}$:Tie2-Cre, SKA-31 dilations were also not attenuated by L-NA and indomethacin and responses were indistinguishable between $Cx40^{fl/fl}$ and $Cx40^{fl/fl}$:Tie2-Cre (Fig.2B). The vascular effects of the solvent cremophor were negligible at all conditions investigated (Fig.2). These results suggest that the dilation 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 wild-type mice (n=40 arterioles in n=5 mice) the effect of the nonspecific gap junction blocker carbenoxolone (Cbx, 30 µmol/L) on SKA-31 induced dilation was studied in the presence of L-NA and indomethacin. Cbx itself caused dilations 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 dilations remained unaffected (Fig.S2) indicating that these dilation were independent of gap junctional coupling. Likewise, dilations upon ACh and sodium-nitroprusside were not attenuated (3 µmol/L, 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 3-4 animals in each genotype, P=0.15). From this level, 1 µmol/L 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 (Fig.3A,B). Addition of a specific $K_{Ca}3.1$ blocker (TRAM-34, 1 µmol/L) 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). The membrane potential changes after addition of TRAM-34 and UCL1684 were similar in all 3 genotypes (Fig.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 (Fig.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) to uncouple cells, capacitance decreased in both genotypes within 1 minute to values ($Cx40^{fl/fl}$: 11±1, $Cx40^{fl/fl}$: 10±1 pF) corresponding to single murine endothelial cells (Schmidt *et al.*, 2010).

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

Five days after transmitter implantation arterial pressure and heart rate was measured in conscious mice starting 30 min before and continuing for 2 h after intraperitoneal 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 heart rate (HR) of 601±11 bpm. 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 bpm). 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 bpm. Intraperitoneal injection of SKA-31 at all dosages as well as injection of the vehicle induced a slight increase in pressure (Fig.4) and a transient increase in heart rate in all genotypes (Fig.5, Fig.6B) that most likely reflects excitement and sympathetic activation upon animal handling. At 1 and 3 mg/kg, SKA-31 did not produce a significant change from MAP baseline in any genotype (Fig.4). SKA-31 was also without significant effect at 10 mg/kg in WT or $Cx40^{fl/fl}$:Tie2-Cre but significantly reduced MAP in $Cx40^{-/-}$ mice (Fig.4B). At the higher dosage of 30 mg/kg, SKA-31 lowered MAP in all genotypes starting at 20 min after injection and lasting for about 35 min (Fig.4). Absolute changes in MAP were similar in all genotypes (Fig.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 SKA-31 induced a more prolonged pressure drop that was similar in all genotypes and lasted for up to 120 min after injection (Fig.4, Fig.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 except for $Cx40^{fl/fl}$:Tie2-Cre mice (P<0.05 vs. 30 mg/kg). HR decreased significantly from ~600 to 350 bpm in all genotypes after 100 mg/kg but remained mostly unaltered after 30 mg/kg SKA-31 (Fig.5, Fig.6B).

Discussion

This study demonstrates *in vivo* that: 1) the $K_{Ca}3.1/K_{Ca}2$ activator SKA-31 induces arteriolar dilations that require the presence of $K_{Ca}3.1$ channels but not Cx40; 2) concomitant stimulation by ACh and SKA-31 produces additive dilatory effects; 3) SKA-31 hyperpolarizes endothelial cells and the SKA-31-induced dilation resembles an EDH-type dilation that is independent of Cx40; 4) SKA-31 lowers mean arterial pressure in wild-type 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; 5) a high dosage of SKA-31 (100 mg/kg i.p.) lowers heart rate 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. Since 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 dilations. The fact that arteriolar dilations and pressure lowering effects following local or systemic SKA-31 application are fully intact in animals lacking Cx40, suggests that dilations initiated through endothelial $K_{Ca}3.1$ channels *in vivo* do not require myoendothelial gap junctions formed by Cx40. Since deficiency of Cx40 concomitantly impairs the presence of Cx37 in endothelial cell membranes (Simon and McWhorter, 2003; de Wit, 2010; Jobs *et al.*, 2012) it is therefore questionable whether EDH-type dilations *in vivo* do indeed require myoendothelial gap junctions as *in vitro* studies have suggested (Mather *et al.*, 2005).

SKA-31 activates $K_{Ca}3.1$ channels with an EC_{50} value of 0.26 µmol/L and $K_{Ca}2.3$ channels with a ten-fold lower potency (2.9 µmol/L). 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 dilation of murine carotid arteries and of canine mesenteric arteries in the presence of a vasospasmic 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 dilation is strictly $K_{Ca}3.1$-dependent since it was absent in $K_{Ca}3.1^{-/-}$ mice. The SKA-31 mediated dilation was potent, amounting to about 40% of the vessel's maximal diameter, but did not reach the maximal levels achieved by ACh stimulation (range of 75%) (Koeppen *et al.*, 2004; Wölfle and de Wit, 2005). This lower efficacy for SKA-31 may be due to its limited access to the endothelial cells when applied with the superfusion solution onto the cremaster muscle and may also explain the need for higher concentrations compared to the reported EC_{50} values of 0.26 µmol/L determined by measuring channel activity in transfected HEK293 cells or isolated endothelial cells. The SKA-31 induced dilation is of a similar magnitude as those reported for the less selective activator of K_{Ca} activator DCEBIO which also induces dilations 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 dilation (Fig.1). Nonetheless, it may well be, that SKA-31 increases NO production as reported for rat arteries (Sheng *et al.*, 2009; Stankevicius *et al.*, 2011). However, if so, this does not seem to contribute to the dilation in murine arterioles in the *in vivo* experiments presented here as is true for stimulation using ACh (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 of impaired ACh-dilation (Grgic *et al.*, 2009; Kohler *et al.*, 2010) or in case of an upregulated $K_{Ca}3.1$-mediated pathway (Simonet *et al.*, 2012) because SKA-31 potentiated ACh-induced dilations also in other species or 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 dilation without the need to stimulate G-protein coupled receptors may render it a novel pharmacological tool to investigate pure EDH-type dilations and related electrical mechanisms of dilation 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 dilations. Our results clearly show that arteriolar dilations 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 dilation even in the absence of endothelial Cx40 thus excluding that Cx40-dependent myoendothelial gap junctions are a prerequisite for $K_{Ca}3.1$-mediated (resembling EDH-type) dilations in murine skeletal muscle arterioles as was reported for rat mesenteric vessels (Mather *et al.*, 2005). We further suggest that EDH-type dilations may be even completely independent of myoendothelial gap junctions, since Cx40-deficient endothelial cells also exhibit strongly reduced Cx37 expression (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 (Fig.3C). The fact that the nonspecific gap junction blocker carbenoxolone did not affect SKA-31 dilations supports further the idea that these dilations do not require gap junctional coupling. This conclusion is also corroborated by our previous observations in Cx40-deficient animals which exhibit intact ACh-induced EDH-type dilations in the *in vivo* setting (de Wit *et al.*, 2003; Boettcher and de Wit, 2011). We therefore suggest that other mechanisms than the mere transmission of an endothelial hyperpolarization towards the vascular smooth muscle need to be reevaluated to explain EDH-type dilator responses at least in murine skeletal arterioles.

Intraperitoneal injections of SKA-31 has been previously reported to lower 24h mean arterial pressure 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 intravenous 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 about the time course of SKA-31´s systemic cardiovascular actions. Administration of 30 and 100 mg/kg SKA-31 lowered pressure by 20 to 32 mmHg within 2h after intraperitoneal injection in normo- and hypertensive genotypes. Lower concentrations of 1 and 3 mg/kg had no effect, while 10 mg/kg only produced a depressor response in hypertensive Cx40-deficient mice. Most likely, these depressor responses were elicited by a decrease of peripheral resistance due to dilation 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 2h after a single intraperitoneal injection (Sankaranarayanan *et al.*, 2009). The pressure drop was not accompanied by significant changes in heart rate in wild-type mice at 30 mg/kg SKA-31 suggesting that neither the pressure drop was due to a change in heart rate nor that the return to baseline is driven predominantly by a reflective increase in heart rate. 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, e.g. 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 dose of 30 mg/kg without a drop in heart

rate. In fact, heart rate was moderately enhanced in this group. Hypertensive ubiquitous Cx40-deficient mice responded in a similar fashion to 30 mg/kg and even at 10 mg/kg without changes in heart rate. 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) exerted a strong and long-lasting depressor response but this was accompanied by a significant slowing of heart rate. To our knowledge K_{Ca}3.1-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 shortened the action potential in pacemaker tissue and enhanced firing rate and, conversely, its ablation decreased spontaneous firing (Zhang *et al.*, 2008) suggesting that K_{Ca}2 channel modulation may affect electrical behaviour in pacemaker tissue. Overexpressed K_{Ca}2 is still responsive to physiological regulation and consequences are different from phramacological channel activation. Although speculative, the potentiated activity of K_{Ca}2 channels by SKA-31 may 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 heart rate. Additionally, a central sedative effect with a resulting decrease of sympathetic drive may add to the slowing of the heart rate. K_{Ca}2 channels underlie the medium afterhyperpolarization in neurons and SKA-31 is brain penetrant. In fact, SKA-31 exerts a sedative effect at higher doses independent of K_{Ca}3.1 since it was also observed in animals deficient for K_{Ca}3.1 (Lambertsen *et al*., 2012). Thus, we suggest that the bradykardia observed at the highest SKA-31 dose in mice is likely due to activation of K_{Ca}2 channels through an effect on pacemaker tissue in conjunction with a potential sedative effect.

However, telemetric pulse wave recordings as conducted in the present study cannot resolve these questions, but our findings highlight the fact that specific activation of $K_{Ca}3.1$ is desirable to induce dilation and pressure decrease.

In conclusion, the endothelial $K_{Ca}3.1$ channel provides an attractive pharmacological target to initiate EDH-type dilations. Upon activation, endothelial cells hyperpolarize and induce an arteriolar dilation that is independent of NO and prostaglandins. *In vivo*, this dilation 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 fact that $K_{Ca}3.1$ activation can still induce EDH-type dilations 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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Figure 1

Concentration-dependent arteriolar dilation upon superfusion of SKA-31 in wild-type (WT) mice before (Control, A) and after L-NA and indomethacin treatment (3 and 30 µmol/L, LN+Indo, B). In marked contrast, SKA-31 did not dilate arterioles in $K_{Ca}3.1$-deficient mice ($K_{ca}3.1^{-/-}$). The solvent (cremophor) did not change diameters significantly. WT: n=42 arterioles in 5 animals, $K_{Ca}3.1^{-/-}$: n=45 arterioles in 5 animals; *: P<0.05, **: P<0.01, ***: P<0.001 vs. solvent, #: P<0.05, ###: P<0.001 vs. $K_{Ca}3.1^{-/-}$, paired and unpaired t-test, respectively.

Figure 2

SKA-31 induced arteriolar dilations in mice deficient for Cx40 in endothelial cells ($Cx40^{fl/fl}$:Tie2-Cre) without (Control, A) and after blockade of NO-synthase and cyclooxygenase (LN+Indo, B). These dilations were not attenuated compared to controls ($Cx40^{fl/fl}$). Inhibition of NO-synthase and cyclooxygenase did not attenuate the dilations in both genotypes. The solvent (cremophor) was without dilatory 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 vs. solvent, ##: P<0.01 vs. $Cx40^{fl/fl}$:Tie2-Cre, paired and unpaired t-test, respectively.

Figure 3

SKA-31-induced membrane hyperpolarization in endothelial cell clusters. SKA-31 (1 µmol/L) hyperpolarized endothelial cells to a similar extend 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) and completely abrogated after additionally blocking of $K_{Ca}2.3$ (UCL1684, 1 µmol/L) 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, triangles) capacitance values dropped, corresponded 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 either genotype in untreated cells and after uncoupling. n=12-14 endothelial cell clusters derived from carotid arteries of 3 animals of each genotype; *: P<0.05, **: P<0.01, ***: P<0.001 vs. initial value.

Figure 4

Mean arterial pressure measured telemetrically in conscious mice before and after intraperitoneal application of SKA-31 (at 0 min) in wild-type (A: WT), $Cx40^{fl/fl}$:Tie2-Cre (B), and $Cx40^{-/-}$ mice(C), the latter being significantly hypertensive. At low SKA-31 dose (1 to 10 mg/kg) pressure remained unchanged in all genotypes except for a significant depressor effect in $Cx40^{-/-}$ mice. SKA-31 (30 and 100 mg/kg) 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. n=6-8 mice each genotype; *, **, ***: indicates a significant difference (P<0.05, P<0.01, P<0.001, respectively) from constant mean at applied dosage (univariate repeated measures ANOVA, the first 2 time points were excluded from analysis).

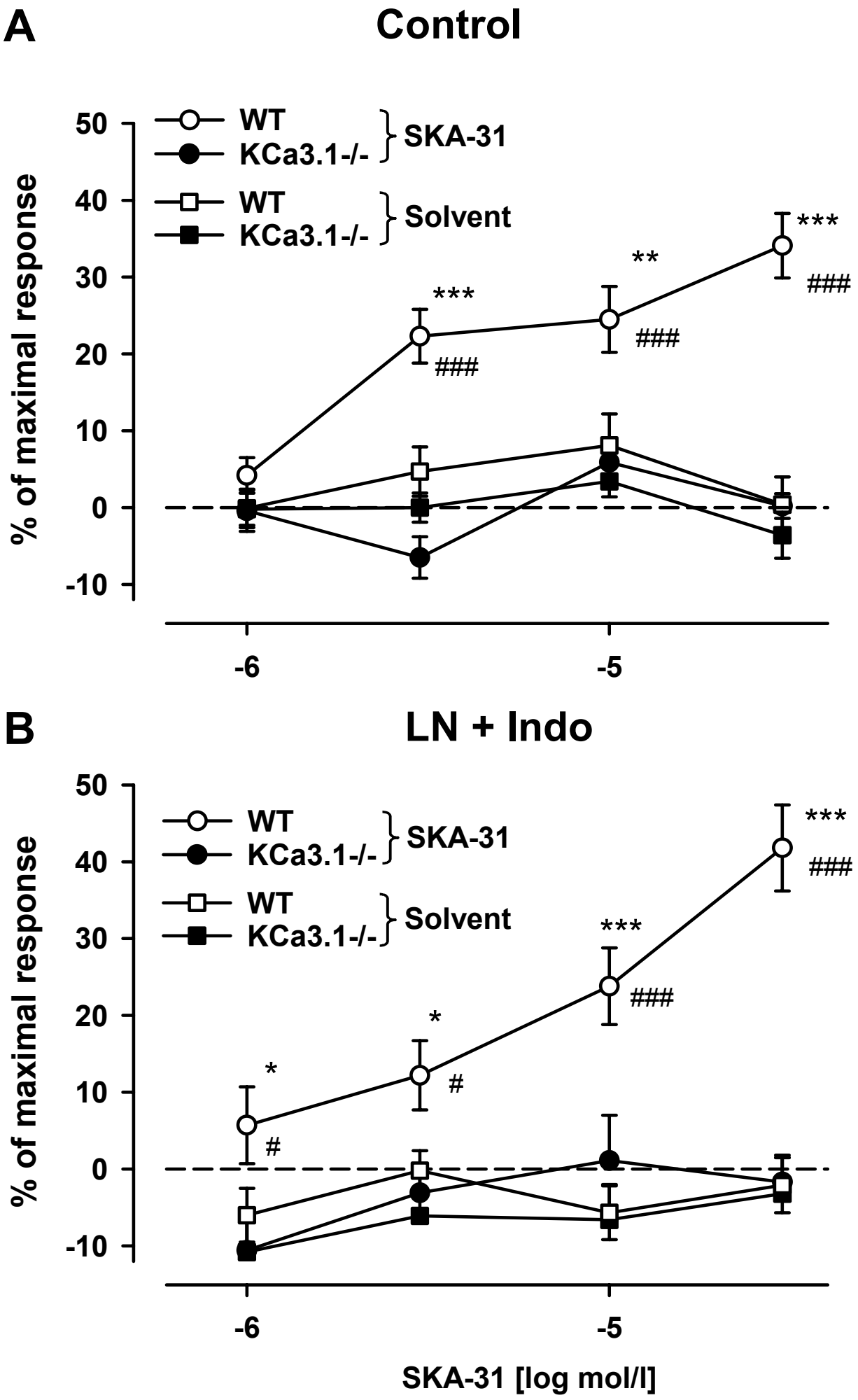
Figure 5

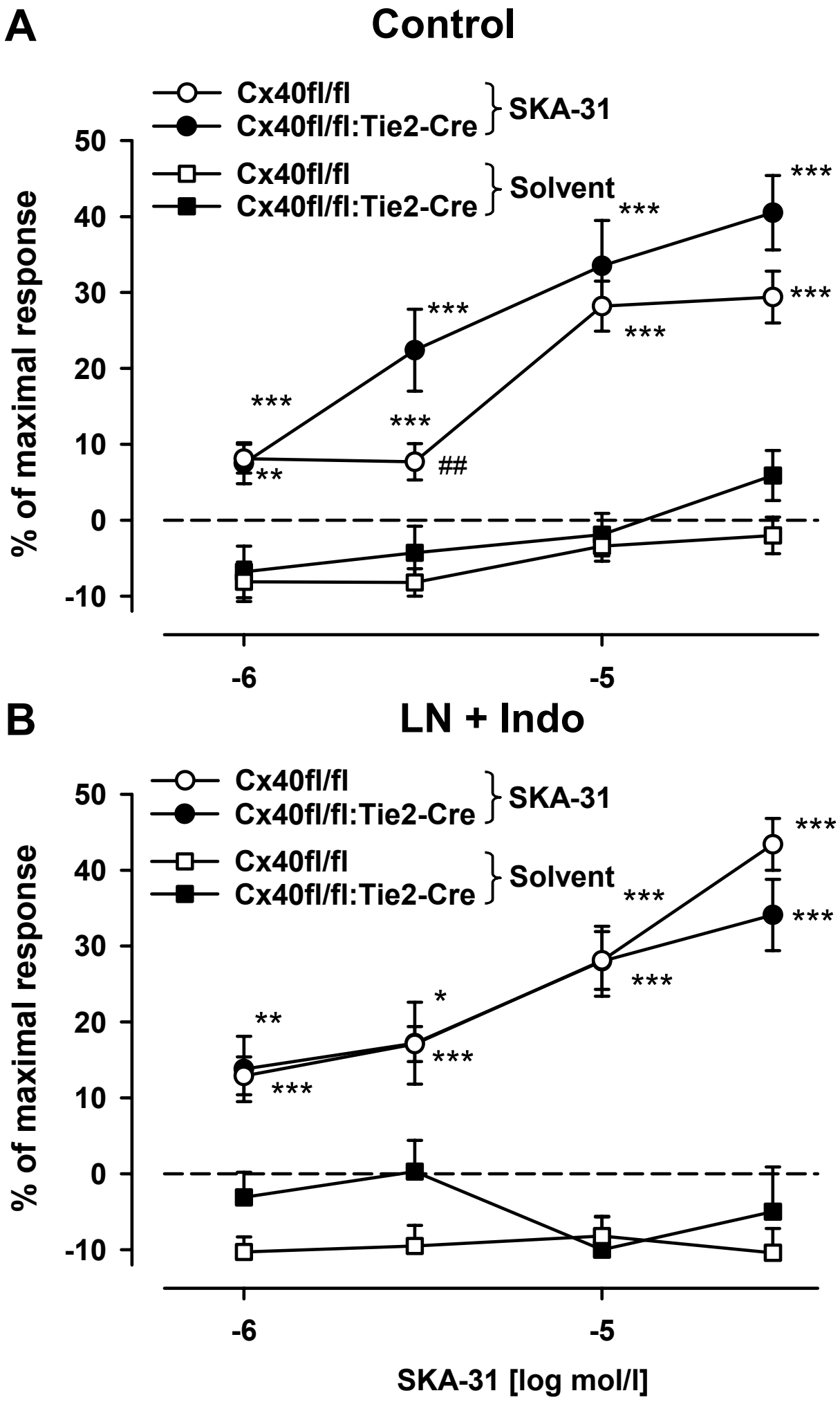
Heart rate (HR) measured in conscious mice before and after intraperitoneal application of SKA-31 (at 0 min) in wild-type (WT, A), Cx40fl/fl:Tie2-Cre (B), and Cx40-/- mice (C). In all genotypes at every dosage of SKA-31 a transient increase in HR was evident, most likely due to animal handling. Thus, the first 2 data points were excluded from statistical analysis. Low dosages of SKA-31 (1 to 10 mg/kg, white symbols) did not lower HR. Likewise, HR remained unchanged in WT and Cx40-/- during the measurement period after 30 mg/kg SKA-

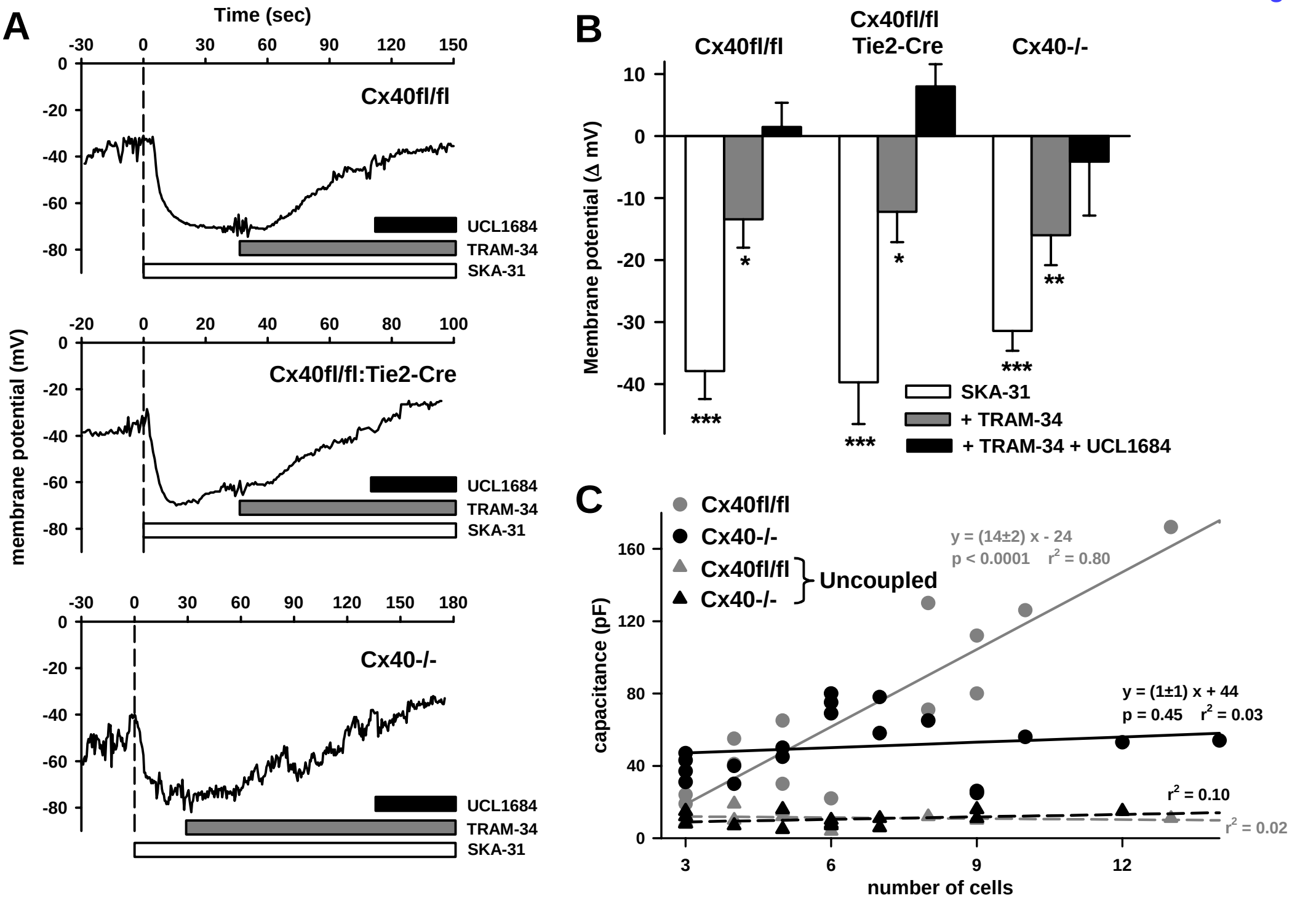
31. However, in Cx40fl/fl:Tie2-Cre animals a significant change was evident (a decrease followed by an increase). The highest dosage of SKA-31 (100 mg/kg) depressed HR in all genotypes similarly. n=6-8 mice each genotype; **, ***: indicates a significant difference (P<0.01, P<0.001, respectively) from 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 of mean arterial pressure (A) and heart rate (B) by SKA-31 (30 and 100 mg/kg) in wild-type (WT), $Cx40^{fl/fl}$:Tie2-Cre, and $Cx40^{-/-}$ mice. The amplitudes of pressure changes were similar in different genotypes for each SKA-31 dose. 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. SKA-31 at 100 mg/kg decreased heart rate significantly in all genotypes. *, **, ***: indicates a significant difference (P<0.05, P<0.01, P<0.001, respectively) from constant mean at the applied dosage (univariate repeated measures ANOVA, the first 2 time points were excluded from analysis). Evidence for non-parallel curves between genotypes was not found (repeated measures ANOVA, at least P=0.41).


A
Control
WT
KCa3.1-/-
SKA-31
WT
KCa3.1-/-
Solvent
% of maximal response
50
40
30
20
10
0
-10
-6
-5
***
**
***
###
###
###
B
LN + Indo
WT
KCa3.1-/-
SKA-31
WT
KCa3.1-/-
Solvent
% of maximal response
50
40
30
20
10
0
-10
-6
-5
*
*
#
#
***
###
***
###
SKA-31 [log mol/l]


A
Control
Cx40fl/fl
Cx40fl/fl:Tie2-Cre
SKA-31
Cx40fl/fl
Cx40fl/fl:Tie2-Cre
Solvent
% of maximal response
50
40
30
20
10
0
-10
-6
-5
***
**
##
B
LN + Indo
Cx40fl/fl
Cx40fl/fl:Tie2-Cre
SKA-31
Cx40fl/fl
Cx40fl/fl:Tie2-Cre
Solvent
% of maximal response
*
SKA-31 [log mol/l]


A
Time (sec)
Cx40fl/fl
UCL1684
TRAM-34
SKA-31
membrane potential (mV)
Cx40fl/fl:Tie2-Cre
UCL1684
TRAM-34
SKA-31
Cx40-/-
UCL1684
TRAM-34
SKA-31
B
Cx40fl/fl
Cx40fl/fl
Tie2-Cre
Cx40-/-
Membrane potential (Δ mV)
SKA-31
+ TRAM-34
+ TRAM-34 + UCL1684
C
Cx40fl/fl
Cx40-/-
Cx40fl/fl
Cx40-/-
Uncoupled
y = (14±2) x - 24
p < 0.0001 r2 = 0.80
y = (1±1) x + 44
p = 0.45 r2 = 0.03
r2 = 0.10
r2 = 0.02
capacitance (pF)
number of cells

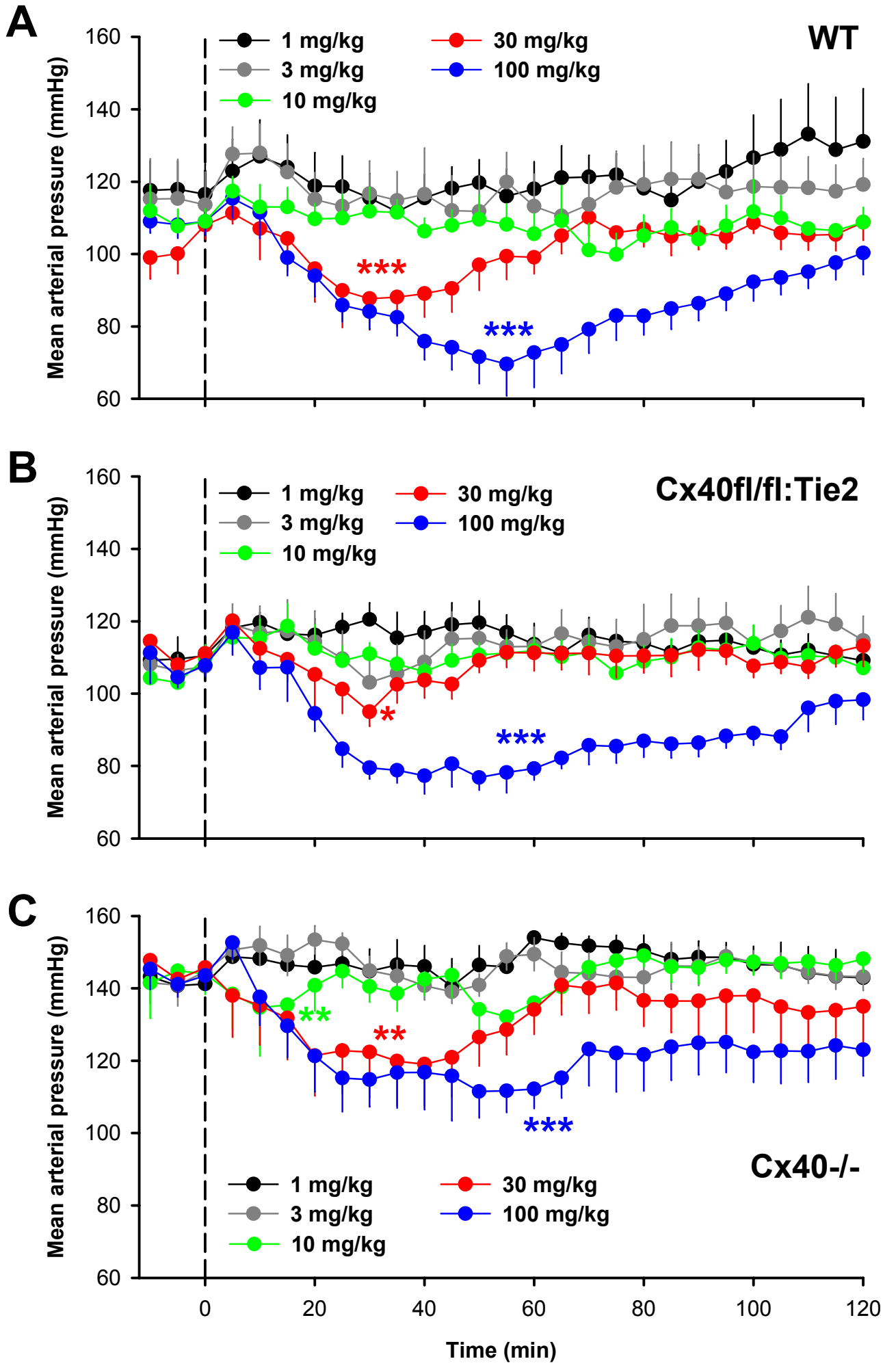

A
WT
1 mg/kg
3 mg/kg
10 mg/kg
30 mg/kg
100 mg/kg
Mean arterial pressure (mmHg)
160
140
120
100
80
60
***
***
B
Cx40fl/fl:Tie2
1 mg/kg
3 mg/kg
10 mg/kg
30 mg/kg
100 mg/kg
Mean arterial pressure (mmHg)
160
140
120
100
80
60
*
***
C
Cx40-/-
1 mg/kg
3 mg/kg
10 mg/kg
30 mg/kg
100 mg/kg
Mean arterial pressure (mmHg)
160
140
120
100
80
60
**
**
***
0
20
40
60
80
100
120
Time (min)

Fig. 5

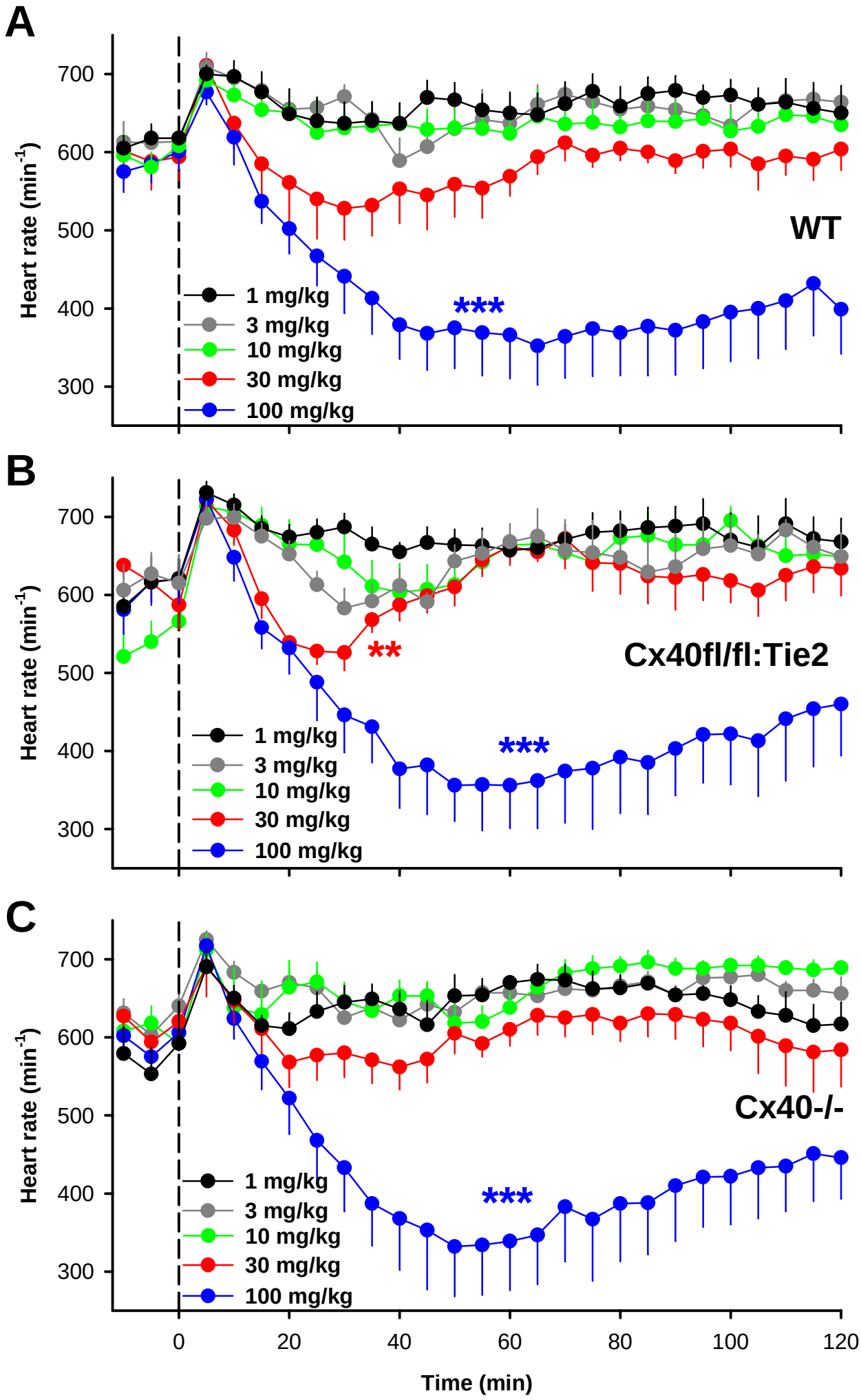
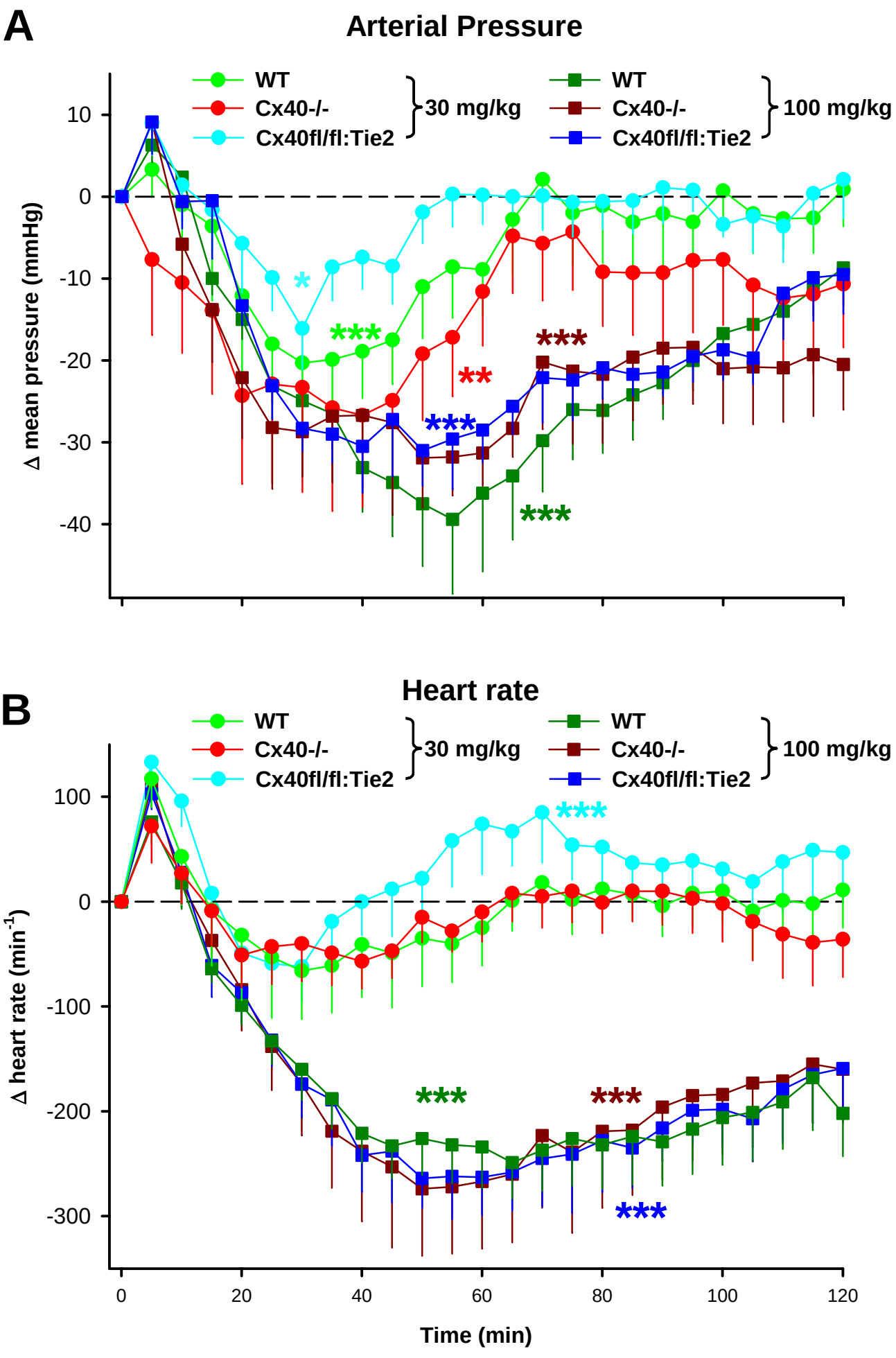

A
B
C
Heart rate (min-1)
700
600
500
400
300
WT
Cx40fl/fl:Tie2
Cx40-/-
1 mg/kg
3 mg/kg
10 mg/kg
30 mg/kg
100 mg/kg
***
**
0
20
40
60
80
100
120
Time (min)

Fig. 6


A
Arterial Pressure
WT
Cx40-/-
Cx40fl/fl:Tie2
30 mg/kg
100 mg/kg
Δ mean pressure (mmHg)
10
0
-10
-20
-30
-40
*
***
**
***
***
***
B
Heart rate
Δ heart rate (min-1)
100
0
-100
-200
-300
***
***
***
0
20
40
60
80
100
120
Time (min)

Online Supplement

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

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Methods

Animals:

Animal care and experiments were in accordance with the German Animal Welfare Act and approved by local authorities and are described in accordance with the ARRIVE guidelines. Mice carrying a Cre-recombinase under the control of the Tie2 promoter were interbred with mice (mixed 129/Sv-C57BL/6 background) expressing a floxed Cx40 gene ($Cx40^{fl}$) to generate mice with endothelium-specific Cx40 deficiency ($Cx40^{fl/fl}$:Tie2-Cre) (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 control littermates were derived from our breeding colonies and genotyped as described previously (de Wit *et al.*, 2003; Si *et al.*, 2006). Overall, 63 mice were studied.

Endothelial cell isolation and measurements of membrane potential:

Endothelial cells were isolated from the carotid artery and measured as described (Brähler *et al.*, 2009; Schmidt *et al.*, 2010). After dissection, mounting, and incubation of the arteries with trypsin, endothelial cells, mainly as cell clusters (8±4 cells, range: 3-21), were detached by gentle scrapping and transferred to a culture dish containing DMEM medium and cover slips to allow settling (2-4 h). After establishing electrical access by seal rupture, we measured membrane capacitance in cell clusters using an Axopatch patch-clamp amplifier (Axon Instruments, Foster City, CA) and recorded thereafter membrane potential in the current-clamp. Recordings were stable for 3-10 min. To activate K_{Ca}-channels, SKA-31 (1 µmol/L) was added to the bath solution followed by adding the selective $K_{Ca}3.1$ blocker TRAM-34 (1 µmol/L) (Wulff *et al.*, 2000) and the $K_{Ca}2.X$ blocker UCL1684 (1 µmol/L) (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 docosahexaenoic acid (Schmidt *et al.*, 2010).

Intravital microscopy of the microcirculation:

Mice were anesthetized with intraperitoneal injection of fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidin (0.5 mg/kg). A jugular vein catheter allowed subsequent continuous infusion of the anesthetic drugs. The cremaster muscle was prepared as described (de Wit, 2010) and superfused with a tempered (34° C) buffer containing (mmol/L): 118.4 NaCl, 20 $NaHCO_3$, 3.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$. In each mouse 8-10 arterioles were observed through a 20-fold objective using a video camera-equipped microscope (Eclipse E600, Nikon, Germany). Images were recorded on videotape to allow later measurement of the inner arteriolar diameter with an application using LabVIEW7.0. Arteriolar diameters were measured before and during superfusion of SKA-31 (1–30 µmol/L, dissolved at 20 mmol/L in CremophorEL and further diluted using saline). Before studying the next concentration of SKA-31 or the solvent, arterioles were allowed to return to their resting diameter for 4 min. This protocol was repeated during superfusion of N^G-nitro-*L*-arginine (L-NA, 30 µmol/L) and indomethacin (indo, 3 µmol/L) to block NO-synthase and cyclooxygenase. In a subset of experiments, dilations induced by SKA-31 were studied before and after addition of the nonspecific gap junction blocker carbenoxolone (30 µmol/L, dissolved in water at 3 mmol/L and further diluted in the superfusion buffer) in the continuous presence of L-NA and indomethacin. In a second protocol, SKA-31 (3, 30 µmol/L) and ACh (0.03-10 µmol/L) were superfused in the continuous presence of L-NA and indomethacin either alone or together and arteriolar diameters were assessed as described above. At the end of all experiments the maximal diameter of the arterioles was determined by combined superfusion of ACh, adenosine, and sodium nitroprusside (each 30 µmol/L). Animals were then sacrificed by an overdose of pentobarbital.

Blood pressure measurement:

Mice were anesthetized by inhalation of isoflurane (2% in oxygen) and received fentanyl (0.07 mg/kg intraperitoneally for pain relief) for implantation of telemetric pressure transducers (Data Sciences International, s'Hertogenbosch, Netherlands). The catheter was introduced into the left carotid artery and the transmitter positioned subcutaneously at the abdomen (Jobs *et al.*, 2012). The animals recovered for 5 days before measurements were started. SKA-31 was dissolved in warmed (37°C) peanut oil by vigorous stirring and administered intraperitoneally (150 µL) at 7 pm. Measurements were started 30 min before application and continued thereafter. Increasing concentrations of SKA-31 (1–100 mg/kg) were studied in all animals nonrecurringly during consecutive nights with a 48 h interval between applications of higher SKA-31 concentrations. Finally, mice were sacrificed following an overdose of pentobarbital. Data were analyzed using DSI software.

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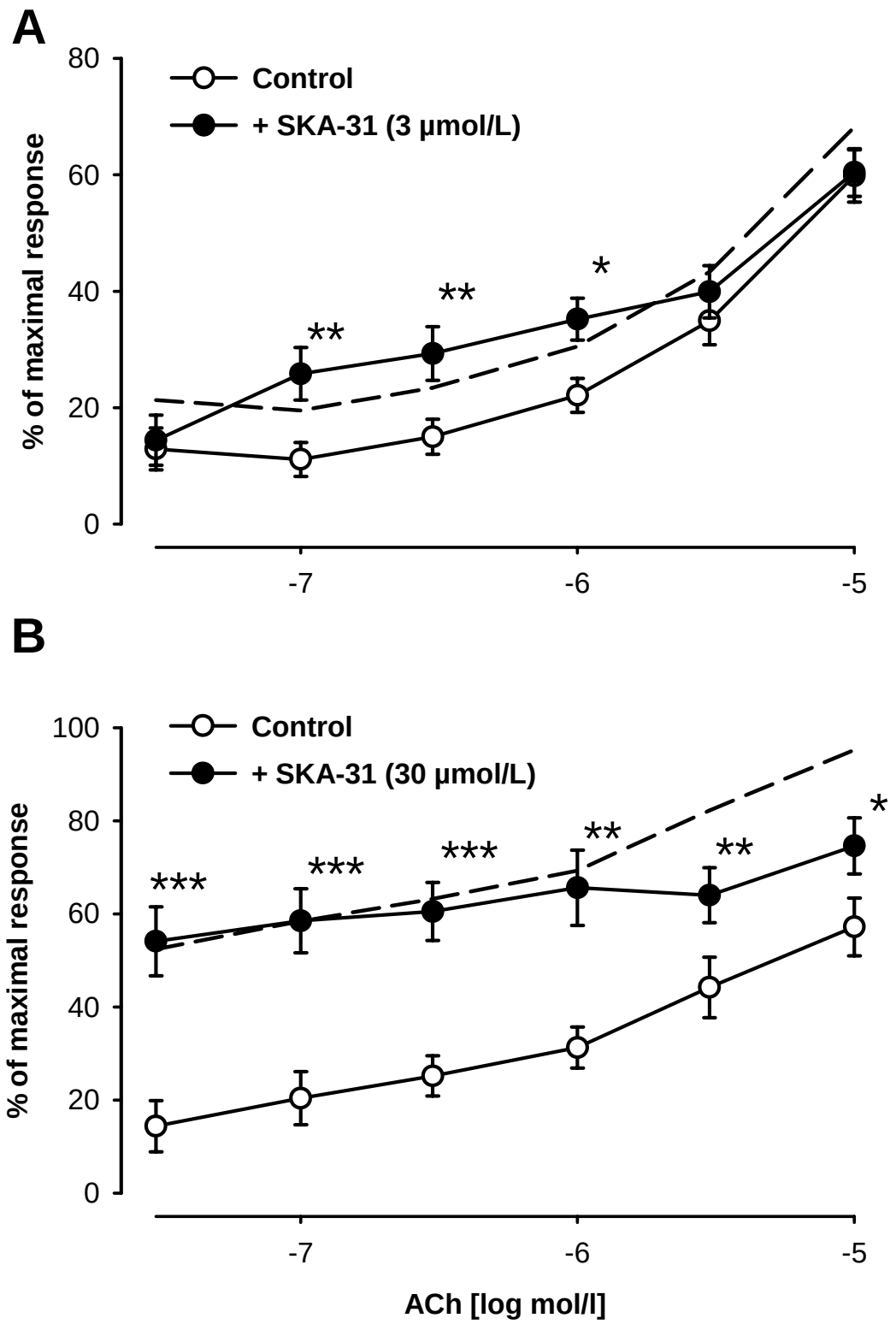
Figure S1

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

Figure S2

SKA-31 induced a concentration dependent dilation during inhibition of NO-synthase and cyclooxygenase (LN and Indo, 3 and 30 µmol/L) in arterioles of wild-type mice that was unaffected in the presence of the nonspecific gap junction blocker carbenoxolone (Cbx, 30 µmol/L). 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.

Figure S1



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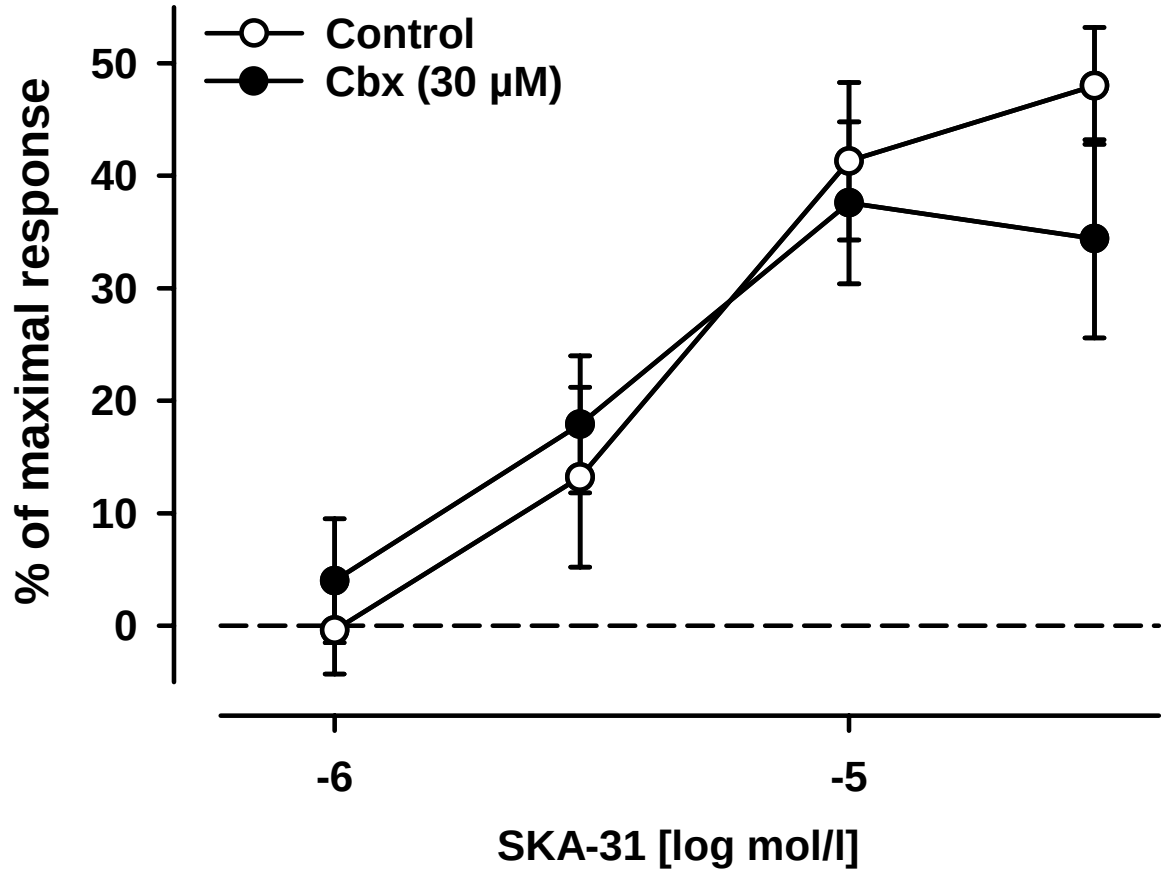

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