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RESEARCH PAPER

Pharmacological activation of KCa3.1/KCa2.3 channels produces endothelial hyperpolarization and lowers blood pressure in conscious dogs

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BACKGROUND AND PURPOSE

In rodents, the endothelial KCa channels, KCa3.1 and KCa2.3, have been shown to play a crucial role in initiating endothelium-derived hyperpolarizing factor (EDHF) vasodilator responses. However, it is not known to what extent these channels are involved in blood pressure regulation in large mammals, which would also allow us to address safety issues. We therefore characterized canine endothelial KCa3.1 and KCa2.3 functions and evaluated the effect of the KCa3.1/KCa2.3 activator SKA-31 on blood pressure and heart rate in dogs.

EXPERIMENTAL APPROACH

Canine endothelial KCa3.1/KCa2.3 functions were studied by patch-clamp electrophysiology and wire myography in mesenteric arteries. Systemic cardiovascular actions of acute SKA-31 administration were monitored in conscious, unstressed beagle dogs.

KEY RESULTS

Mesenteric endothelial cells expressed functional KCa3.1 and KCa2.3 channels that were strongly activated by SKA-31. SKA-31 hyperpolarized the endothelial membrane and doubled endothelial hyperpolarization-dependent vasodilator responses in mesenteric arteries. SKA-31 (2 mg·kg^{-1}, i.v.) rapidly decreased the MAP by 28 ± 6 mmHg; this response was transient (8 ± 1 s), and the initial drop was followed by a fast and pronounced increase in HR (+109 ± 7 beats min^{-1}) reflecting baroreceptor activation. SKA-31 significantly augmented similar transient depressor responses elicited by ACh (20 ng·kg^{-1}) and doubled the magnitude of the response over time.

CONCLUSIONS AND IMPLICATIONS

Activation of endothelial KCa3.1 and KCa2.3 lowers arterial blood pressure in dogs by an immediate electrical vasodilator mechanism. The results support the concept that pharmacological activation of these channels may represent a potential unique endothelium-specific antihypertensive therapy.

Abbreviations

CMEC, canine mesenteric artery endothelial cells; EDHF, endothelium-derived hyperpolarizing factor; KCa2.3, small-conductance Ca^{2+}-activated potassium channel, subtype 3; KCa3.1, intermediate-conductance Ca^{2+}-activated potassium channel; SKA-31, naphtho[1,2-*d*]thiazol-2-ylamine; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; UCL1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo[b,n][1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide

Introduction

The endothelium-derived hyperpolarizing factor (EDHF) system (Feletou and Vanhoutte, 2009; Grgic *et al.*, 2009; Edwards *et al.*, 2010) is the third major endothelium-dependent vasodilator mechanism besides NO and prostacyclin. Disturbances of this system have been shown to contribute to endothelial dysfunction in experimental models of cardiovascular disease, chronic renal failure (Köhler *et al.*, 2005), diabetes (Ding *et al.*, 2005; Brondum *et al.*, 2009), hypercholesteraemia (Morikawa *et al.*, 2005) and after angioplastic intervention (Köhler *et al.*, 2001), cardiopulmonary bypass surgery (Feng *et al.*, 2008; Liu *et al.*, 2008) and in other human cardiovascular pathologies (Grgic *et al.*, 2009; Feletou *et al.*, 2010; Köhler *et al.*, 2010). Moreover, enhanced EDHF functions seem to compensate for the absence of NO in endothelial NOS-deficient mice (Brandes *et al.*, 2000) and in several clinical conditions (Giachini *et al.*, 2009; Grgic *et al.*, 2009; Feletou *et al.*, 2010). The EDHF system may thus constitute an attractive target for improving endothelial function in cardiovascular disease states and for antihypertensive therapy (Feletou *et al.*, 2010; Köhler *et al.*, 2010).

In most vascular beds, the function of the EDHF system requires activation of endothelial Ca^{2+}-activated K^+ channels (KCa) (Edwards *et al.*, 1998), i.e. KCa3.1 (Ishii *et al.*, 1997) and KCa2.3 (Köhler *et al.*, 1996; Wei *et al.*, 2005), thereby producing hyperpolarization of the endothelium; this is then transmitted to the underlying smooth muscle cells leading to closure of voltage-gated Ca^{2+} channels and relaxation (Grgic *et al.*, 2009). In mice, genetic loss of endothelial KCa3.1 (Si *et al.*, 2006) and KCa2.3 (Brähler *et al.*, 2009) has been shown to impair endothelial function *in vivo* and to increase systemic blood pressure, thus highlighting the role of the EDHF–vasodilator system in regulating the circulation (Köhler and Ruth, 2010). Conversely, the KCa3.1/KCa2.3 activator naphtho[1,2-*d*]thiazol-2-ylamine (SKA-31) augmented endothelial KCa functions, and i.p. injections of a SKA-31 depot lowered the blood pressure of normotensive mice over 24 h, as well as that in hypertensive, angiotensin II-infused mice (Sankaranarayanan *et al.*, 2009; Hasenau *et al.*, 2011). These data suggest that KCa3.1 and KCa2.3 serve as targets for endothelium-specific antihypertensive therapy (Feletou *et al.*, 2010; Köhler *et al.*, 2010).

However, this interpretation of data derived from mouse experiments may be premature because cardiovascular parameters in mice [i.e. heart rate (HR) of ≈700 beats min^{-1} and high sympathetic input] differ substantially from those in larger mammals and humans (HR ≈70 beats min^{-1}). We therefore tested the hypothesis that SKA-31 augments endothelial KCa3.1/KCa2.3 functions and lowers blood pressure in a large unstressed mammal. For this purpose, we identified and pharmacologically characterized canine endothelial KCa3.1/KCa2.3 functions and the systemic cardiovascular actions of acute i.v. injections of SKA-31 in conscious, trained and unstressed dogs. Our findings in dogs support the concept that the KCa3.1/KCa2.3 EDHF dilator system could represent a new target for antihypertensive therapy.

Methods

Animals

The blood pressure monitoring was performed in five unstressed, conscious, female beagles weighing 14–16 kg. The dogs were trained for 6 months and tolerated handling during blood pressure monitoring in a quiescent and unstressed fashion. The dogs were kept in a group and fed a commercial maintenance diet (daily intake of Na^+/K^+: 2.3/2.9 $mmol \cdot kg^{-1} \cdot day^{-1}$). Before the study, the dogs underwent two-stage surgery during general anaesthesia under antiseptic conditions as previously described (Wamberg *et al.*, 2003). Firstly, both common carotid arteries were displaced to skin loops to facilitate arterial puncture. Secondly, bilateral ovariectomy and a hysterectomy were carried out to prevent spontaneous changes in sex hormone concentrations from affecting the results. After full recovery, the dogs were investigated 18–20 h after their latest meal and 8 h after water withdrawal. All animal care and experimental procedures complied with the guidelines of the Danish Animal Experiments Inspectorate (No. 2009/561-1753) and were approved by Dyreforsøgstilsynet of the Danish Ministry of Justice.

Experimental protocols

On the night before the experiments, the supply of drinking water was interrupted at midnight by a timer-controlled electric valve to ensure a uniform degree of hydration in the morning. Baseline conditions, therefore, are characterized following 9 h of water deprivation. For experimentation, dogs were placed in a sling and instrumented by sterile i.a. and i.v. catheters allowing blood pressure measurements and i.v. infusions, respectively. Arterial blood pressure and heart rate were measured continuously by computer based on 100 Hz sampling, as described previously (Kjolby *et al.*, 2005). For any one dog, experiments were conducted at intervals of no less than 2 weeks. Each experiment lasted for 2 h; before any drugs were injected, the dogs were allowed 1 h to equilibrate after instrumentation. One day after *in vivo* experimentation, the dogs were killed by exsanguination under deep isoflurane anaesthesia and the mesenteric arteries were collected.

Endothelial cell isolation and patch-clamp electrophysiology

Isolation of endothelial cells from mesenteric artery was carried out using previously described protocols (Brähler *et al.*, 2009). In brief, 1–2 cm segments of first order mesenteric arteries (MA) were dissected from the mesenteric bed and cleaned from fat and connective tissue. Segments were mounted on a small glass capillary and filled with a trypsin (0.25%)/EDTA buffer (Biochrom KG, Berlin, Germany). After this, MA were sutured and incubated for 45 min at 37°C. Thereafter, the vessel was cut open, and the luminal surface was gently scraped with a 10 µL pipette tip. Detached single endothelial cells and endothelial cell clusters were aspirated and transferred to a culture dish containing DMEM medium supplemented with 10% fetal calf serum and penicillin/streptomycin (all from Biochrom KG) and cover slips. Cells were allowed to settle down for 2–4 h and used for electrophysiological measurements within 4 h.

Whole-cell membrane currents in single CMEC (9.1 ± 0.3 pF, n = 8) were recorded using an Axopatch patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) and the Clampex 9.2 data acquisition software, and analysed by the Clampfit 9.2 software (Axon Instruments). For activation of KCa currents, CMEC were dialysed with a KCl pipette solution containing 3 μmol·L^{-1} $[Ca^{2+}]_{free}$ (in mmol·L^{-1}): 140 KCl, 1 Na_2ATP, 1 $MgCl_2$, 2 EGTA, 1.91 $CaCl_2$ and 5 HEPES, pH 7.2. The NaCl bath solution contained (mmol·L^{-1}) 137 NaCl, 4.5 Na_2HPO_4, 3 KCl, 1.5 KH_2PO_4, 0.4 $MgCl_2$, 0.7 $CaCl_2$ and 10 glucose (pH 7.4). For potentiation of currents, the KCa3.1/KCa2.X activator SKA-31 (1 μmol·L^{-1}) was added to the bath solution. For blocking experiments, we applied the selective KCa3.1 blocker TRAM-34 (Wulff *et al.*, 2000) (1 μmol·L^{-1}) and the KCa2.X blocker UCL1684 (Rosa *et al.*, 1998) (1 μmol·L^{-1}); 1 mmol·L^{-1} stock solution of TRAM-34, UCL1684 and SKA-31 were prepared with dimethyl sulphoxide (DMSO). The final DMSO concentration did not exceed 0.3%. Membrane potentials were measured in endothelial cell clusters (consisting of 7 ± 2 cells, range 3–20 cells) using the current-clamp mode of the Axopatch-amplifier (Axon Instruments). The pipette solution contained 0.1 μM Ca^{2+}. After rupture of the cell membrane, high capacitance values (on average 53 ± 9 pF, n = 13) indicated intact inter-endothelial electrical coupling. Capacitance values correlated strictly with the cell count in a positive fashion (data not shown). Recordings were stable over a time period of 4–15 min. EC clusters were stimulated with acetylcholine (100 nmol·L^{-1}) followed by stimulation with the KCa3.1/KCa2.X activator SKA-31 (1 μmol·L^{-1}) (Sankaranarayanan *et al.*, 2009), or in reverse order. In a subset of experiments, 1 μmol·L^{-1} TRAM-34 followed by 1 μmol·L^{-1} UCL1684 (Tocris Bioscience, Bristol, UK) were added to the bath solution. Drugs did not modulate inter-endothelial electrical coupling as capacitance values were unchanged at the end of the experiment.

mRNA expression studies

mRNA was extracted and purified from freshly dissected mesenteric arteries and from CMEC after 7 days of primary culture using the RNeasy Mini Kit (Qiagen, Copenhagen, Denmark). RT-PCR was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Copenhagen, Denmark) for cDNA synthesis and Taq DNA polymerase (Invitrogen, Hellerup, Denmark) for PCR. A standard PCR protocol was used with initial denaturing at 95°C for 3 min and subsequently 40 cycles of denaturing at 95°C for 25 s, annealing at 57°C for 20 s and extending at 72°C for 40 s. A final step of extension was done at 72°C for 3 min. PCR products were analysed by gel electrophoresis using 1.5% agarose in TBE buffer and staining with GelRed™ (Biotium, Bromma, Sweden). Specific RT-PCR primers were designed to span intronic sequences. The expected product lengths were 306 bp for eNOS, 163 bp for KCa3.1, 319 bp for KCa2.3, 267 bp for KCa2.2 and 269 bp for KCa2.1. Semiquantitative RT-PCR was done using iQ™ CYBR® Green Supermix (Bio-Rad) and a Stratagene MX3000p cycler (Stratagene, La Jolla, CA, USA). qPCR was done using an initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 30 s, annealing at 56°C for 45 s and an extension step at 72°C for 40 s. Thereafter, a three-step series of 95°C for 60 s, 55°C for 30 s, and a gradual increase to 95°C was run to determine melting points of qPCR products. Signals were normalized to expression of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are given as % GAPDH. The identity of all PCR products was verified by sequencing. Primer sequences were as follows: eNOS F, 5′-TTACCAGCTGGCCAAAGTGA; eNOS R, 5′-CCC ATGAGTGAGGCAGAGAT; KCa3.1 F, 5′-CCCATCACATTC CTGACCAT; KCa3.1 R, 5′-GCACATGCTTCTCTGCCTTA; KCa2.3 F, 5′-ATTGGTTACGGGGACATGGT; KCa2.3 R, 5′-CGC TCCTCAGTTGGTGAATG; KCa2.2 F, 5′-CGACAAGGCGT CACTGTATT; KCa2.2 R, 5′-GTGGATGGGGCATAGGAGAA; KCa2.1 F, 5′-GACTCAGGGAAGACCCCAAA; KCa2.1 R, 5′-TCCACCATGAACAGCTGGAT. The efficiency of primer pairs was determined by qPCR on serial dilutions of known cDNA amounts. Efficiency values for all primer pairs were ≥95%. Expression levels were normalized to expression of GAPDH as reference gene by calculating the ΔC_t values [ΔC_t = C_t(target) – C_t(GAPDH)] and the ratios to GAPDH (ratio = $2^{-\Delta Ct}$). Results are expressed as percentage of GAPDH (% GAPDH).

Isometric vessel studies

First-order MA (600–800 μm in diameter) were dissected into 2 mm rings and mounted isometrically in a wire myograph (DMT, Aarhus, Denmark). The gassed (95% artificial air, 5% CO_2) bath solution was composed of (in mmol·L^{-1}) 115 NaCl, 25 $NaHCO_3$, 2.5 KH_2PO_4, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES and 5.5 glucose and contained 300 μmol·L^{-1} N^G-nitro-L-arginine (L-NA) and indomethacin (INDO, 10 μmol·L^{-1}) to block NOS and COXs (Hristovska *et al.*, 2007). Isometric precontractions were produced by addition of 10 nmol·L^{-1} of the thromboxane mimetic U46619 (Sigma-Aldrich, Copenhagen, Denmark) followed by stimulation with increasing concentrations of ACh (1 nmol·L^{-1} to 10 μmol·L^{-1}) in the absence or presence of SKA-31 (1 μmol·L^{-1}). At the end of the experiments, maximal vasocontraction was determined by switching to a 60 mmol·L^{-1} KCl solution. After washout, maximal vasorelaxation was determined by addition of 10 μmol·L^{-1} sodium nitroprusside (SNP) to the bath solution. Responses to ACh are expressed as a percentage of the vasorelaxation induced by SNP.

I.v. injections of SKA-31

Appropriate amounts of SKA-31 for 0.4 and 2 mg·kg^{-1} injections were dissolved in 1 mL DMSO and diluted 1:1 by addition of 1 mL 0.9% NaCl. A total volume of 2 mL was injected through a caval catheter inserted via the right saphenous vein within 1–2 s. ACh was dissolved in 0.9% NaCl, and an appropriate amount was added to the 2 mL DMSO/0.9% NaCl with or without SKA-31, to give 20 ng·kg^{-1} ACh per injection. Injections of 2 mL of DMSO/0.9% NaCl alone served as vehicle controls. In experiments testing ACh alone and in combination with SKA-31, the ACh injection was given first, followed by the second injection of ACh + SKA-31 after 5–10 min. In experiments testing two dosages of SKA-31, the smaller dose was given first, followed after 5–30 min by injection of the higher dose. When giving two consecutive ACh injections, the second injection gave rise to equal or somewhat smaller responses (n = 2, data not shown), excluding sensitization to repetitive ACh administration.

Determination of SKA-31 plasma levels

Blood samples (2–5 mL) were taken via the carotid artery catheter before and 1, 10, 30 or 60 min after injection, transferred into EDTA-containing tubes and centrifuged at 500× *g* for 15 min at 4°C. Plasma samples were stored at –80°C pending analysis. Plasma samples were purified using C18 solid phase extraction (SPE) cartridges. After conditioning of the cartridges plasma samples (200 µL) were loaded, and the interfaces were removed by washing the columns twice with 0.5 mL H_2O. SKA-31 was eluted with two times 0.5 mL of a 50:50 mixture of methanol and acetonitrile containing 1% ammonium hydroxide. The eluted SKA-31 fractions were evaporated to dryness and reconstituted in 200 µL of a 50:50 mixture of methanol and acetonitrile containing 1% ammonium hydroxide and used for the quantification of SKA-31 levels by LC/MS.

HPLC/MS analysis. This analysis was performed with a Hewlett Packard 1100 series HPLC stack (Agilent Technologies, Santa Clara, CA) and a Finnigan LCQ Classic MS (Thermo Fisher Scientific, Waltham, MA, USA). A Zorbax SB-C18 column (3.5 µm, 2.1 × 150 mm; Agilent) was used. The mobile phase consisted of H_2O (0.2% formic acid) and acetonitrile (0.2% formic acid). Starting from 60:40 H_2O/acetonitrile, the gradient was ramped from 2.5 min to 8 min to 20:80 H_2O/acetonitrile and from 8 min to 12 min back to 60:40 H_2O/acetonitrile. The run time was 15 min at a flow rate of 0.5 mL·min^{-1}. The column temperature was maintained at 25°C. SKA-31 eluted at ~6.98 min and was detected at a mass of 201.3 (molecular weight plus H^+) using electrospray ionization (positive ion mode; capillary temperature 250°C; capillary voltage 33 V; tube lens offset 20 V). SKA-31 concentrations were calculated with a seven-point calibration curve from 0 to 10 µmol·L^{-1} of SKA-31. After log transformation, plasma concentrations fitted well to a monoexponential decay [$Y = Y_0 \times \mathrm{Exp}(-k_e t)$] (see also Figure S1), and the plasma concentration of SKA-31 at time of injection (C_0) was estimated from the *Y*-intercept. The volume of distribution was calculated using the following formula: V_D = Dose/C_0. We further tested the plasma activity of SKA-31 by determining its effect on the electrophysiology of cloned human KCa3.1 expressed in HEK-293 cells (Sankaranarayanan *et al.*, 2009) using protocols and buffers as stated above (see also Figure S1C for further details). Plasma protein binding was determined as described previously by us (Sankaranarayanan *et al.*, 2009).

Evaluation of blood pressure data

Continuous blood pressure data were averaged for every 3 s. A change in mean arterial blood pressure (MAP) or heart rate (HR) was defined as a variation from baseline that exceeded the SD of the baseline value (determined by a 1 min recording ahead of injection) and lasted at least 3 s. The end of the response was defined as the time point at which values returned to nearby baseline levels. The magnitude of the response over time was determined by calculating the area under the curve (AUC) with the value of the 1 min baseline recording serving as baseline. Delay time for onset of HR alterations in response to blood pressure decrease was measured on a beat-to-beat basis for each experiment. Data analysis was performed using Origin9 (Originlab, Northampton, MA, USA).

Statistics

Data are given as mean ± SEM. For comparison of data sets, Student's paired or unpaired *t*-test was used where appropriate. Plasma concentrations and qRT-PCR data are given as mean ± SD.

Results

Expression of KCa3.1 and KCa2.3 channels in canine mesenteric endothelial cells

Functional expression of KCa3.1 and KCa2.3 was characterized by whole-cell patch clamp in canine single, freshly isolated mesenteric endothelial cells (CMEC). Infusion of 3 µmol·L^{-1} Ca^{2+} via the patch-pipette activated slightly inwardly rectifying outward currents and shifted the reversal potential towards the K^+ equilibrium potential (see Figure 1A for representative traces and Figure 1C for statistics). These currents – being submaximally or maximally pre-activated by infusion of 3 Ca^{2+} µmol·L^{-1} – were strongly potentiated by 1 µmol·L^{-1} of the KCa3.1/KCa2.X activator SKA-31 (Figure 1A,C), a finding similar to previous observations using SKA-31 (Sankaranarayanan *et al.*, 2009) or the related activator NS309 (Li *et al.*, 2009). The currents were blocked by the selective KCa3.1-blocker TRAM-34 (Wulff *et al.*, 2000) (1 µmol·L^{-1}) and the KCa2.X blocker UCL1684 (Rosa *et al.*, 1998) (1 µmol·L^{-1}) (Figure 1B, C). These electrophysiological and pharmacological properties of KCa in CMEC resemble the characteristics of KCa3.1 and KCa2.3 in native endothelial cells from mice and humans (Grgic *et al.*, 2009; Köhler *et al.*, 2010). In mRNA extracts from dog mesenteric arteries and primary CMEC, RT-PCR showed considerable amounts of KCa3.1 mRNA as well as mRNAs for KCa2.3 and, interestingly, also for KCa2.2 and KCa2.1 (Figure 2A). For results of semiquantitative RT-PCR analysis on mesenteric arteries, see Figure 2B.

Membrane hyperpolarization mediated by KCa3.1 and KCa2.3 channels in canine mesenteric endothelial cells

In current-clamp experiments on CMEC clusters, we determined endothelial membrane potential changes in response to ACh, SKA-31, and combinations of both. ACh alone (100 nmol·L^{-1}) produced strongly fluctuating hyperpolarizations (Figure 3A), which most likely reflects brief Ca^{2+} release events from IP_3-sensitive stores, recently termed Ca^{2+} pulsar (Ledoux *et al.*, 2008), and accompanying KCa activation. SKA-31 (1 µmol·L^{-1}) significantly potentiated this hyperpolarization towards a more negative and stable level (Figure 3A) and abolished fluctuations. This hyperpolarization response was greatly reversed by TRAM-34 (1 µmol·L^{-1}) with a partial re-occurrence of spiking and was completely abolished by the combination of TRAM-34 and UCL1684 (1 µmol·L^{-1}) (Figure 3A). CMEC exhibited no or only small spontaneous hyperpolarization events in the absence of ACh (Figure 3B). However, even under these conditions, SKA-31 produced sustained hyperpolarization (Figure 3B), which likewise was strongly inhibited by TRAM-34, and abolished by the combination of TRAM-34 and UCL1684. SKA-31-induced hyperpo-

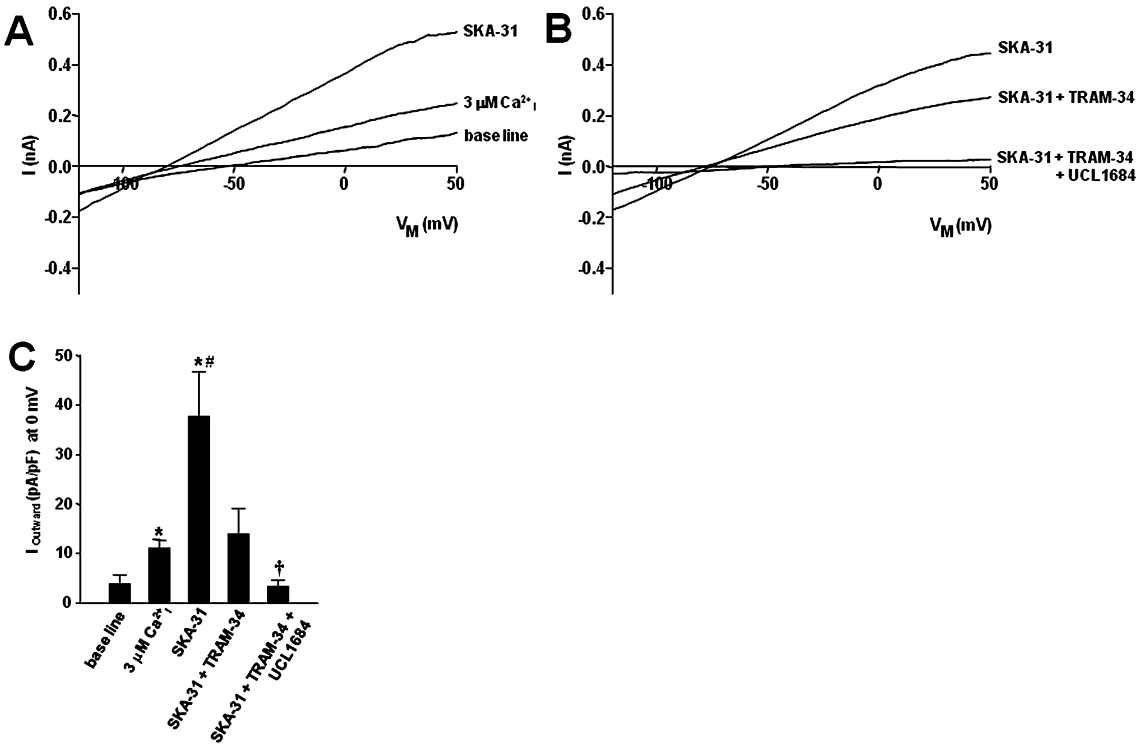

Figure 1

KCa3.1 and KCa2.3 currents in CMEC. (A) Current–voltage relationship (n = 8 experiments) at base line (initial currents after achieving electrical access) and during infusion of a CMEC with 3 µmol·L^{-1} $[Ca^{2+}]_i$. KCa currents were potentiated by the KCa3.1/KCa2.X activator SKA-31 (1 µmol·L^{-1}, n = 5 experiments). (B) SKA-31-activated currents were inhibited by the KCa3.1 blocker TRAM-34 (1 µmol·L^{-1}, n = 3 experiments) and further reduced by additional application of the KCa2.X blocker UCL1684 (1 µmol·L^{-1}, n = 3 experiments). (C) Summary of data for K^+ currents at 0 mV. Values are given as mean ± SEM. *P < 0.05 versus base line, #P < 0.05 versus 3 µmol·L^{-1} $[Ca^{2+}]_i$, †P < 0.05 versus SKA-31, Student's unpaired t-test.

larization was further increased by 100 nmol·L^{-1} ACh (by –13 ± 5 mV, n = 3). These data suggest that (i) ACh and SKA-31 elicit hyperpolarization via activation of KCa3.1 and to a lesser extent KCa2.3, and (ii) that ACh and SKA-31 have additive hyperpolarizing effects.

Potentiation of EDHF-type vasorelaxation by SKA-31

Next we tested whether SKA-31 augments endothelium-dependent EDHF-type vasorelaxations, which are initiated by activation of endothelial KCa3.1 and KCa2.3. Using wire myography, we studied EDHF-mediated relaxation responses in first-order mesenteric artery rings (MA) from three dogs. Experiments were performed in the presence of L-NA (300 µmol·L^{-1}) and INDO (10 µmol·L^{-1}). A precontraction was induced by the thromboxane mimetic U46619 (10 nmol·L^{-1}), which generated a force of 47 ± 3 mN. Subsequent stimulation with ACh (1 µmol·L^{-1}) caused a small but appreciable EDHF-type relaxation by –1.4 ± 1.1 mN (Figure 3C). At 1 µmol·L^{-1}, SKA-31 enhanced the EDHF-type relaxation produced by ACh by 2.5-fold (–3.5 ± 1.3 mN, P < 0.05, Student's paired t-test; Figure 3C). This result corresponds to the potentiating effects of SKA-31 on ACh-induced EDHF-type vasodilatations in murine carotid arteries previously reported by us (Sankaranarayanan *et al.*, 2009). Of note, SKA-31 did not induce an EDHF-type relaxation in canine MA on its own or alter the relaxation to 10 µmol·L^{-1} SNP (–39 ± 3 mN vs. SNP ± SKA-31: –38 ± 6 mN).

Blood pressure-lowering actions of SKA-31 in conscious, unstressed dogs

In keeping with the potentiating effects of SKA-31 on canine endothelial KCa3.1 and KCa2.3 functions, its ability to produce strong endothelial hyperpolarization and the SKA-31-evoked augmentation of EDHF-type vasorelaxation, we next tested whether SKA-31 causes a systemic depressor response or enhances the ACh-induced depressor responses *in vivo*. For this purpose, we monitored blood pressure in trained, conscious and unstressed female beagle dogs before and after i.v. administration of SKA-31, ACh or combinations of the two compounds (for traces, see Figure 4; for statistics, see Table 1). Bolus i.v. injections of the vehicle (1:1 DMSO/0.9%NaCl, n = 2) did not alter the MAP or HR. At 0.4 mg·kg^{-1} SKA-31 produced a visible and immediate depressor response of low amplitude, which was transient and difficult to discriminate from baseline variations (see trace in Figure 4). Nonetheless, this small depressor response was accompanied by a small decrease in pulse pressure (–4 ± 3 mmHg) and a substantial increase in HR (≈+61 beats min^{-1}, from baseline 75 ± 1 beats min^{-1} to 135 ± 8 beats min^{-1}). At 2 mg·kg^{-1}, SKA-31 produced strong depressor responses of ≈–28 mmHg (from

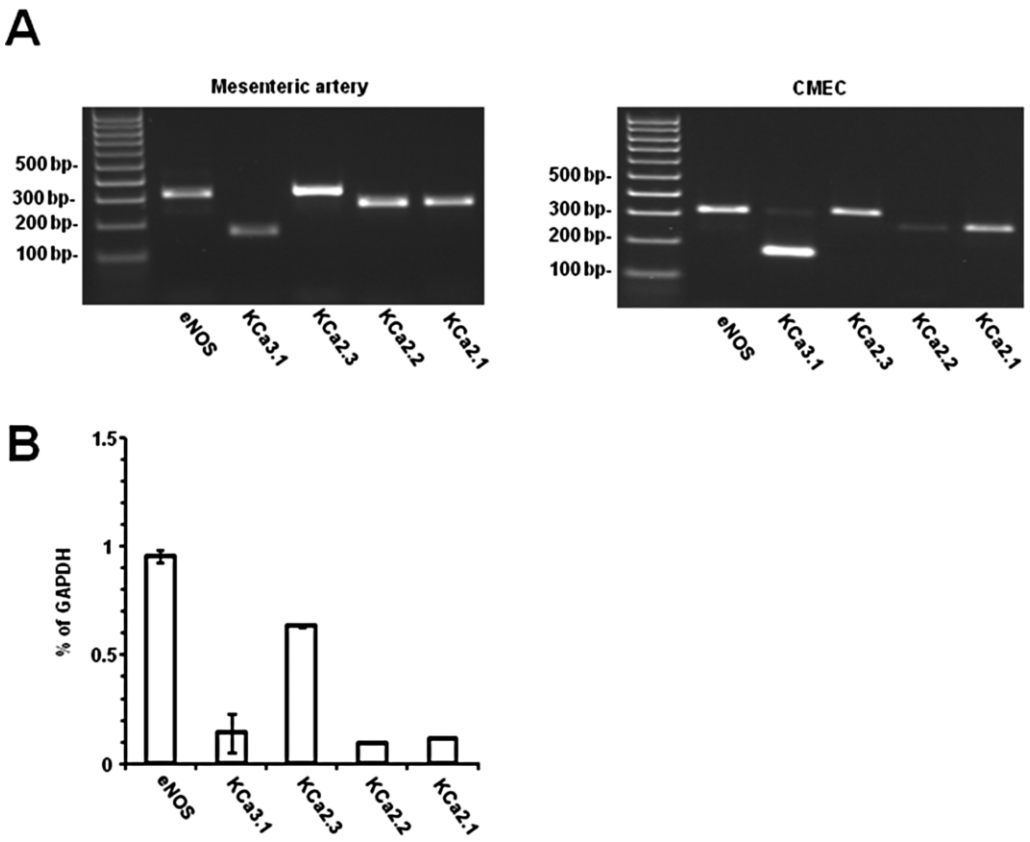

Figure 2

Expression pattern of KCa3.1 and KCa2 genes in canine mesenteric artery and endothelial cells. (A) mRNA-expression pattern of KCa3.1 and KCa2.X genes, and of eNOS as an endothelial cell-specific expression marker in freshly dissected mesenteric arteries (on left) and in primary CMEC (on right). (B) Quantitative RT-PCR analysis of KCa3.1 and KCa2.X gene expression in freshly dissected canine mesenteric arteries. Data are given as mean ± SD of duplicates.

baseline MAP 130 ± 7 mmHg to 100 ± 10 mmHg; time to peak after injection 7 ± 2 s). Similar to the low dose of SKA-31, this depressor response was short-lived (8 ± 1 s), accompanied by a decrease in pulse pressure (–7 ± 3 mmHg) and a rapid and pronounced increase in HR (≈+109 beats min^{-1}; time to peak after injection 10 ± 2 s). In contrast to the lower dose, HR remained slightly increased thereafter (+8 ± 2 beats min^{-1}; 78 ± 2 before vs. 86 ± 1 beats min^{-1} for at least 3 min after injection; $P < 0.05$, Student's paired *t*-test; see trace in Figure 4). This reflected immediate and considerable baroreceptor activation (delay time, 2.4 ± 0.3 s). Likewise, bolus i.v. injections of ACh (20 $ng{\cdot}kg^{-1}$) produced a similarly transient depressor response of ≈–33 mmHg (13 ± 1 s, time to peak: 7 ± 1 s), which was also accompanied by a decrease in pulse pressure (–17 ± 3 mmHg) and an increase in HR (≈+91 bpm, time to peak: 10 ± 1 s; delay time 2.3 ± 0.2 s; Table 1 and traces in Figure 4). HR returned to baseline levels thereafter (79 ± 4 before vs. 82 ± 4 beats min^{-1} after injection; n.s.). In experiments where ACh was first administered alone and then followed by ACh in combination with SKA-31, the combination of ACh and 0.4 $mg{\cdot}kg^{-1}$ SKA-31 ($n = 4$) resulted in depressor responses of similar amplitude, but of significantly higher magnitude over time (AUC). HR increased similarly and returned to baseline levels thereafter (81 ± 4 before vs. 75 ± 4 beats min^{-1} after injection; n.s.). The combination of ACh and 2 $mg{\cdot}kg^{-1}$ SKA-31 ($n = 6$) resulted in an even stronger depressor response with a significantly increased amplitude (≈–46 mmHg) and magnitude over time. The combination of ACh with 2 $mg{\cdot}kg^{-1}$ SKA-31 also produced a stronger increase in HR, and, similar to SKA-31 alone, HR remained, after the initial increase, at a slightly higher level (76 ± 3 before vs. 84 ± 4 beats min^{-1} after injection; $P < 0.05$; see trace in Figure 4). For mean ΔMAP, ΔHR and mean AUC values in all groups, see Table 1. The acute injections of SKA-31 or the combinations of SKA-31/ACh did not produce obvious gastrointestinal or behavioural side effects such as sedation or motor disturbances.

SKA-31 plasma concentrations

Subsequent analysis of total SKA-31 plasma concentrations by HPLC/MS (see also Figure S1A,B) revealed that injection of 0.4 $mg{\cdot}kg^{-1}$ yielded SKA-31 arterial plasma concentrations of 2.6 ± 0.3 $\mu mol{\cdot}L^{-1}$ ($n = 5$) after 1 min, 1.5 ± 0.2 $\mu mol{\cdot}L^{-1}$ ($n = 5$) after 10 min and 1.0 ± 0.1 $\mu mol{\cdot}L^{-1}$ ($n = 2$) after 30 min. The higher dose of 2 $mg{\cdot}kg^{-1}$ produced plasma concentrations of 12.1 ± 2.0 $\mu mol{\cdot}L^{-1}$ ($n = 3$) after 1 min, 7.4 ± 0.6 $\mu mol{\cdot}L^{-1}$ ($n = 2$) after 10 min, 4.0 ± 0.4 $\mu mol{\cdot}L^{-1}$ ($n = 2$) after 30 min and 1.7 ± 0.1 $\mu mol{\cdot}L^{-1}$ ($n = 2$) after 60 min. Plasma protein binding of

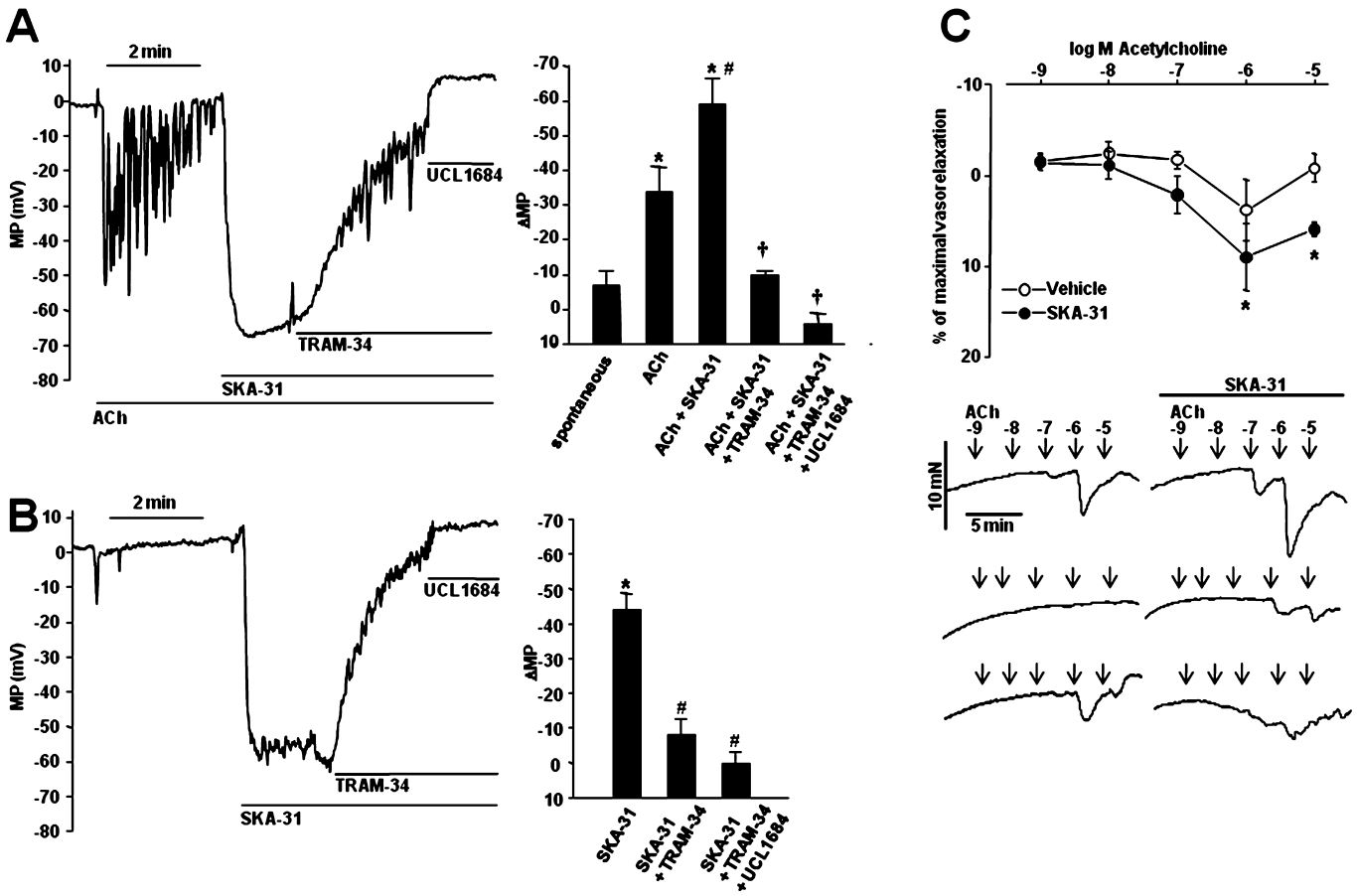

Figure 3

Hyperpolarization responses to ACh and SKA-31 in CMEC clusters. (A) In current clamp recordings of endothelial membrane potentials (MP), ACh (100 nmol·L^{-1}) produced fluctuating hyperpolarization from depolarized base line levels (n = 5 experiments from two dogs). Hyperpolarizations were strongly potentiated by SKA-31 (1 μmol·L^{-1}, n = 4 experiments) and blocked by the KCa3.1 blocker TRAM-34 (1 μmol·L^{-1}, n = 3 experiments) and further reduced by additional application of the KCa2.X blocker UCL1684 (1 μmol·L^{-1}, n = 3 experiments). In three experiments, CMEC showed small spontaneous events (see also trace in panel B). CMEC clusters consisted of 7 ± 2 cells (range 3–20 cells) with a capacitance of 53 ± 9 pF (n = 13). (B) Hyperpolarization responses to SKA-31 (1 μmol·L^{-1}, n = 7 experiments) alone and the strong inhibition of these responses by TRAM-34 (1 μmol·L^{-1}, n = 7 experiments) and complete inhibition by the combination of TRAM-34 and UCL1684 (1 μmol·L^{-1}, n = 6 experiments). Summary of data in right panel. Data are given as mean ± SEM. *P < 0.01 versus base line [–1 ± 1 mV (n = 12), #P < 0.01 vs. ACh alone in (A). †P < 0.01, vs. ACh + SKA-31 in (A). #P < 0.05 vs. SKA-31 in (B); Student's unpaired t-test]. (C) SKA-31 improves EDHF-type vasorelaxations. Wire myography of ACh-induced EDHF-type relaxation responses of U46619 (10 nmol·L^{-1}) precontracted first-order canine mesenteric arteries (600–800 μm in diameter, 2 mm segments) in the presence of L-NA and INDO (300 and 10 μmol·L^{-1}, respectively) and in the absence or presence of SKA-31 (1 μmol·L^{-1}, n = 3, dogs). Upper panel: representative traces from three dogs. Lower panel: summary of data (% of maximal relaxation) representing the average from mesenteric artery preparations (±SKA-31) from three dogs and are given as mean ± SEM. Maximal relaxation was determined by addition of 10 μmol·L^{-1} SNP. *P < 0.05, Student's paired t-test.

SKA-31 was found to be 36 ± 9% (n = 4), a value similar to SKA-31's plasma protein binding of ~40% in rats (Sankaranarayanan *et al.*, 2009). Considering this protein binding and SKA-31's EC_{50} values for KCa3.1 (250 nmol·L^{-1}) and KCa2.3 (3 μmol·L^{-1}) (Sankaranarayanan *et al.*, 2009), these plasma concentrations should have been sufficient to activate both endothelial KCa3.1 and KCa2.3 channels for more than 30 min at the higher dose. Although the total plasma concentrations of SKA-31 were above the EC_{50} values for KCa3.1 channels for more than 60 min (Figure S1A,B), the calculated large volume of distribution (6.9 ± 1.5 L·kg^{-1}, n = 4) suggested that there might not be a direct relationship between the plasma concentration and the hypotensive effect, and that there could be additional binding sites or distribution into other compartments. However, plasma collected 1 min after injection of 0.4 mg·kg^{-1} and 10 min after injection of 2 mg·kg^{-1} was found to be capable of producing significant activation of cloned human KCa3.1 channels (Sankaranarayanan *et al.*, 2009) *in vitro* (Figure S1C), suggesting considerable plasma activity of SKA-31.

Discussion

EDHF-type vasodilatation involving the activation of endothelial KCa3.1/KCa2.3 channels has been proposed to contrib-

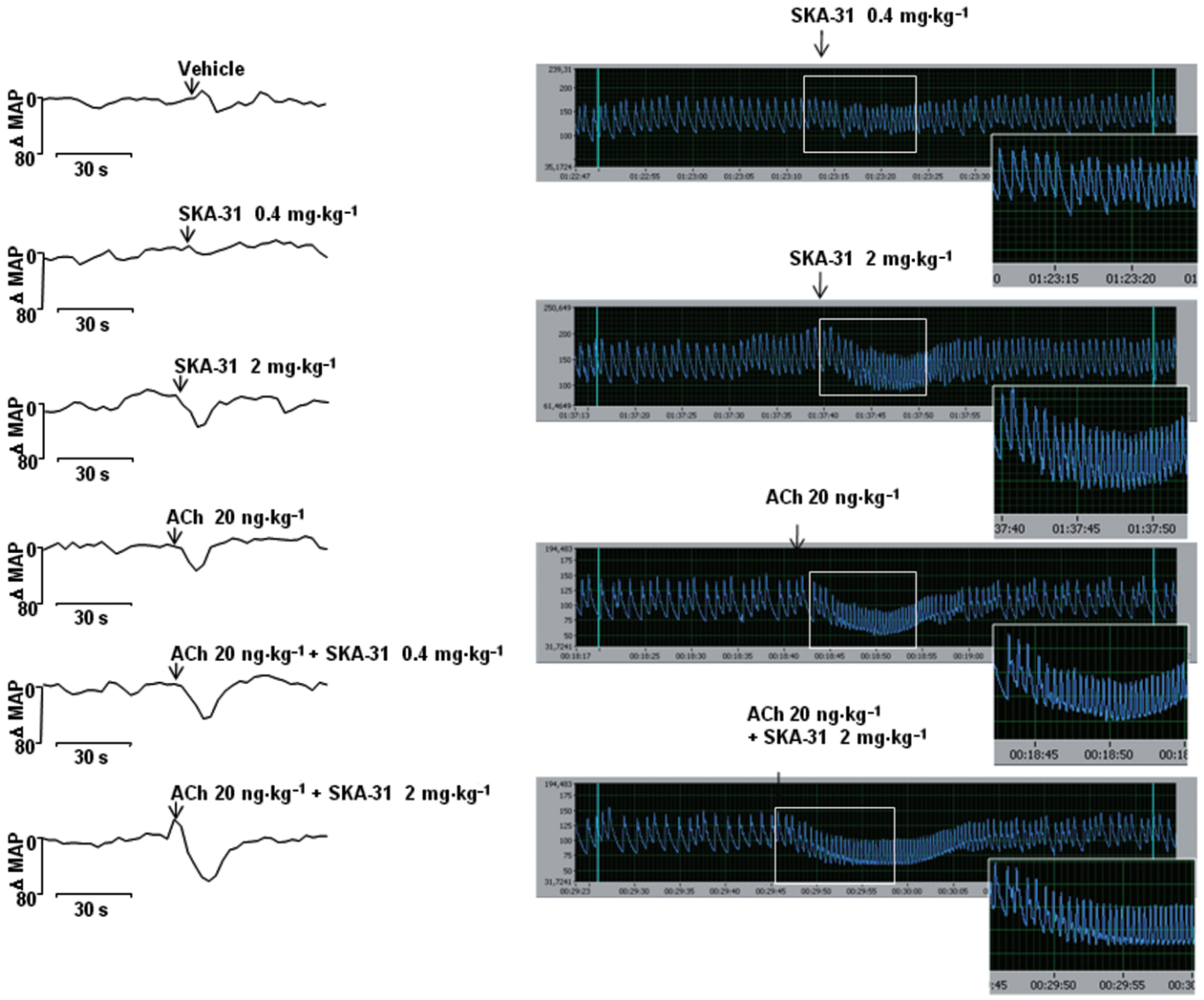

Figure 4

Effects of SKA-31 on blood pressure and ACh-induced depressor responses in conscious and unstressed dogs. Left panel: graphs show representative changes of MAP (ΔMAP, on left) from baseline after i.v. injections of vehicle, 0.4 and 2 mg·kg^{-1} SKA-31, ACh 20 ng·kg^{-1} and combinations hereof. Right panel: original recordings of depressor effects and increases in heart rate. Enlarged cut-outs illustrate that the pressure drops are followed by increases in heart rate within 2–3 s. For statistics see Table 1 and text.

ute to blood pressure regulation (Brähler *et al.*, 2009), and pharmacological activation of these channels has been shown to lower blood pressure in mice (Sankaranarayanan *et al.*, 2009). However, it is not known whether activation of the KCa3.1/KCa2.3-EDHF-system can also lower blood pressure in larger mammals with cardiovascular parameters more comparable with humans. Our present study in dogs showed that the KCa3.1/KCa2.X activator SKA-31 doubled canine endothelial KCa currents. These currents had electrophysiological characteristics similar to those of KCa3.1 and KCa2.3 in carotid endothelial cells from mice (Sankaranarayanan *et al.*, 2009) and in mesenteric EC from humans (Köhler *et al.*, 2000; 2010). The sensitivity of these currents to the selective KCa3.1 blocker TRAM-34 (Wulff *et al.*, 2000) and to the KCa2.X blocker UCL1684 (Rosa *et al.*, 1998) at a concentration of 1 μmol·L^{-1} further supports this interpretation. Likewise, the mRNA expression analysis provided proof of the expression of KCa3.1 and KCa2.3, which is in line with previous findings in human and murine endothelia (Köhler *et al.*, 2000; Brähler *et al.*, 2009). Notably, we observed mRNA for KCa2.1 and KCa2.2 channel genes in mRNA extracts from whole-mount mesenteric artery preparations and from isolated endothelial cells kept in primary culture. Whether the expression of these subtypes contributed to the functional KCa currents in the canine EC or smooth muscle remains unclear. Interestingly, KCa.2.2 expression was previously observed in endothelium and smooth muscle cells of porcine coronary arteries, although the channel proteins appeared to

Table 1

Blood-pressure-lowering effects of SKA-31 in conscious dogs

Treatment	*n*	ΔMAP (mmHg)	AUC MAP (mmHg·min^{-1})	ΔHR (beats min^{-1})	AUC HR (bmp·min^{-1})
Vehicle	2	−2 ± 2	1 ± 10	13 ± 13	−20 ± 5
SKA-31 (0.4 mg·kg^{-1})	2	−2 ± 1	−11 ± 4	61 ± 8#	108 ± 45
SKA-31 (2 mg·kg^{-1})	3	−28 ± 6#	−72 ± 15#	109 ± 7##	354 ± 20##
ACh (20 ng·kg^{-1})	11	−33 ± 3##	−76 ± 11#	91 ± 7##	264 ± 41#
ACh (20 ng·kg^{-1}) + SKA-31 (0.4 mg·kg^{-1})	4	−37 ± 3##	−106 ± 14*##	111 ± 6##	292 ± 60#
ACh (20 ng·kg^{-1}) + SKA-31 (2 mg·kg^{-1})	6	−46 ± 6*##	−177 ± 39*#	111 ± 6##	539 ± 64*##

Data are mean ± SEM, *$P < 0.05$ versus ACh; Student's paired *t*-test. #$P < 0.05$, ##$P < 0.01$ versus vehicle; Student's unpaired *t*-test. ΔMAP, change in MAP; ΔHR, change in HR.

be localized intracellularly (Burnham *et al.*, 2002). The current clamp experiments were performed to selectively study endothelial hyperpolarization responses. These experiments revealed that SKA-31 strongly potentiated endothelial hyperpolarization to ACh, and it also produced hyperpolarization on its own in freshly isolated endothelial cell clusters exhibiting small or no spontaneous hyperpolarization events. These electrophysiological data demonstrated that SKA-31 was able to activate KCa3.1 and KCa2.3 channels from a low basal activity level and, thereby, to elicit strong hyperpolarization from a depolarized membrane potential in this EC preparation. Our wire myography on canine mesenteric arteries showed that EDHF-type vasorelaxations to ACh were small but appreciable in the presence of a potent vasospasmic agent (the thromboxane mimetic, U46619). Nevertheless, SKA-31 doubled this response in all preparations. Interestingly, SKA-31 did not induce vasorelaxation on its own in the presence of a strong vasospasmic agent, suggesting that endothelial KCa channels are incompletely activated under basal conditions in wire myography experiments. This observation is in line with our previous findings in murine carotid arteries using pressure myography (Sankaranarayanan *et al.*, 2009). In summary, we here provided proof of the expression and pharmacological excitability of KCa3.1/KCa2.3 in canine endothelium and of the potentiating effects of SKA-31 on the ACh-induced EDHF system in dogs.

The major novel finding of the present study derived from testing the effects of SKA-31 on blood pressure in trained, conscious and unstressed beagles. At a dose of 2 mg·kg^{-1}, SKA-31 elicited a large depressor response (≈−28 mmHg), which was associated with a decrease in pulse pressure (≈−7 mmHg) and which was relatively short-lived (≈8 s). Notably, the response was accompanied by a robust tachycardia with a – for baroreceptor activation typical – short delay time of ≈2 s (Figure 4), suggesting that the dogs rapidly responded to the pressure drop by baroreceptor activation and a strong immediate increased heart rate (+109 bpm) followed by a small persistent increase (+8 bpm at least over 3 min) to compensate for the drop in peripheral resistance. A lower dose of SKA-31 (0.4 mg·kg^{-1}) produced only a minor hypotensive effect, which was similarly associated with a sizeable and transient increase in heart rate. The more effective depressor response at the higher dose can be explained by the higher total arterial plasma concentration in the low micromolar range. Thus, these findings suggested that SKA-31-activation of KCa3.1 and KCa2.3 and the ensuing endothelial hyperpolarization (Figure 3) can produce an acute and strong depressor response, which is counterbalanced by immediate baroreceptor activation and possibly volume compensation from the abdominal circulation, which in dogs is known to contain about 25% of total blood volume (Delorme *et al.*, 1951; Johnstone, 1956). In this regard, the acute depressor responses reported here were similar to the transient depressor responses and reflex tachycardia elicited by bolus injections of Ca^{2+} antagonists in dogs (Nakaya *et al.*, 1983), although the amplitude of the hypotensive effect elicited by SKA-31 was more pronounced but less long-lasting in this experimental setting.

Our observations further suggested that *in vivo* (and unlike during wire myography experimentation and in the presence of vasospasmic agents), KCa3.1and/or KCa2.3 channels exhibited basal activity, which could be potentiated by SKA-31.

Bolus injections of ACh (20 ng·kg^{-1}), a neurotransmitter with established depressor effects, caused similar short-lasting depressor responses and increases in heart rate owing to a decrease in total peripheral resistance (Gardier *et al.*, 1963) but did not lead to persisting alterations in heart rate, which can be explained by its rapid metabolization and ineffectiveness on cardiac neurotransmission at this low concentration. SKA-31 was able to further enhance the amplitude and duration of this ACh depressor response and of the increase in heart rate and produced, similar to that seen with SKA-31 alone, a persisting higher heart rate (+8 bpm). Supra-additive (potentiating) effects of SKA-31 (0.4 and 2 mg·kg^{-1}) on the ACh depressor response were not evident, however. This observation fits with the notion that each compound on its own is able to produce significant endothelial hyperpolarization, albeit different in kinetics (ACh: fluctuating hyperpolarization vs. SKA-31: stable hyperpolarization; Figure 3).

The large animal model used in the present study allowed us also to address safety issues. One of the main concerns with SKA-31 in the present study was that it increased heart rate. KCa2.X channels were found in mouse and human cardiac

tissue with predominant expression in atrial tissue of single and/or heteromultimers of the channels, and they were suggested to contribute to cardiac repolarization (Tuteja *et al.*, 2005; 2010). However, others re-addressed the role of KCa2.X channels in canine and human atrial and ventricular myocardial strips and found that apamin failed to modify repolarization (Nagy *et al.*, 2009). Nevertheless, NS8593, a negative gating modulator of KCa2.3, and UCL1684 were shown to treat arterial fibrillation in hypertensive and normotensive rats (Diness *et al.*, 2011). Although we cannot exclude a direct effect of SKA-31 on cardiac KCa2.x channels, the simultaneous measurements of HR and MAP in the present study and the apparent delay in the increase in heart rate (Figure 4) after the blood pressure reduction induced by SKA-31, but also by ACh, suggest that baroreceptor activation is involved in the strong immediate increase and the small persisting increase in heart rate associated with SKA-31 bolus injection. A possible limitation of the present study is that it was performed in a small group of trained dogs. Nonetheless, the described depressor response and increase in heart rate caused by SKA-31 were robust and clear-cut.

In conclusion, the present study revealed for the first time that endothelial KCa3.1/KCa2.3 can be used to elicit a depressor response in a large conscious and unstressed mammal with anatomical and functional characteristics similar to the human circulatory system, particularly a very similar HR. The results of the present study further support the concept that pharmacological activation of KCa3.1 (and KCa2.3) produces vasodilatation and decreases blood pressure *in vivo* by causing endothelial hyperpolarization and an EDHF-type vasodilator response. Thus, a small molecule activator of these channels with an improved bioavailability and pharmacokinetic profile (Köhler *et al.*, 2010) may offer a novel type of endothelium-specific treatment of acute severe hypertension and could also be of use for long-term treatment in essential hypertension and/or ischaemic diseases, perhaps as part of a combination therapy with β-adrenoceptor antagonists.

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Conflict of interest statement

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Total SKA-31 plasma concentrations (A) and mean log transformed plasma concentrations (B) plotted against time. Based on data from four dogs, concentrations at time of injection C_0 was extrapolated to the *Y*-intercept, and the volume of distribution (V_D) was calculated to be 6.9 ± 1.5 L·kg^{-1} with the 0.4 mg·kg^{-1} dose. Shown with broken lines are half maximal activation (EC_{50}) values for SKA-31 of KCa2.3 and KCa3.1 channels. Activity testing of plasma (*C*) from SKA-31-treated dogs revealed that 1:75 diluted plasma collected after 1 min following injection of 0.4 mg·kg^{-1} produced significant activation of cloned human KCa3.1 in HEK293 cells (n = 4). The current was approx. 50% of the current activated by 1 μM SKA-31 and was fully blocked by 10 μM TRAM-34. 1:75 diluted plasma collected after 10 min failed to produce sizeable current activation (n = 3) Plasma collected 10 min after injection of 2 mg·kg^{-1} also produced significant activation (n = 4). For statistics, see bar chart in

lower panel. These data indicate that plasma concentrations of SKA-31 1 min after injection of 0.4 mg·kg^{-1} and 10 min after injection of 2 mg·kg^{-1} were high enough for KCa3.1 activation *in vitro*. Solutions and conditions are the same as those for current-clamp experiments.