

Vascular Reactivity Profile of Novel $K_{Ca}3.1$ -Selective Positive-Gating Modulators in the Coronary Vascular Bed

Aida Oliván-Viguera^{1,†}, Marta Sofía Valero^{2,†}, Estéfano Pinilla³, Sara Amor⁴, Ángel Luis García-Villalón⁴, Nichole Coleman⁵, Celia Laría², Víctor Calvín-Tienza², Ángel-Luis García-Otín², José M. Fernández-Fernández⁶, M^a Divina Murillo⁷, José A. Gálvez⁸, María D. Díaz-de-Villegas⁸, Ramón Badorrey⁸, Ulf Simonsen³, Luis Rivera⁹, Heike Wulff⁵ and Ralf Köhler^{1,10}

¹Aragon Institute of Health Sciences & IIS, Zaragoza, Spain, ²Department of Pharmacy, Faculty of Health Sciences, Universidad San Jorge, Villanueva de Gállego, Spain, ³Department of Biomedicine, Pulmonary and Cardiovascular Pharmacology, Aarhus University, Aarhus, Denmark, ⁴Department of Physiology, Faculty of Medicine, Universidad Autónoma de Madrid, Madrid, Spain, ⁵Department of Pharmacology, University of California, Davis, CA, USA, ⁶Laboratori de Fisiologia Molecular i Canalopaties, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain, ⁷Department of Pharmacology and Physiology, Veterinary Faculty, University of Zaragoza, Zaragoza, Spain, ⁸Departamento de Catálisis y Procesos Catalíticos, Instituto de Síntesis Química y Catálisis Homogénea (ISQCH), CSIC - Universidad de Zaragoza, Zaragoza, Spain, ⁹Department of Physiology, Faculty of Pharmacy, Universidad Complutense de Madrid, Madrid, Spain and ¹⁰Aragon Agency for Research and Development (ARAID), Zaragoza, Spain

(Received 27 August 2015; Accepted 17 January 2016)

Abstract: Opening of intermediate-conductance calcium-activated potassium channels ($K_{Ca}3.1$) produces membrane hyperpolarization in the vascular endothelium. Here, we studied the ability of two new $K_{Ca}3.1$ -selective positive-gating modulators, SKA-111 and SKA-121, to (1) evoke porcine endothelial cell $K_{Ca}3.1$ membrane hyperpolarization, (2) induce endothelium-dependent and, particularly, endothelium-derived hyperpolarization (EDH)-type relaxation in porcine coronary arteries (PCA) and (3) influence coronary artery tone in isolated rat hearts. In whole-cell patch-clamp experiments on endothelial cells of PCA (PCAEC), K_{Ca} currents evoked by bradykinin (BK) were potentiated ≈ 7 -fold by either SKA-111 or SKA-121 (both at 1 μ M) and were blocked by a $K_{Ca}3.1$ blocker, TRAM-34. In membrane potential measurements, SKA-111 and SKA-121 augmented bradykinin-induced hyperpolarization. Isometric tension measurements in large- and small-calibre PCA showed that SKA-111 and SKA-121 potentiated endothelium-dependent relaxation with intact NO synthesis and EDH-type relaxation to BK by ≈ 2 -fold. Potentiation of the BK response was prevented by $K_{Ca}3.1$ inhibition. In Langendorff-perfused rat hearts, SKA-111 potentiated coronary vasodilation elicited by BK. In conclusion, our data show that positive-gating modulation of $K_{Ca}3.1$ channels improves BK-induced membrane hyperpolarization and endothelium-dependent relaxation in small and large PCA as well as in the coronary circulation of rats. Positive-gating modulators of $K_{Ca}3.1$ could be therapeutically useful to improve coronary blood flow and counteract impaired coronary endothelial dysfunction in cardiovascular disease.

Altered function of the intermediate-conductance calcium-activated potassium channels ($K_{Ca}3.1$) [1,2] has been suggested to accompany endothelial dysfunction observed in many cardiovascular disease states including diabetes [3] (for extensive review, see [4,5]). However, preserved $K_{Ca}3.1$ functions and ensuing EDH-mediated relaxations have also been reported [6–8]. So, regardless of whether the EDH system is impaired or preserved, pharmacological activation of $K_{Ca}3.1$ might constitute a therapeutic strategy to improve endothelial function in diseased coronary arteries and other vascular beds and may provide cardiovascular protection [4,5,9–12]. This strategy may be advantageous over strategies targeting other vascular and smooth muscle potassium channels such as K_{ATP} channels [13,14], K_{2P} channels [15,16] and large-conductance $K_{Ca}1.1$ channels [17,18] because

$K_{Ca}3.1$ activation particularly favours endothelium-dependent relaxation [5].

The current most selective and potent activator of $K_{Ca}3.1$, the benzothiazole SKA-31 [19] (for recent review, see [5]), which has 10 times higher potency on $K_{Ca}3.1$ channels than on $K_{Ca}2$ channels, has been shown to produce vasodilation in coronary and skeletal muscle vascular beds [20,21], to lower blood pressure in normotensive and angiotensin-II-infused hypertensive mice [19] and to produce a short-lived depressor response in conscious dogs and pigs [22,23]. However, at least in rodents, blood pressure-lowering doses of SKA-31 also produce severe bradycardia [21] and sedation [24]. This may be attributed to the activation of cardiac and neuronal $K_{Ca}2$ channels and therefore limits the use of SKA-31 for cardiovascular conditions.

In the present study, we tested the hypothesis that selective pharmacological activation of $K_{Ca}3.1$ is capable of potentiating endothelial hyperpolarization and endothelium-dependent relaxation to bradykinin (BK). For this purpose, we investigated the effect of the benzothiazole SKA-111 and the benzoxazole SKA-121, two new positive-gating modulators, in porcine large and small coronary arteries (PCA). SKA-111

Author for correspondence: Aida Oliván-Viguera, Aragon Institute of Health Sciences, Unidad de Investigación Traslacional, Hospital Universitario Miguel Servet, Paseo Isabel la Católica, 1-3, Edificio General, Planta Baja, 50009 Zaragoza, Spain (fax: +34 976 769 566, e-mail: aidaolivánviguera@gmail.com).

[†]Contributed equally and share first authorship (in alphabetical order).

and SKA-121 have, respectively, 120 times and 40 times higher selectivity for $K_{Ca3.1}$ channels over K_{Ca2} channels [25]. We show that selective positive-gating modulation of $K_{Ca3.1}$ by SKA-111 and SKA-121 elicited $K_{Ca3.1}$ currents and hyperpolarization in porcine coronary artery endothelial cells (PCAEC) and selectively potentiated BK-induced endothelium-dependent and, particularly, EDH-type relaxation of PCA. In Langendorff-perfused rat hearts, SKA-111 potentiated bradykinin-induced reduction in coronary perfusion pressure (CPP).

Materials and Methods

Porcine coronary artery endothelial cells. PCAEC were isolated from hearts kindly provided by the local slaughterhouse (Matadero Mercazaragoza, Zaragoza) as described previously [26]. In brief, PCAs, with an inner diameter of about 1–2 mm and an outer diameter of 2–3 mm, were carefully dissected and cleaned of surrounding tissue and fat, were cut open longitudinally and incubated in trypsin/EDTA (0.25%/0.02%) in PBS without Ca^{2+}/Mg^{2+} (Biochrom KG, Berlin, Germany) for 30 min. Subsequently, PCAEC were scraped and aspirated from the luminal side using a pipette tip and seeded on cover slips. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (Biochrom KG, Berlin, Germany). Before performing patch-clamp experiments, cover slips were transferred into the NaCl bath solution and the cells were used for electrophysiological measurements within ≈ 36 hr.

Patch-clamp electrophysiology. Whole-cell currents were measured using an EPC10-USB patch-clamp amplifier (HEKA Electronics, Germany) and Patchmaster™ software as described in detail previously [26]. Currents were recorded using voltage ramps (U-ramps, -100 to 100 mV, 1 sec.) and a holding potential of 0 mV. Leak subtraction was omitted during data acquisition, although 'ohmic' leak of up to 0.3 nS was subtracted during analysis, if appropriate. Amplitudes of K^+ -outward currents were measured at 0 mV and normalized to cell capacitance. The standard pipette solution contained calcium to activate K_{Ca} channels and was composed of (in mM): 140 KCl, 1 MgCl₂, 2 EGTA, 1.71 CaCl₂ (1 μ M $[Ca^{2+}]_{free}$) and 5 HEPES (adjusted to pH 7.2 with KOH). In another series of whole-cell experiments, we used a lower calcium concentration in the pipette solution of 0.7 mM ($[Ca^{2+}]_{free}$ 0.1 μ M) to demonstrate that K_{Ca} activation occurs only at a calcium concentration, which is above the threshold for $K_{Ca2.3}/K_{Ca3.1}$ activation. In other experiments, we patch-clamped endothelial cell clusters using the pipette solution with low calcium to monitor current activation during BK and/or 5-HT stimulation in electrically coupled cells. In current-clamp experiments, we measured variations in membrane potential. Here, the calcium concentration in the pipette solution was 0.7 mM ($[Ca^{2+}]_{free}$ 0.1 μ M). In all experiments, the NaCl bath solution was composed of (mM): 140 NaCl, 5 KCl, 1 MgSO₄, 1 CaCl₂, 10 glucose and 10 HEPES (adjusted to pH 7.4 with NaOH). Regarding cell capacitance, single cells had a mean capacitance of 9.4 ± 0.7 pF ($n = 29$ experiments). Cells in clusters were electrically coupled and exhibited higher capacitance values of 101 ± 13 pF ($n = 30$ experiments).

Gene expression studies. RNA isolation and reverse transcription: Total RNA from primary freshly isolated pig aortic endothelial cells was isolated with TriReagent (Sigma, St. Louis, USA) following the manufacturer's protocol and further purified using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany). Quantity and purity of extracted RNA samples were analysed by spectrophotometry

(NanoDrop 1000; ThermoFisher, Waltham, MA, USA) before using them to reverse transcription or storing them at $-80^{\circ}C$ for a later use. Isolated RNA samples were also analysed for integrity and genomic DNA contamination by gel electrophoresis prior to their use in reverse transcription.

RT-PCR was performed by using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Random hexamers were used to synthesize cDNA. In order to detect mRNA expression of the channel genes (K_{Ca} 2.1, K_{Ca} 2.2, K_{Ca} 2.3, K_{Ca} 3.1) and endothelial nitric oxide synthase (eNOS, gene name NOS3), we used a standard PCR protocol with an initial denaturation step at $94^{\circ}C$ for 2 min., 40 cycles ($94^{\circ}C$ denaturation for 15 sec., $53^{\circ}C$ annealing for 30 sec. and $72^{\circ}C$ extension for 30 sec.), followed by a final extension step at $72^{\circ}C$ for 5 min., using a MyCycler thermal cycler (BioRad, Hercules, USA) and Taq polymerase (Stratagene, Birkenfeld, Germany).

Intron-spanning primers were designed by using Primer3 software.

Primer sequences:

β -Actin F, 5'-CACGCCATCCTGCGTCTGGA-3';
 β -Actin R, 5'-AGCACCGTGTGGCGTAGAG-3';
 eNOS F, 5'-AGCCTCCAGAACTCTTTGCT-3';
 eNOS R, 5'-TGCCAATCTCTGTGCTCATG-3';
 $K_{Ca2.1}$ F, 5'-CCAGGACCAGGAAGAGGAAG-3';
 $K_{Ca2.1}$ R, 5'-TGAGTGCAAATGAGTACAGCG-3';
 $K_{Ca2.2}$ F, 5'-TCCTGCTCGGTCTGATCATC-3';
 $K_{Ca2.2}$ R, 5'-GTGGATGGGGCATAGGAGAA-3';
 $K_{Ca2.3}$ F, 5'-GACAACCATGCCATCAGAC-3';
 $K_{Ca2.3}$ R, 5'-TCAGGGCCAACGAAAACATG-3';
 $K_{Ca3.1}$ F, 5'-TCCTGCTCAACGTCTCTCTAC-3';
 $K_{Ca3.1}$ R, 5'-GGTCAGGAATGTGATGGGGA-3'.

PCR products were analysed by gel electrophoresis using 2% agarose in TAE buffer and stained with GelRed (Biotium, Bromma, Sweden). The expected product lengths were 380 bp for β -actin, 198 bp for eNOS, 239 bp for $K_{Ca2.1}$, 208 bp for $K_{Ca2.2}$, 283 bp for $K_{Ca2.3}$ and 233 bp for $K_{Ca3.1}$.

Myography on porcine coronary arteries. Isometric myography on large PCA rings was performed as described previously [27,28]. In brief, rings were mounted onto an isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, UK). The bath solution was a Krebs buffer ($37^{\circ}C$; equilibrated with 95% O₂/5% CO₂) and consisted of (mM): 120 NaCl, 24.5 NaHCO₃, 2.4 CaCl₂, 4.7 KCl, 1.2 MgSO₄, 1 KH₂PO₄ and 5.6 glucose, pH 7.4. Rings were pre-stretched to an initial tension of 1 g (10 mN). Changes in force were recorded using a Mac Lab System/8e program (AD Instruments Inc, Milford, MA, USA) at a sample rate of 0.5 sec. Small coronary arteries (internal lumen diameters of 250–400 μ m) isolated from the left ventricle of hearts obtained at Danish Crown (Horsens, Denmark) were mounted in microvascular myographs and stretched as previously described for lamb coronary arteries [29]. To measure EDH-type relaxation in large PCA, the buffer contained the NO-synthase blocker, *N*- ω -nitro-L-arginine (L-NNA, 300 μ M), and the cyclooxygenase blocker, indomethacin (INDO) (10 μ M). In experiments on small PCA, we used 100 μ M NG-nitro-L-arginine methyl ester (L-NAME) instead of L-NNA. After an equilibration period of 40 min., compounds, alone or in combination, or vehicle (DMSO), were tested as follows: 1) pre-contraction of rings with one or two compounds for 5 min.; 2) pre-contraction with 5-HT (1 μ M, 10 min.); 3) cumulative relaxation with increasing doses of BK (10 nM, 100 nM and 1 μ M, over 10 min.); 4) washout over 10 min. Finally, rings were contracted with KCl (60 mM) buffer for 10 min., and relaxation was induced by adding sodium nitroprusside (10 μ M). In other experimental series, we conducted the experiments in the absence of L-NNA (or L-NAME)

and INDO. Under these conditions, 5-HT caused smaller contractions above the low spontaneous tone in the large PCA (data not shown).

Data analysis. We evaluated absolute increases in force to 5-HT or to 60 mM KCl. EDH-type relaxations were determined as % change of pre-contraction relative to the totally relaxed state (absence of the contracting agents).

Isolated rat heart (Langendorff). Male Sprague Dawley rats were used for these studies (Harlan Interfauna Ibérica S.A., Barcelona, Spain). All animal protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Institute's Animal Care and Use Committee (Comité de Ética de la Investigación, Universidad Autónoma de Madrid). Hearts were perfused in a Langendorff apparatus as described previously [30]. Briefly, hearts were removed from Sprague Dawley rats (300–350 g) under anaesthesia with i.p. sodium pentobarbital (100 mg/kg) and after i.v. injection of heparin (1000 UI). Next, the ascending aorta was cannulated and the heart was subjected to retrograde perfusion with Krebs buffer at 37°C. Perfusion was kept at a constant flow rate of 11–15 ml/min. to provide a basal CPP of 60–70 mmHg. CPP was measured through a lateral connection in the perfusion system. Changes in CPP were recorded using a Mac Lab System/8e program (AD Instruments Inc, Milford, MA, USA) at a sample rate of 0.5 sec. After a 15-min. equilibration period with constant flow perfusion, U46619 (30–60 nM) was infused to achieve a sufficiently high CP. CPP increased from low basal levels (<70 mmHg) to 144 ± 3 mmHg ($n = 10$)., and when the CPP was stable, BK (0.01–1 nM) alone or in combination with SKA-111 (1 μ M) was added to the perfusate and falls of CPP were recorded. In other series, SKA-111 was infused alone at a higher concentration of 10 μ M.

Compounds and chemicals. Compounds were purchased from Sigma/Aldrich, Tocris, Fluorochem, Alfa Aesar, or synthesized in house (HW's laboratory: TRAM-34 [31], SKA-111, SKA-121 [25]; RB's laboratory: RA-2 [32]). Stock solutions (at 1 or 10 mM) were prepared with DMSO, and the final DMSO concentration was always <0.5%, if not stated otherwise.

Statistics. Data are given as mean \pm S.E.M. For comparison of data sets, we used unpaired or paired two-tailed Student's t-test or, in case of multiple comparisons, we used one-way ANOVA followed by the Tukey *post hoc* test. Statistical significance was considered for *p*-values of <0.05.

Results

Gene expression of $K_{Ca3.1}$ and K_{Ca2} channels in PCAEC.

RT-PCR analysis showed that freshly isolated PCAEC express the $K_{Ca3.1}$ and $K_{Ca2.3}$ subtypes as well as $K_{Ca2.2}$ (fig. 1), providing additional evidence for mRNA expression of these channels in PCA endothelial cells [33,34].

Patch-clamp electrophysiology on primary PCAEC.

We performed 'whole-cell' patch-clamp experiments on primary PCA endothelial cells (PCEC). In single cells, infusion of calcium into the cells via the patch-pipette activated K^+ -outward currents that were voltage independent and showed inward rectification at positive membrane potentials (fig. 2A), thus resembling key electrophysiological properties of $K_{Ca3.1}$ channels [1,2,35]. Such current activation was not seen

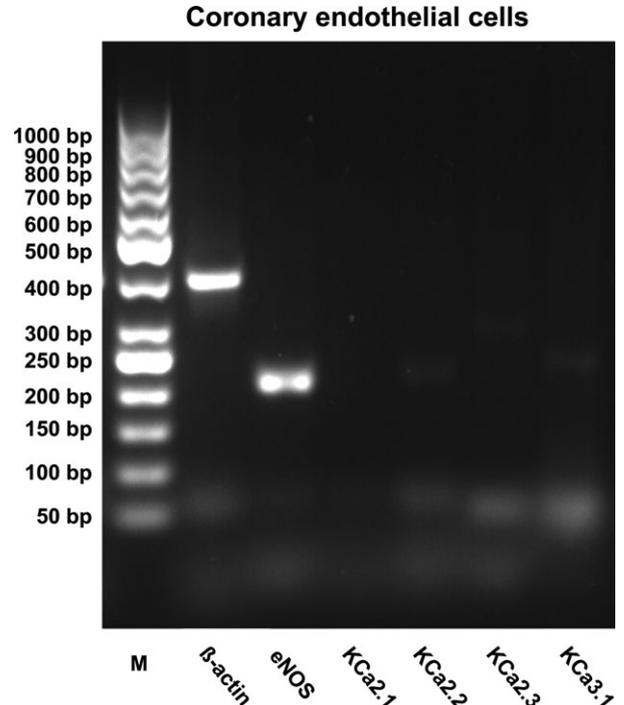


Fig. 1. Analysis of mRNA expression of $K_{Ca2/3}$ subtypes in freshly isolated porcine coronary endothelial cells. The GelRed-stained 2% agarose gel shows RT-PCR products for $K_{Ca2.2}$, $K_{Ca2.3}$ and $K_{Ca3.1}$ (lanes on right) that match expected amplicon sizes (208 bp for $K_{Ca2.2}$, 283 bp for $K_{Ca2.3}$ and 233 bp for $K_{Ca3.1}$). Expression of $K_{Ca2.1}$ (expected amplicon size, 239 bp) is not detected. Expression of beta-actin (expected amplicon, size, 380 bp; first lane from left) serves as positive control. Expression of eNOS (expected amplicon size, 198 bp, second lane from left) indicates endothelial origin of mRNA (lanes on left). M = molecular weight marker. Note the presence of primer dimers in the lower part of the gel.

(fig. 2A) when we used a pipette solution with a free calcium concentration of 100 nM, which is below the threshold for $K_{Ca3.1}$ channel activation (fig. 2A).

The novel positive-gating modulator, SKA-111, with 120 times higher selectivity for $K_{Ca3.1}$ over K_{Ca2} channels [25] potentiated the K_{Ca} -outward currents by 7-fold (fig. 2B, panel on left for traces and panel on right for summary data). SKA-121 potentiated these currents in a similar way (fig. 2C, panel on left for traces and panel on right for summary data).

Potentiated currents were sensitive to the classical $K_{Ca3.1}$ blocker, TRAM-34 [31] (1 μ M, $52 \pm 5\%$ blockage of SKA-111-activated currents, fig. 2B, and $78 \pm 7\%$ blockage of SKA-121-activated currents, summary data in fig. 2C). The K_{Ca2} blocker UCL-1684 [36] (1 μ M) had small additional blocking effects (fig. 2B,C). The negative-gating modulator, RA-2 (1 μ M), that directly suppresses positive-gating modulation [32] potently blocked all K_{Ca} current (not shown) and a small TRAM-34/UCL-1684-resistant K_{Ca} current (fig. 2B,C), which may suggest incomplete block of porcine $K_{Ca3.1}$ by the pore blocker, TRAM-34 [37].

We conducted a series of whole-cell patch-clamp experiments to show that calcium mobilizations to BK or 5-HT were also able to activate $K_{Ca3.1}$ (fig. 3A) and that positive-gating

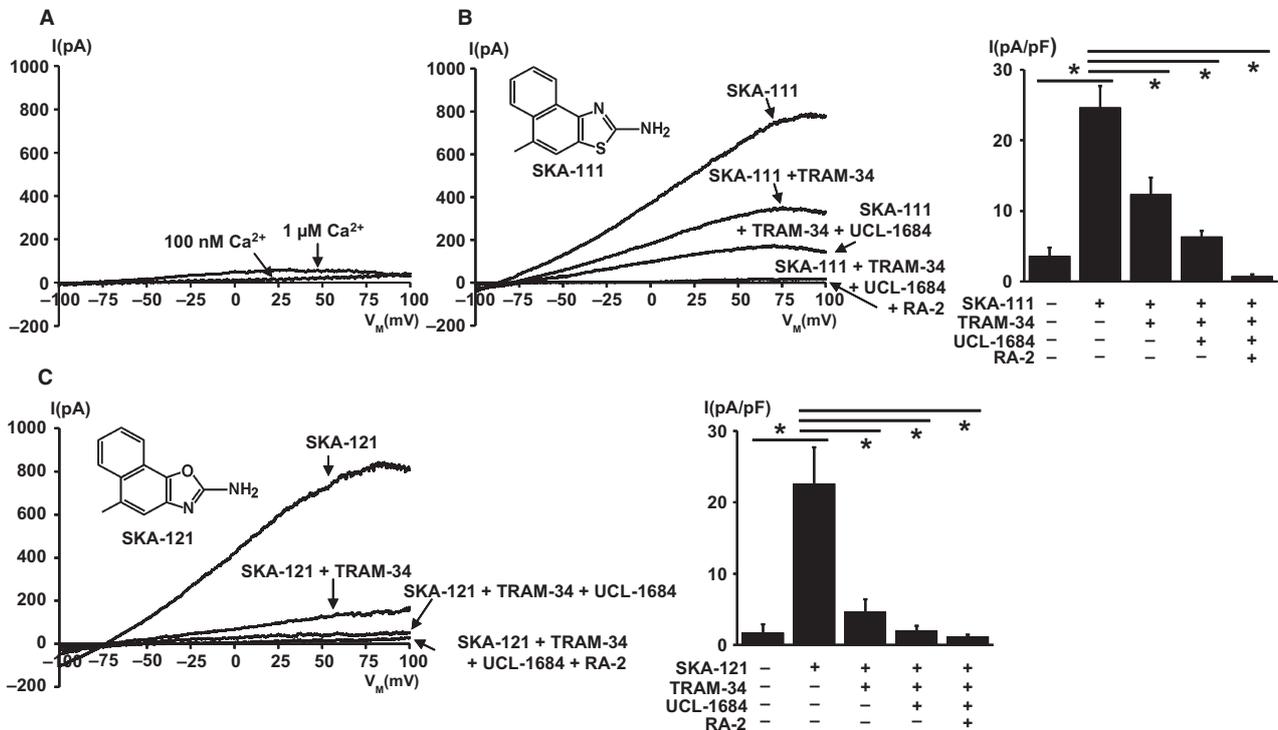


Fig. 2. Whole-cell patch-clamp recordings of K_{Ca} currents in freshly isolated endothelial cells of PCA. (A) Representative recording showing the activation of K_{Ca} currents during infusion of $1 \mu M Ca^{2+}$ into the cells via the patch-pipette, and no activation during infusion of $100 nM Ca^{2+}$. (B) On left: Potentiation of $K_{Ca3.1}$ currents by SKA-111 ($1 \mu M$, $n = 9$) and current inhibition by the $K_{Ca3.1}$ blocker, TRAM-34 ($1 \mu M$, $n = 8$). Note that TRAM-34 in combination with the K_{Ca2} blocker UCL-1684 ($1 \mu M$, $n = 5$) had additional effects by blocking a small K_{Ca2} current. Complete block of TRAM-34/UCL-1684-resistant K currents by the negative-gating modulator, RA-2 ($1 \mu M$, $n = 5$). On right: summary of current data (currents at $0 mV$ normalized to cell capacitance). (C) On left: Potentiation of $K_{Ca3.1}$ currents by SKA-121 ($1 \mu M$, $n = 9$) and block by TRAM-34 ($1 \mu M$, $n = 9$). Note a small additional effect by the combination of TRAM-34 and UCL-1684 ($1 \mu M$, $n = 4$). Complete inhibition of TRAM-34/UCL-1684-resistant K currents by RA-2 ($1 \mu M$, $n = 3$). On right: Current data (at $0 mV$ normalized to cell capacitance). Data points are means \pm S.E.M. $*p < 0.05$. Inserts: Chemical structures of the $K_{Ca3.1}$ -selective positive-gating modulators, the benzothiazole, SKA-111, and the benzoxazole, SKA-121.

modulation of $K_{Ca3.1}$ further potentiated these currents. We found that BK (fig. 3A, left) caused transient activation of outward currents that were long-lastingly augmented by SKA-121. In contrast, 5-HT produced only small outward currents under these conditions. Interestingly, SKA-121 produced large $K_{Ca3.1}$ currents also independently of BK and 5-HT (fig. 3A, right) that suggested some minor basal $K_{Ca3.1}$ activity in these isolated cells, which could be potentiated by SKA-121.

Next, we conducted a series of current-clamp experiments on primary PCAEC clusters to investigate whether $K_{Ca3.1}$ positive-gating modulators augment membrane potential changes in response to BK and 5-HT. As shown in fig. 3B, we first tested the effects of 5-HT and BK alone and found that 5-HT ($1 \mu M$) or BK ($1 \mu M$) shifted membrane potentials from depolarized values ($\approx 1 mV$) to negative values, with 5-HT producing small responses ($\approx \Delta -10 mV$) and BK producing stronger responses ($\approx \Delta -27 mV$). The negative-gating modulator, RA-2, reversed the responses. In contrast, the positive-gating modulator, SKA-121, augmented the agonist-induced response ($\approx \Delta -65 mV$, $p < 0.05$; for trace, see fig. 3B). This augmented response was likewise sensitive to RA-2. Importantly, SKA-121 was capable of producing hyperpolarization by itself. However, the amplitude of this hyperpolarization was smaller

($\approx \Delta -42 mV$) than the amplitudes seen after stimulation of BK in combination with SKA-121 (see traces in fig. 3B). Moreover, the time course of hyperpolarization was different: BK elicited maximal responses within a few seconds ('time-to-peak', fig. S1), which can be expected for an agonist that binds to its GPCR and causes rapid IP_3 -mediated calcium release from the endoplasmic reticulum. In contrast, maximal responses to SKA-121 were achieved after approximately half a minute. The ability of SKA-121 to produce hyperpolarization in its own right can be explained by the activation of a few $K_{Ca3.1}$ channels exhibiting basal activity and normally only causing insignificant membrane potential changes but giving rise to a slow hyperpolarization response when potentiated by SKA-121.

EDH-type relaxation.

Isometric tension measurements on large PCA in the presence of L-NNA and INDO to block NO and prostacyclin syntheses showed that contractions of PCA in response to 5-HT ($1 \mu M$) were of similar amplitude in the presence of SKA-111 ($1 \mu M$) or SKA-121 ($1 \mu M$) as those in the presence of the vehicle, DMSO (fig. S2A). Thus, SKA-111 and SKA-121 at

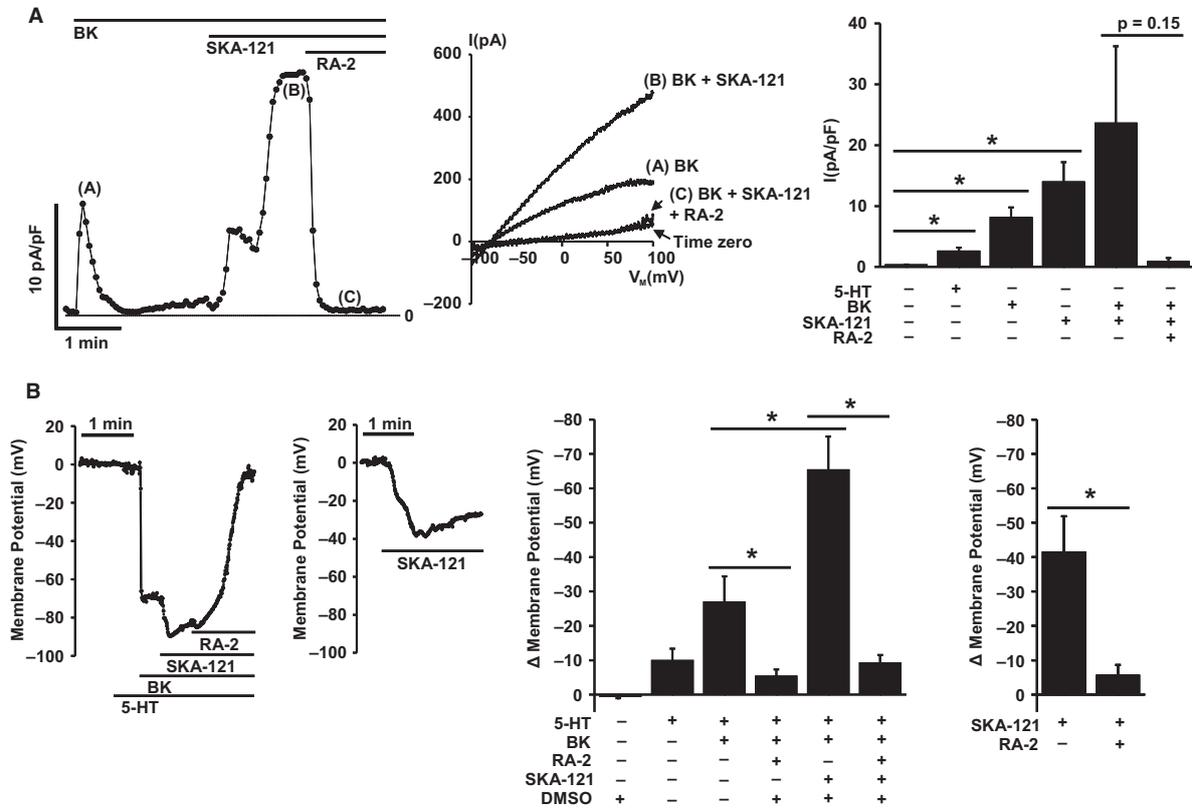


Fig. 3. (A) Whole-cell patch-clamp recordings of K_{Ca} currents in PCAEC clusters. On left, time course of the effect of BK, SKA-121 and RA-2 on $K_{Ca3.1}$ currents. Note the double peak with the second peak being larger in some of the experiments. This could be interpreted as a positive feedback of K_{Ca} -induced hyperpolarization on the calcium influx that further increases K_{Ca} currents. In middle, representative recording of activation of $K_{Ca3.1}$ by BK, BK + SKA-121 and blockade of currents by RA-2. On right, summary data (currents at 0 mV normalized to cell capacitance); 5-HT (1 μ M, $n = 3$), BK (1 μ M, $n = 4$), SKA-121 (1 μ M, $n = 3$), BK + SKA-121 ($n = 3$) and current inhibition by RA-2 (1 μ M, $n = 3$). (B) Membrane potential measurements in PCAEC cluster in the current-clamp mode. On left, representative traces. On right: summary data: 5-HT (1 μ M, $n = 15$), BK (1 μ M, $n = 13$), RA-2 (1 μ M, $n = 7$), SKA-121 (1 μ M, $n = 6$), SKA-121 after 5-HT and BK (1 μ M each, $n = 5$). The latter hyperpolarization responses were reversed by RA-2 ($n = 3$ after 5-HT+BK and $n = 8$ after 5-HT+BK+SKA-121). Data points are means \pm S.E.M.; * $p < 0.05$.

concentrations $\leq 1 \mu$ M at which the compounds selectively activate $K_{Ca3.1}$ did not influence contraction or produce vasorelaxation on their own.

With respect to endothelium-dependent EDH-type relaxation, SKA-111 and SKA-121 significantly augmented the BK-induced EDH-type relaxation in large PCA (fig. 4A, for summary data; fig. S3 for original traces). Fitting of the data revealed a left shift of the concentration–response curve (see table S1 for EC_{50} values). TRAM-34 (1 μ M) reduced the SKA-111-potentiated response (fig. 4B) and the combination of TRAM-34 and UCL-1684 (1 μ M) had no additional effect. Data for SKA-121 are shown in fig. S4A. RA-2, a pan-negative-gating modulator of both $KCa3.1$ and $KCa2$ channels, was equally effective in inhibiting the SKA-111-potentiated BK response (fig. S4B). BK-induced relaxation in the absence of SKA-111 and SKA-121 (fig. S4C,D) was inhibited by the combination of TRAM-34 and UCL-1684 as well as by RA-2.

None of the compounds significantly altered KCl-induced contractions and sodium nitroprusside-induced relaxations in large PCA (fig. S2B,C).

In small porcine coronary arteries, we found essentially the same: 1 μ M SKA-111 by itself did not change U46619-induced contractions ($n = 6$) (fig. S5), but SKA-111 markedly leftward-shifted concentration–response curves for BK-induced EDH responses (fig. 4C; for EC_{50} values, see table S1). In the presence of TRAM-34 or the combination of TRAM-34 and UCL1684, SKA-111 did not change the concentration responses for BK (fig. 4C).

Together, these data demonstrate that BK selectively activates $K_{Ca3.1}$ to produce EDH-type relaxation and that positive-gating modulation of $K_{Ca3.1}$ is capable of potentiating this response in large- and small-calibre PCA.

Endothelium-dependent relaxation with NO and prostaglandin syntheses intact.

In another series of experiments, we omitted blockers of NO and prostaglandin syntheses from the bath solution in order to evaluate the effects of the channel modulators with uncompromised synthesis of NO and the vasorelaxant prostacyclin. Positive-gating modulation of $K_{Ca3.1}$ showed a trend to potentiate

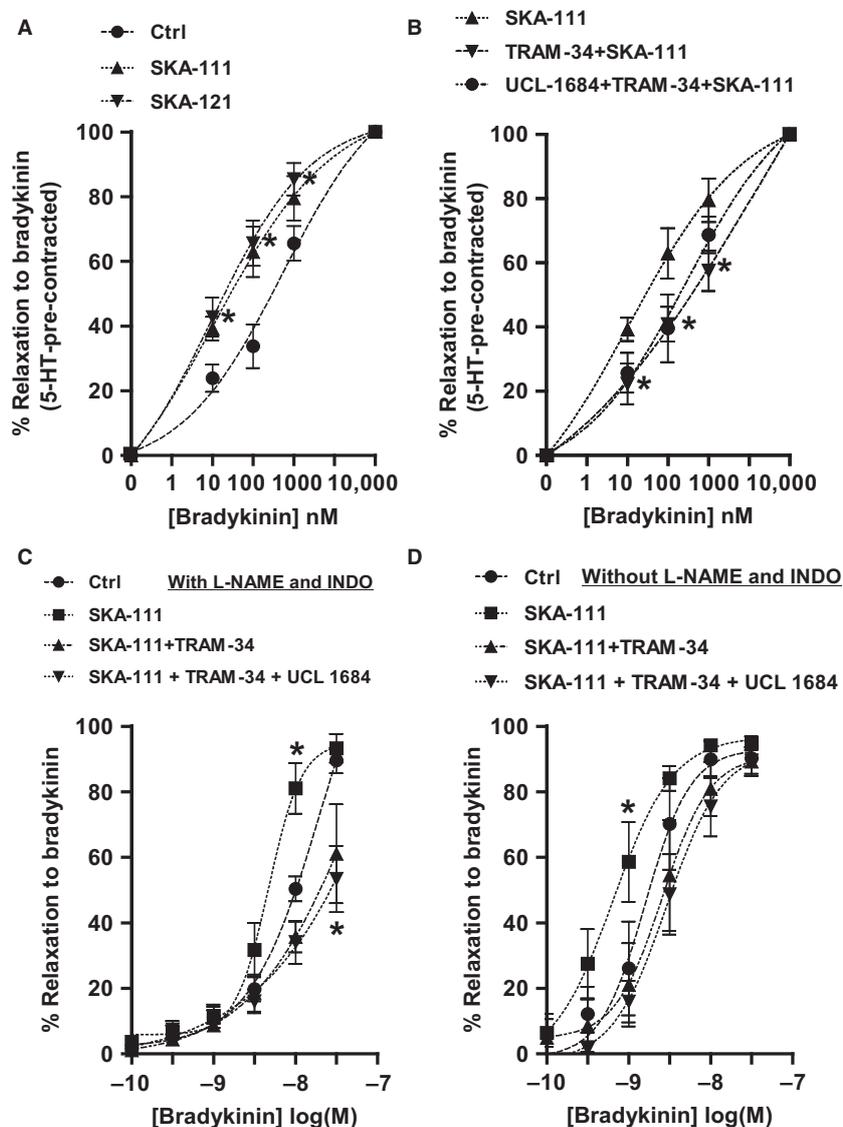


Fig. 4. Potentiation of endothelium-dependent relaxation in large- and small-calibre porcine coronary artery. (A) EDH-type relaxation: SKA-111 and SKA-121, both at 1 μ M, potentiated BK-induced relaxation in the presence of L-NNA (300 μ M) and INDO (10 μ M). Control (Ctrl) was BK-induced relaxation in the presence of the vehicle, DMSO (<0.5%). (B) TRAM-34 (1 μ M) reduced the SKA-111-potentiated BK-induced EDH-type relaxations. The combination of TRAM-34 and UCL-1684 (1 μ M) had similar effects. (C) Similarly, SKA-111 at 1 μ M potentiated BK-induced relaxation of small-calibre PCA in the presence of L-NAME and INDO. Inhibition of potentiation by TRAM-34 or by the combination of TRAM-34 and UCL-1684. (D) Potentiation of BK-induced endothelium-dependent relaxation at intact NO and prostaglandin syntheses in small-calibre PCA and inhibition of potentiation by TRAM-34 or by the combination of TRAM-34 and UCL-1684. Data points (each 5–8) are means \pm S.E.M. and were fitted with the Boltzmann equation. For clarity and mathematical reasons (curve fit and EC_{50} calculation), curves for large PCA were forced through 0% and 100% as indicated by black squares. * p < 0.05 versus control (DMSO).

BK-induced relaxations in the large PCA, although the difference did not reach a statistical significance (fig. S6A). Yet, such a trend was not seen when testing a positive-gating modulator of $K_{Ca3.1}$ in combination with the negative-gating modulator of $K_{Ca3.1}$, RA-2 (fig. S6B; for calculated EC_{50} values, see table S1). In contrast, in the small PCA, SKA-111 effectively leftward-shifted concentration–response curves for BK under these conditions (fig. 4D) as it did in the presence of L-NAME and INDO. TRAM-34 alone or the blocker combination, TRAM-34 and UCL1684, suppressed this potentiation (fig. 4D).

Together, these data suggested that $K_{Ca3.1}$ -selective positive-gating modulation also caused a significant potentiation of endothelium-dependent relaxations in small-calibre PCA when NO/prostaglandin synthesis was intact.

Experiments in isolated rat hearts.

We next performed Langendorff experiments on rat hearts and tested whether positive-gating modulation of $K_{Ca3.1}$ potentiates BK-induced vasodilation in the coronary vascular bed (measured as a decrease in coronary perfusion pressure

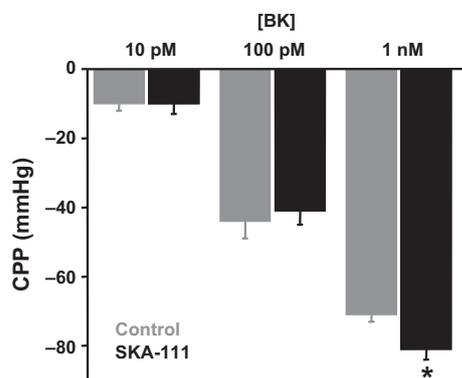


Fig. 5. Langendorff experiments on rat heart. At 1 μ M, SKA-111 potentiates significantly the fall in coronary perfusion pressure (CPP) induced by 1 nM BK in the presence of the vasoconstrictor, U46619. Data points are means \pm S.E.M. Control = BK w/o SKA-111. * $p < 0.05$.

(CPP). BK evoked reduction in CPP in a concentration-dependent fashion (fig. 5). Co-infusion of SKA-111 at 1 μ M significantly potentiated this response at 1 nM BK by 14% (≈ 10 mmHg), but not at lower BK concentrations (fig. 5). Like in myography experiments, infusion of 1 μ M SKA-111 alone did not change perfusion pressure (not shown). However, at a higher concentration of 10 μ M, at which SKA-111 becomes less selective and can activate K_{Ca2} channels, SKA-111 caused a substantial fall in CPP (-61 ± 2 mmHg, $n = 4$) that was clearly different from vehicle control (0.1% DMSO, -12 ± 2 mmHg, $n = 2$). This fall in CPP was as pronounced as the fall caused by a high BK concentration of 100 nM (fig. S7).

In sum, these data suggest that selective positive-gating modulation of $K_{Ca3.1}$ by SKA-111 is capable of improving BK-induced coronary dilations in the isolated rat heart.

Discussion

The goal of the present study was to test whether two new positive-gating modulators, SKA-111 and SKA-121, with an improved selectivity profile for $K_{Ca3.1}$ over K_{Ca2} channels could improve endothelial hyperpolarization and endothelium-dependent vasorelaxation in coronary arteries. We found that (1) PCAEC exhibited calcium-dependent activity of $K_{Ca3.1}$ currents and $K_{Ca3.1}$ gene expression. SKA-111 and SKA-121 potentiated calcium- as well as BK-induced $K_{Ca3.1}$ currents, while TRAM-34 inhibited the current. (2) Positive-gating modulation of $K_{Ca3.1}$ augmented hyperpolarization of PCAEC to BK and elicited slow hyperpolarization by itself. (3) Positive-gating modulation of $K_{Ca3.1}$ potentiated BK-induced endothelium-dependent relaxation and, particularly, EDH-type relaxations in small- and large-calibre PCA. (4) In Langendorff-perfused hearts, positive-gating modulation of $K_{Ca3.1}$ potentiated BK-induced changes in CPP as a measure of coronary vasodilation. Together, the present data suggest that $K_{Ca3.1}$ -selective positive-gating modulators have potential

utility for pharmacological manipulation of blood flow in the coronary circulation.

Endothelial $K_{Ca3.1}$ and $K_{Ca2.3}$ channels have been reported to mediate a significant part of EDH-type relaxations in response to receptor stimulation in rodents, pigs and human beings (for in-depth review, see [12,38]). However, subtype-specific roles of $K_{Ca3.1}$ in endothelium-dependent relaxations in the coronary circulation have not been established so far, and it remains unclear whether selective positive-gating modulation of $K_{Ca3.1}$ elicits vasorelaxation in the coronary arteries and/or potentiates agonist-induced vasorelaxation. The main outcome of the present *in vitro* study on PCA was that we could demonstrate that $K_{Ca3.1}$ channels contribute to BK-induced EDH-type relaxation in small-calibre PCA in a substantial fashion and that this contribution could be further potentiated by SKA-111 and SKA-121. A major role of $K_{Ca2.3}$ was not evident for these EDH-type responses in PCA because the $K_{Ca3.1}$ blocker TRAM-34 inhibited the response, while the K_{Ca2} blocker, UCL-1684, in combination with TRAM-34 had no additional effects on either the potentiated response or the non-potentiated response. Yet, it is worth to mention that inhibition of $K_{Ca3.1}$ did not completely suppress EDH-type relaxation, suggesting that other endothelium-derived relaxation factors such as prostacyclin or diffusible EDHF(s) [4,39,40] may play additional roles here. We further found that positive-gating modulation of $K_{Ca3.1}$ was still able to augment BK-induced relaxation when nitric oxide and prostaglandin syntheses were intact. This was particularly evident in small-calibre PCA while potentiation was only seen by trend in the large-calibre PCA. This suggests that positive-gating modulation of $K_{Ca3.1}$ in the porcine heart is an effective way to improve endothelium-dependent relaxation in a physiological setting as well as under conditions of compromised NO or prostacyclin synthesis or action.

It is worth mentioning that SKA-111 and SKA-121 were unable to produce relaxation by themselves in PCA. This was unexpected because positive-gating modulation by SKAs was capable of producing significant hyperpolarization of PCAEC *in vitro*. However, it needs to be considered that freshly isolated endothelial cells are in an altered state (as indicated by their depolarized membrane potential) and slow hyperpolarization to SKAs may result from potentiating background $K_{Ca3.1}$ activity due to elevated basal $[Ca^{2+}]_i$. Alternatively, positive-gating modulation of $K_{Ca3.1}$ may have a stronger vasorelaxant impact in a situation of endothelial depolarization in order to restore the endothelial resting membrane potential and to provide a negative feedback (repolarization) on agonist-induced depolarization and tone as suggested previously [41]. The Langendorff experiments on isolated rat heart largely agree with our findings from vessel myography by showing that $K_{Ca3.1}$ -selective positive-gating modulation was capable of potentiating BK-induced reduction in coronary resistance.

Conclusions

Coronary artery disease is associated with compromised coronary blood flow and myocardial ischaemia. Therefore, from the

translational clinical perspective, selective positive-gating modulators of $K_{Ca3.1}$ could be of therapeutic value to reverse ischaemia and improve selectively endothelium-dependent and/or agonist-induced vasodilation in the coronary circulation.

Authorship Contributions

AOV, MSV, JMFF, LR, HW, US and RK participated in research design. AOV, MSV, SA, ALGV, CL, VCT, EP and LR conducted the experiments. AOV, MDDV, JAG, RB, MDM, ALGO, ALGV, NC, LR, HW and RK contributed new reagents or analytic tools. AOV, MSV, SA, ALGV, EP, LR, VCT, ALGO, US, HW and RK performed data analysis. AOV, MSV, ALGO, ALGV, JMFF, LR, US, HW and RK wrote or contributed to the writing of the manuscript.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft [KO1899/11-1 to RK]; the Danish Heart Foundation (to RK and US), European Community [FP7-PEOPLE Project 321721]; Department of Industry & Innovation, Government of Aragon [GIPASC-B105 to ALGO, RK, AOV]; and the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III [RIC RD12/0042/0014 Red HERACLES, to RK]. This study received Grant from the Government of Aragón [GA E-102 to JAG, MDD, RB]; Grant of the Spanish Ministry of Economy and Competitiveness plus FEDER Funds [no SAF2012-31631 to LR and SAF2012-31089 to JMFF]; Grants from the National Institute of Neurological Disorders and Stroke [R21NS072585 to HW]; and from the National Heart, Lung & Blood Institute T32 Training Program in Basic and Translational Cardiovascular Science [T32HL086350 to NC].

Disclosures and Competing Interests

HW, NC, RK and AOV are named as inventors on a University of California patent claiming SKA-121 and related benzoxazoles as antihypertensives.

JAG, MDDV, RB, AOV and RK are named as inventors on an international patent (PCT/ES2015/070662) filed by the Aragon Institute of Health Sciences and joining institutions claiming RA-2 as tool for negative-gating modulation of K_{Ca} channels.

References

- Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J. A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci U S A* 1997;**94**:11651–6.
- Wei AD, Gutman GA, Aldrich R, Chandy KG, Grissmer S, Wulff H. International Union of Pharmacology. LII. Nomenclature and molecular relationships of calcium-activated potassium channels. *Pharmacol Rev* 2005;**57**:463–72.
- Brondum E, Kold-Petersen H, Simonsen U, Aalkjaer C. NS309 restores EDHF-type relaxation in mesenteric small arteries from type 2 diabetic ZDF rats. *Br J Pharmacol* 2010;**159**:154–65.
- Feletou M, Köhler R, Vanhoutte PM. Endothelium-derived vasoactive factors and hypertension: possible roles in pathogenesis and as treatment targets. *Curr Hypertens Rep* 2010;**12**:267–75.
- Wulff H, Köhler R. Endothelial small-conductance and intermediate-conductance KCa channels: an update on their pharmacology and usefulness as cardiovascular targets. *J Cardiovasc Pharmacol* 2013;**61**:102–12.
- Thollon C, Fournet-Bourguignon MP, Saboureau D, Lesage L, Reure H, Vanhoutte PM *et al.* Consequences of reduced production of NO on vascular reactivity of porcine coronary arteries after angioplasty: importance of EDHF. *Br J Pharmacol* 2002;**136**:1153–61.
- Waeckel L, Bertin F, Clavreul N, Damery T, Köhler R, Paysant J *et al.* Preserved regulation of renal perfusion pressure by small and intermediate conductance K channels in hypertensive mice with or without renal failure. *Pflügers Arch* 2015;**467**:817–31.
- Climent B, Moreno L, Martinez P, Contreras C, Sanchez A, Perez-Vizcaino F *et al.* Upregulation of SK3 and IK1 channels contributes to the enhanced endothelial calcium signaling and the preserved coronary relaxation in obese Zucker rats. *PLoS ONE* 2014;**9**:e109432.
- Simonet S, Isabelle M, Bousquenaud M, Clavreul N, Feletou M, Vayssettes-Courchay C *et al.* KCa 3.1 channels maintain endothelium-dependent vasodilatation in isolated perfused kidneys of spontaneously hypertensive rats after chronic inhibition of NOS. *Br J Pharmacol* 2012;**167**:854–67.
- Yang Q, Huang JH, Man YB, Yao XQ, He GW. Use of intermediate/small conductance calcium-activated potassium-channel activator for endothelial protection. *J Thorac Cardiovasc Surg* 2011;**141**:501–10.
- Hasenau AL, Nielsen G, Morisseau C, Hammock BD, Wulff H, Köhler R. Improvement of endothelium-dependent vasodilations by SKA-31 and SKA-20, activators of small- and intermediate-conductance Ca^{2+} -activated K^{+} -channels. *Acta Physiol (Oxf)* 2011;**203**:117–26.
- Feletou M. Calcium-activated potassium channels and endothelial dysfunction: therapeutic options? *Br J Pharmacol* 2009;**156**:545–62.
- Abdel-Raheem IT, Taye A, Abouzied MM. Cardioprotective effects of nicorandil, a mitochondrial potassium channel opener against doxorubicin-induced cardiotoxicity in rats. *Basic Clin Pharmacol Toxicol* 2013;**113**:158–66.
- Zingman LV, Alekseev AE, Hodgson-Zingman DM, Terzic A. ATP-sensitive potassium channels: metabolic sensing and cardioprotection. *J Appl Physiol* 2007;**103**:1888–93.
- Nielsen G, Wandall-Frostholm C, Sadda V, Oliván-Viguera A, Lloyd EE, Bryan RM Jr *et al.* Alterations of N-3 polyunsaturated fatty acid-activated K2P channels in hypoxia-induced pulmonary hypertension. *Basic Clin Pharmacol Toxicol* 2013;**113**:250–8.
- Judge SI, Smith PJ. Patents related to therapeutic activation of K (ATP) and K(2P) potassium channels for neuroprotection: ischemic/hypoxic/anoxic injury and general anesthetics. *Expert Opin Ther Pat* 2009;**19**:433–60.
- Singh H, Lu R, Bopassa JC, Meredith AL, Stefani E, Toro L. MitoBK(Ca) is encoded by the *Kcnnml* gene, and a splicing sequence defines its mitochondrial location. *Proc Natl Acad Sci USA* 2013;**110**:10836–41.
- Kiraly I, Pataricza J, Bajory Z, Simonsen U, Varro A, Papp JG *et al.* Involvement of large-conductance $Ca(2+)$ -activated $K(+)$ channels in both nitric oxide and endothelium-derived hyperpolarization-type relaxation in human penile small arteries. *Basic Clin Pharmacol Toxicol* 2013;**113**:19–24.
- Sankaranarayanan A, Raman G, Busch C, Schultz T, Zimin PI, Hoyer J *et al.* Naphtho[1,2-d]thiazol-2-ylamine (SKA-31), a new activator of $KCa2$ and $KCa3.1$ potassium channels, potentiates the endothelium-derived hyperpolarizing factor response and lowers blood pressure. *Mol Pharmacol* 2009;**75**:281–95.
- Mishra RC, Belke D, Wulff H, Braun AP. SKA-31, a novel activator of SK(Ca) and IK(Ca) channels, increases coronary flow in male and female rat hearts. *Cardiovasc Res* 2013;**97**:339–48.
- Radtke J, Schmidt K, Wulff H, Köhler R, de Wit C. Activation of $KCa3.1$ by SKA-31 induces arteriolar dilatation and lowers blood

- pressure in normo- and hypertensive connexin40-deficient mice. *Br J Pharmacol* 2013;**170**:293–303.
- 22 Damkjaer M, Nielsen G, Bodendiek S, Staehr M, Gramsbergen JB, de Wit C *et al.* Pharmacological activation of KCa_{3.1}/KCa_{2.3} channels produces endothelial hyperpolarization and lowers blood pressure in conscious dogs. *Br J Pharmacol* 2012;**165**:223–34.
- 23 Mishra RC, Mitchell JR, Gibbons-Kroeker C, Wulff H, Belenkie I, Tyberg JV *et al.* A pharmacologic activator of endothelial KCa channels increases systemic conductance and reduces arterial pressure in an anesthetized pig model. *Vascul Pharmacol* 2015 Aug 1. pii: S1537-1891(15)00176-7. doi: 10.1016/j.vph.2015.07.016. [Epub ahead of print].
- 24 Lambertsen KL, Gramsbergen JB, Sivasaravanaparan M, Ditzel N, Sevelsted-Moller LM, Oliván-Viguera A *et al.* Genetic KCa_{3.1}-deficiency produces locomotor hyperactivity and alterations in cerebral monoamine levels. *PLoS ONE* 2012;**7**:e47744.
- 25 Coleman N, Brown BM, Oliván-Viguera A, Singh V, Olmstead MM, Valero MS *et al.* New positive Ca²⁺-activated K⁺ channel gating modulators with selectivity for KCa_{3.1}. *Mol Pharmacol* 2014;**86**:342–57.
- 26 Oliván-Viguera A, Valero MS, Murillo MD, Wulff H, Garcia-Otin AL, Arbones-Mainar JM *et al.* Novel phenolic inhibitors of small/intermediate-conductance Ca²⁺-activated K⁺ channels, KCa_{3.1} and KCa_{2.3}. *PLoS ONE* 2013;**8**:e58614.
- 27 Alda JO, Valero MS, Pereboom D, Gros P, Garay RP. Endothelium-independent vasorelaxation by the selective alpha estrogen receptor agonist propyl pyrazole triol in rat aortic smooth muscle. *J Pharm Pharmacol* 2009;**61**:641–6.
- 28 Valero MS, Pereboom D, Barcelo-Batlloay S, Brines L, Garay RP, Alda JO. Protein kinase A signalling is involved in the relaxant responses to the selective beta-oestrogen receptor agonist diarylpropionitrile in rat aortic smooth muscle in vitro. *J Pharm Pharmacol* 2011;**63**:222–9.
- 29 Simonsen U, Garcia-Sacristan A, Prieto D. Apamin-sensitive K⁺ channels involved in the inhibition of acetylcholine-induced contractions in lamb coronary small arteries. *Eur J Pharmacol* 1997;**329**:153–63.
- 30 Garcia-Villalón AL, Granado M, Monge L, Fernandez N, Carreno-Tarragona G, Amor S. Purinergic component in the coronary vasodilatation to acetylcholine after ischemia-reperfusion in perfused rat hearts. *J Vasc Res* 2014;**51**:283–9.
- 31 Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, Chandy KG. Design of a potent and selective inhibitor of the intermediate-conductance Ca²⁺-activated K⁺ channel, IKCa₁: a potential immunosuppressant. *Proc Natl Acad Sci USA* 2000;**97**:8151–6.
- 32 Oliván-Viguera A, Valero MS, Coleman N, Brown BM, Laria C, Murillo MD *et al.* A novel pan-negative-gating modulator of KCa_{2/3} channels, the fluoro-di-benzoate, RA-2, inhibits EDH-type relaxation in coronary artery and produces bradycardia in vivo. *Mol Pharmacol* 2015;**87**:1–12.
- 33 Bychkov R, Burnham MP, Richards GR, Edwards G, Weston AH, Feletou M *et al.* Characterization of a charybdotoxin-sensitive intermediate conductance Ca²⁺-activated K⁺ channel in porcine coronary endothelium: relevance to EDHF. *Br J Pharmacol* 2002;**137**:1346–54.
- 34 Burnham MP, Bychkov R, Feletou M, Richards GR, Vanhoutte PM, Weston AH *et al.* Characterization of an apamin-sensitive small-conductance Ca(2+)-activated K(+) channel in porcine coronary artery endothelium: relevance to EDHF. *Br J Pharmacol* 2002;**135**:1133–43.
- 35 Köhler M, Hirschberg B, Bond CT, Kinzie JM, Marrion NV, Maylie J *et al.* Small-conductance, calcium-activated potassium channels from mammalian brain. *Science* 1996;**273**:1709–14.
- 36 Rosa JC, Galanakis D, Ganellin CR, Dunn PM, Jenkinson DH. Bis-quinolinium cyclophanes: 6,10-diaza-3(1,3),8(1,4)-dibenzena-1,5(1,4)-diquinolincyclodecaphane (UCL 1684), the first nanomolar, non-peptidic blocker of the apamin-sensitive Ca²⁺-activated K⁺ channel. *J Med Chem* 1998;**41**:2–5.
- 37 Wulff H, Gutman GA, Cahalan MD, Chandy KG. Delineation of the clotrimazole/TRAM-34 binding site on the intermediate conductance calcium-activated potassium channel, IKCa₁. *J Biol Chem* 2001;**276**:32040–5.
- 38 Köhler R, Ruth P. Endothelial dysfunction and blood pressure alterations in K⁺-channel transgenic mice. *Pflugers Arch* 2010;**459**:969–76.
- 39 Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I *et al.* Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature* 1999;**401**:493–7.
- 40 Campbell WB, Gauthier KM. Inducible endothelium-derived hyperpolarizing factor: role of the 15-lipoxygenase-EDHF pathway. *J Cardiovasc Pharmacol* 2013;**61**:176–87.
- 41 Crane GJ, Gallagher N, Dora KA, Garland CJ. Small- and intermediate-conductance calcium-activated K⁺ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. *J Physiol* 2003;**553**:183–9.

Supporting Information

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

Fig. S1. Time to maximal hyperpolarization (time to peak) during stimulation with 5-HT (1 μM, n = 10), BK (1 μM, n = 11), and SKA-121 (1 μM, n = 12).

Fig. S2. Isometric tension recording on PCA rings: (A) SKA-111 and SKA-121 (1 μM) did not modulate 5-HT-induced tension in the presence of L-NNA and INDO. Ctrl was the vehicle control (DMSO).

Fig. S3. Original recordings of EDH-type relaxation to bradykinin (BK) in 5-HT-pre-contracted PCA and of the potentiating effect of the KCa_{3.1} positive-gating modulator, SKA-111 (upper panel, on right).

Fig. S4. (A) TRAM-34 (1 μM) reduced SKA-121-potentiated BK-induced EDH-type relaxations.

Fig. S5. Lack of a direct vasorelaxing effect of SKA-111 in pre-contracted small-caliber (internal diameter 300–400 μm) PCA.

Fig. S6. Effects of the KCa_{3.1}-selective positive-gating modulator, SKA-121, on vasorelaxation of large PCA with intact NO and prostaglandin synthesis.

Fig. S7. Langendorff experiments on rat heart. With similar efficacies, SKA-111 (10 μM) and BK (100 nM) reduced coronary perfusion pressure (CPP) in the presence of the vasoconstrictor, U46619, to almost basal levels.

Table S1. BK-induced vasorelaxation in PCA: pEC₅₀ values.