Sexual dimorphism in prostacyclin-mimetic responses within rat mesenteric arteries: A novel role for $K_V7.1$ in shaping IP-receptor mediated relaxation.

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Author contribution

S.N.B designed and implemented all experiments. S.N.B, E.A.F and L.M performed experiments, generated and analysed data. S.N.B and I.A.G drafted the manuscript. I.A.G oversaw the project and prepared the submission of the paper. I.A.G provided funding.

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Conflict of interest

The authors declare no conflict of interest.

Data availability

The data generated herein is available upon reasonable request to the corresponding author.

Ethics approval statement

Animals used within the following investigation were handled in strict accordance with the Animal (Scientific Procedures) Act 1986.

Abstract

Background and purpose- Prostacyclin mimetics express potent vsoactive effects via prostanoid receptors which are not defined unequivocally, as to date no study has considered sex as a factor. The aim of this study was to determine the contribution of IP and EP_3 prostanoid receptors to prostacyclin mimetic Iloprost mediated responses, whether $K_V7.1$-5 channels represent downstream targets of selective prostacyclin-IP-receptor agonist MRE-269 and the impact of the oestrus cycle on vascular reactivity.

Experimental approach- Within 2nd order mesenteric arteries (MAs) from male and female Wistar rats, we determined; 1.) relative mRNA transcript for $EP_{1\text{-}4}$ (*Ptger*$_{1\text{-}4}$), IP (*Ptgi*) and TXA2 (*Tbxa*) prostanoid receptors via RT-qPCR; 2.) The effect of Iloprost, MRE-269 and isoprenaline on pre-contracted arterial tone in the presence of inhibitors of prostanoid receptors, potassium channels and molecular interreference of $K_V7.1$ via wire-myograph; 3.) Oestrus cycle stage was via histological changes in cervical cell preparations.

Key results- Iloprost evoked a bi-phasic response in male MAs, at concentrations ≤100nmol-L^{-1} relaxing, then contracting the vessel at concentration ≥300nmol-L^{-1} in a process attributed to IP and EP_3 receptors respectively. Secondary contraction was absent in the females, which was associated with a reduction in *Ptger3*. Pharmacological inhibition and molecular interference of $K_V7.1$ significantly attenuated relaxations produced by the selective IP-receptor agonist MRE-269 in male and female Wistar in Diestrus / Metoestrous, but not Pro-oestrus / Oestrus.

Conclusions and implications- Stark sexual dimorphisms in Iloprost mediated vasoactive responses are present within MAs. $K_V7.1$ is implicated in IP-receptor mediated vasorelaxation and is impaired by the Oestrus cycle.

What is already known

- The prostacyclin analogue Iloprost evokes vasoactive responses through myriad receptors.
- K_V7 channels are targets of endogenous vasoactive signalling cascades.

What this study adds

- There are sexual dimorphisms in Iloprost evoked responses. Male mesenteric arteries express a bi-phasic relaxant-contractile response that is absent in females.
- MRE-269 mediated relaxation is impaired Male and Female mesenteric arteries by $K_V7.1$ inhibition, a phenomenon impacted by the oestrus cycle.

Clinical significance

- Sex must be considered as a factor when considering prostacyclin mimetics as a therapeutic tool.

Introduction

Prostacyclin (PGI_2), a product cyclooxygenase-1/2 (COX-1/2) metabolism of arachidonic acid, exhibits anti-thrombotic, anti-inflammatory and potent vasodilatory properties in a process attributed to the activation of G_s-coupled IP prostanoid receptor signalling. PGI_2 and stable analogues including Iloprost however bind to a plethora of G-protein coupled receptors (GPCR) including $EP_{1\text{-}4}$, IP, TP, FP and DP_1. (Katusic, Santhanam & He, 2012), though with different affinities. For instance, in human and rat pulmonary arteries Iloprost binds to IP / EP_1 with high affinity, FP > $EP_{3/4}$ with moderate affinity and – DP_1 > EP_2 > TP with low affinity (Whittle *et al.*, 2012). As such, the potential effect of PGI_2 / Iloprost within the vasculature includes both $EP_{1/3}$ / FP prostanoid receptor evoked contraction (Tang *et al.*, 2008; Orie & Clapp, 2011; Kobayashi *et al.*, 2011) and EP_4 / IP prostanoid receptor mediated relaxation (eg Schubert *et al.*, 1997, 1996; Dumas *et al.*, 1997; Lombard *et al.*, 1999). These diverse responses to prostacyclin mimetics remain largely uncharacterised, and moreover, few studies have considered sex as a factor when characterising prostacyclin-memetic mediated vascular responses.

Previously, large conductance calcium activated potassium channels (BK_{Ca} (eg Schubert *et al.*, 1997)), ATP-sensitive potassium channels (K_{ATP} (Lombard *et al.*, 1999; Schubert *et al.*, 1997)) and inwardly-rectifying potassium channels (K_{IR} (Orie *et al*, 2006)) have been identified as the downstream targets of IP-receptor mediated vasorelaxation. Voltage-gated potassium channels encoded by KCNQ genes (termed K_V7 channels) are voltage gated potassium channels with a negative threshold for activation that have well identified roles maintaining resting excitability in neurones, cardiac myocytes, epithelia, and smooth muscle cells (Barrese et al., 2018). Of the 5 subtypes *Kcnq1*, *4* and *5* are robustly expressed in arterial smooth muscle (Yeung *et al.,* 2007, Mackie et al., 2008, Barrese et al., 2018) and blockers of the expressed channels elicit contraction or enhanced vasoconstrictor response (eg Yeung *et al.*, 2007; Mackie et al., 2008). K_V7 channels are also key functional components of vasorelaxations generated by several agonists of G_s-linked receptors including β-adrenoceptor (Chadha *et al.*, 2012b; Stott, Barrese & Greenwood, 2016) calcitonin gene related peptide (Chadha *et al.*, 2014; Stott *et al.*, 2018) and adenosine (Khanamiri *et al.*, 2013). Additionally, novel findings implicate sexual dimorphisms in channel physiology (Alzamora *et al.*, 2011; Abbott & Jepps, 2016) and pathophysiology (Berg, 2018).

As the contribution of K_V7 channels to prostanoid receptor-mediated relaxations is unknown we sought to determine whether K_V7 channels were involved with prostacyclin mimetic mediated responses in rat mesenteric arteries (MAs) from aged-matched male and female rats. This artery was chosen because; 1.) K_V7 expression has been established (Mackie *et al.*, 2008; Jepps *et al.*, 2011, 2015), 2.) K_V7 activators are effective relaxants (Jepps *et al.*, 2014), 3.) role for K_V7 channels in $G_{\alpha s}$-linked responses has been identified (eg Stott *et al.* , 2016; Stott *et al.*, 2018; Lindman *et al.*, 2018); 4.) endothelium dependent production of PGI_2 mediates concomitant IP-receptor mediated relaxation (Liu *et al.*, 2012) and TP/EP_3 mediated contraction (Liu *et al.*, 2012, 2017). Moreover, in line with Docherty *et al.*, (2019) we investigated possible sex difference as nothing is known about the impact of sex on prostanoid mediated vascular responses. To circumvent the short half-life of prostacyclin we characterised the contribution of EP_3 and IP receptors to responses mediated by its stable analogue Iloprost and defined the contribution of K_V7 channels to IP receptor mediated vasorelaxation using a selective IP receptor agonist, MRE-269. Our data demonstrates a striking sex-dependent difference in response to Iloprost and a role for $K_V7.1$ channels in shaping IP-receptor evoked vasorelaxation within rat MAs which is Oestrus cycle-sensitive.

Materials and methods

Animal models

Experiments were performed on arteries from male and female Wistar rats (Charles River, Margate, UK) ages 11-14 weeks (200-350 g) housed at the Biological Research Facility, St George's, London, UK. Animals were kept in cages with free access to water and food (RM1; Dietex Inter-national, UK) with a 12-hour light/dark cycle and constant temperature and humidity (21 ± 1°C; 50% ± 10% humidity) in accordance with the Animal (Scientific Procedures) Act 1986. Animals were kept in LSB Aspen woodchip bedding. Animals were culled by cervical dislocation with secondary confirmation via cessation of the circulation by femoral artery severance in accordance with Schedule 1 of the ASPA 1986.

For the following investigations, 2nd order MAs were used, identified as the second bifurcation of the superior MA. Arteries were dissected, cleaned of fat and adherent tissue and stored on ice in physiological salt solution (PSS) of the following composition (mmol-L^{-1}); 119 NaCl, 4.5 KCl, 1.17 $MgSO_4.7H_20$, 1.18 NaH_2PO_4, 25 $NaHCO_3$, 5 glucose, 1.25 $CaCl_2$.

Oestrus cycle stage determination

Following euthanasia, 50 µL of PSS was inserted into the vaginal canal via a 2-200µL pipette tip and flushed 4-6 times to liberate cells from the surface of the cervix, then stored on ice. 25 µL of the subsequent cell suspension was mounted on a glass slide and examined under light microscopy (x10 – x20 magnification). Previously described changes in cervical cell histology allowed for the determination of Oestrus cycle stage (Cora *et al*, 2015) as either (in order of the 4-5 day cycle); Pro-oestrus, Oestrus, Metoestrus or Dioestrus. Cycle stage determination was performed post-experiment during functional investigation as a means of blinding, this was not possible during molecular techniques.

Wire Myography

For functional investigations, ~2 mm arterial segments were mounted on 40 μm tungsten steel wire within a myograph chamber (Danish Myo Technology, Arhus, Denmark) containing 5 mL of PSS (composition described above) oxygenated with 95% O_2 and 5% CO_2 at 37°C. Vessels then underwent a passive force normalization process to achieve an internal luminal circumference at a transmural pressure of 100 mmHg (13.3 kPa) to standardize pre-experimental conditions (Mulvany, 1977). Force generated was first amplified by a PowerLab (ADInstruments, Oxford, UK), then recorded via LabChart software (ADInstruments, Oxford, UK). Vessels were then challenged with isotonic high K^+ physiological salt solution (K^+PSS) of the following composition (mmol-L^{-1}): 63.5 NaCl, 60 KCl, 1.17 $MgSO_4.7H_20$, 1.18 NaH_2PO_4, 25 $NaHCO_3$, 5 glucose, 1.25 $CaCl_2$, to determine viability. Vessels were then constricted with 10 μmol-L^{-1} methoxamine, an α-1 adrenoreceptor agonist, and endothelial cell integrity was determined via vasorelaxation in response to 10 μmol-L^{-1} synthetic acetylcholine analogue, carbachol, prior to investigation. Subsequently, vessels were pre-constricted with 300 nmol-L^{-1} thromboxane A2 mimetic U46619. Following which single dose responses or cumulative concentration effect curve (CEC) were generated in response to either prostacyclin mimetic Iloprost (0.001-3 μmol-L^{-1}), IP-receptor agonists Selexipag (0.03-3 μmol-L^{-1}) / MRE-269 (0.01-1/0.01-3 μmol-L^{-1}) or β-adrenoreceptor agonist isoprenaline (0.03-3 μmol-L^{-1}). Vessels were pre-incubated in the presence or absence of a combination of solvent control dimethyl sulphoxide (DMSO) or antagonists of the following: IP prostanoid receptor-CAY-10441 (100 nmol-L^{-1}); EP_3 prostanoid receptor-L-798,106 (300 nmol-L^{-1}); pan-K_V7 channel-Linopirdine (10 μmol-L^{-1}); $K_V7.1$ specific HMR-1556 (10 μmol-L^{-1}); adenylate cyclase SQ22,562 (10 μmol-L^{-1}); PKA Rp8 / KT5720 (1 μmol-L^{-1}); EPAC (100 nmol-L^{-1}); $G_{\beta\gamma}$ M119K (10 μmol-L^{-1}); BK_{Ca} iberiotoxin (100 nmol-L^{-1}); K_{ATP}-glibenclamide (1 μmol-L^{-1}) for a period of 10 minutes.

Reverse transcription quantitative polymerase chain reaction

mRNA from whole MAs was extracted using Monarch Total RNA Miniprep Kit (New England BioLabs, Ipswich, Massachusetts, USA) and reverse transcribed via LunaScript RT SuperMix Kit (New

England BioLabs, Ipswich, Massachusetts, USA). Quantitative analysis of relative gene expression was determined via CFX-96 Real-Time PCR Detection System (BioRad, Hertfordshire, UK). Samples were run in BrightWhite qPCR plate (Primer Design, Camberley, UK) in combination with PrecisionPLUS qPCR Master Mix (Primer Design, Camberley, UK), 300 nmol-L^{-1} of gene specific target primer (Thermofisher scientific, Waltham, Massachusetts, USA) and 10ng cDNA as per manufacturers instruction. Quantification cycle (Cq) was determined via Bio-Rad CFX96 Manager 3.0. Cq values are expressed as normalised values to appropriate, stable, housekeeper genes ($2^{-\Delta Cq}$) calnexin (*Canx*) and cytochrome C1 (*Cyc1*) chosen for their stable, and similar Cq values. Housekeeper genes were acquired from Primer Design (Camberley, UK), as such, for proprietary reasons, the sequences are not disclosed. See table 1 for a list of primers used in the following investigation.

Morpholino transfection

Knockdown of $K_V7.1$ in whole MAs was performed by transfection with morpholinos that prohibit protein translation but do not affect transcript levels (see; (Jepps *et al.*, 2015; Barrese *et al.*, 2020)),. Either $K_V7.1$ morpholino nucleotides or mismatch controls (5 µmol-L^{-1}, Genetools, USA) were mixed with Lipofectamine 2000 (ThermoFisher, Paisley, UK) and Opti-MEM (Sigma, UK) and left at room temperature for 2hrs. Morpholino/Lipofectamine/Opti-MEM mixture was added to Dulbecco's modified eagle medium (DMEM) F-12 (Sigma, UK) containing 1%-penicillin/streptomycin. Arteries were added and left for 48hrs at 37°C with 5% CO_2.

Immunocytochemistry

Vascular smooth muscle cells (VSMCs) were isolated from morpholino transfected MAs via incubation in isolation PSS of the following composition (mmol-L^{-1}): 120 NaCl, 6 KCl, 12 glucose, 10 HEPEs and 1.2 $MgCl_2$ supplemented with 1.75 mg/mL Collagenase Type IA, 0.9 mg/mL protease, 1 mg/mL Trypsin inhibitor and 1 mg/mL bovine serum albumin (Sigma, UK) at 37°C for 17 min. Vessels then underwent mechanical trituration by wide bore glass pipette to liberate VSMCs. The subsequent cell

suspension was plated onto 13 mm coverslips in a 24-well plate, supplemented with an equal volume of Ca^{2+} (2.5 mmol-L^{-1}) containing PSS and left to attach for 1hr.

VSMCs were fixed in 3% paraformaldehyde for 15 min, then stored at 4°C in PBS prior to staining. Cells were then incubated in the following: 100 mmol-L^{-1} glycine in PBS, 5mins; blocking solution (PBS containing 0.1% Triton X-100 and 10% FBS in PBS), 45 mins; primary antibody (K_V7.1, 1:100, Rb, Pineda Antikörper-Service, Germany), overnight at 4°C. The following day, cells were incubated in goat anti-rabbit secondary antibody (1:100, conjugated to Alexa Fluro™ 568), then mounted in Vectasheild (Sigma, P4170). Cells were imaged via Nikon A1R confocal microscope (inverted) on Ti2 chassis (Image Resource Facility, St George's University, London) and total cell fluorescence was analysed using ImageJ software.

Cell culture

The Kv7.1 antibody was validated using Chinese Hamster Ovarian (CHO) cells over-expressing KCNQ1. CHO cells were grown in DMEM/F-12 (Sigma, UK) supplemented in 1% penicillin / streptomycin in an incubator with 5% CO_2 at 37°C. CHO cells were incubated with either a total of 3µg of plasmid containing *Kcnq1* (University of Copenhagen, Denmark) in a Lipofectamine 2000 / Opti-MEM mixture (*Kcnq1* transfected CHO), or lipofectamine / Opti-MEM only for 24 hrs (Non-transfected CHO). Cells were mounted onto glass coverslips, fixed and stained for K_V7.1 as above. Anti-body specificity demonstrated by positive staining for K_V7.1 in *Kcnq1* transfected CHO cells (Fig S1.A), but not non-transfected CHO cells (Fig S1.B).

Drugs and reagents

All drugs for the following investigation were procured from Tocris Bioscience (Oxford, UK) unless stated otherwise. CAY-10441 was acquired from Cayman chemical (Michigan, USA).

Data and statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for no less than 5 independent data points, excluding measurement of total cell fluorescence during immunocytochemistry, in which 10 cells were measured per cell. For isometric tension recordings, single dose responses to Iloprost are expressed as (%) change from stable tone in response to 300 nmol-L-1 U46619, contractions from basal tone are expressed as (%) contraction when normalized to vasoconstriction to 10 μmol-L^{-1} methoxamine and all CECs are expressed as (%) stable contraction in response to 300 nmol-L-1 U46619. This is to account for changes in vessel contractility. For functional experiments involving CECs, a transformed data set was generated using; X=Log(X), to reduce representative skew. Following which, either a four parametric linear regression analysis was performed using either (Log(Agonist) vs. response – Variable slope (four parameters Bottom/Hillslope/top/EC_{50})) using GraphPad Prism (Version 9.0.0) to fit a CEC to the figure. When generating data with morpholino transfected arteries, the investigator was blinded, whereby a second researcher would mount the vessels, then post-investigation reveal which arteries had been transfected with scrambled control or *Kcnq1* targeted morpholino. Blinding for the remaining investigations was impractical due researchers working in isolation during the COVID-19 pandemic. For data comparing multiple groups, a paired students T-test or a Two way-ANOVA followed by either a *post hoc* Bonferonni (for comparing one condition against control) test or Dunnet's (for comparing multiple conditions against control) test, was performed for comparison of mean values for investigations. Significance values are represented as; P=<0.05 (*/#). Investigations expressing groups of unequal numbers were gathered due to technical failure or an artefact of cycle stage determination post-experiment during functional investigations. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology in accordance with (Curtis *et al.*, 2018).

Results

Iloprost mediated vasoactive responses

In mesenteric arteries (MAs) from male rats stable contracted by U46619 (300 nmol-L^{-1}), Iloprost (0.1-3 μmol-L^{-1}) evoked a bi-phasic response, producing relaxation at lower concentrations followed by contraction at concentrations >300 nmol-L^{-1} (Fig 1.A,B; black). This phenomenon was notably absent in MAs from female rats, wherein Iloprost only evoked concentration dependent vasorelaxation (*P*=>0.05; Fig 1.A,B; red). See representative traces in Figure 1.A. Application of solvent control had negligible effect on established tone in MAs from either male or female rats (Fig 1.B). For concentration effect curves generated in response to Iloprost on pre-contracted tone, see supplemental figure 2. Prostacyclin-mediated relaxations are conventionally mediated via activation of IP receptors and EP_3 receptors have been implicated in contractile responses to Iloprost in MAs from hypertensive rats (Liu *et al*., 2017). To establish role of these receptors in the bi-phasic or mono-phasic response observed in male and female rats respectively, vessels were pre-incubated in either EP_3 receptor antagonist L-798,106 (300 nmol-L^{-1}) or IP receptor antagonist CAY-10441 (100 nmol-L^{-1}).

In the presence of the EP_3 receptor antagonist L-798,106, 1 μmol-L^{-1} Iloprost mediated contraction was converted to a relaxation (Fig 2.A). In MAs from females, EP_3 receptor inhibition had no effect (Fig 2.A). In the presence of IP receptor antagonist CAY-10441, 0.3 μmol-L^{-1} Iloprost mediated relaxation was ablated in both groups (Fig 3.A,B *P*=>0.05). Similarly, 3 μmol-L^{-1} Iloprost evoked significantly greater contraction from base-line tone in MAs from male rats pre-incubated in CAY-10441 when compared to MAs from female rats (Fig 3.E). No differences were observed in pre-contracted tone in vessels pre-incubated in either L-798,106 or CAY-10441 when compared to DMSO solvent control or between the sexes (Fig 2.B/D). Subsequently, we performed Quantitative PCR to determine the relative expression of prostanoid receptors (table 1) in MAs from both sexes. Figure 2.F shows that expression of *Ptger2/4* ($EP_{2/4}$) was negligible in MA from both sexes compared to, *Ptger3* > *Ptgir* > *Ptger1,* (EP_3; IP; EP_1) which were well expressed (Fig 2.F). However, *Ptger3* was expressed at significantly lower level in MA from female rats compared to MAs from males (Fig 2.F). Thus, our data demonstrates that

Iloprost mediated relaxation in rat MAs occurs predominantly via IP receptors, whilst contraction was driven by EP_3 receptors. Additionally, the absence of a bi-phasic response to Iloprost in female MAs was associated with a comparably smaller effect of EP_3 receptor inhibition on Iloprost mediated relaxation and reduction in *Ptger3* expression and.

Characterising MRE-269 mediated relaxation.

As Iloprost has a plethora of potential targets, we used the clinically available IP receptor agonist, Selexipag (NS-304) to delineate the mechanisms underlying IP-mediated relaxation. Application of Selexipag produced concentration dependent relaxations of pre-contracted MAs from male rats (Fig S3), however this effect was insensitive to pre-incubation with IP receptor antagonist CAY-10441 (Fig S3), thus its effects are non-IP receptor dependent. It is now known that in the body, Selexipag is metabolized into the active compound, MRE-269. Application of MRE-269 to MAs from male rats produced a relaxation that was ablated by CAY-10441 pre-incubation up to threshold of 1 μmol-L^{-1}. At higher concentrations the MRE-269-mediated relaxation was not sensitive to CAY-10441 and therefore not driven by IP receptor activation. This non-IP receptor mediated relaxation is highlighted by the grey box in Figure 3 and in following investigations, MRE-269 was used at concentrations ≤1 μmol-L^{-1} to ensure only IP receptor-mediated effects were investigated.

A novel role for K_V7.1 in shaping IP receptor-specific agonists in mesenteric arteries.

Kv7 channels, especially Kv7.4 and Kv7.5, are functional endpoints for several G_s-linked receptors (see Barrese et al., 2018, Byron & Brueggmann, 2018). As such, we characterised the potential contribution of K_V7 channels to IP- receptor selective MRE-269 mediated relaxation. In MAs from male rats, MRE-269 mediated relaxation was significantly attenuated by pre-incubation with the pan-K_V7 channel inhibitor Linopirdine (10 μmol-L^{-1}; yellow) when compared to DMSO (Fig 4; black; P=<0.05). Strikingly, pre-incubation with the K_V7.1 specific inhibitor HMR-1556 (10 μmol-L^{-1}), also impaired MRE-269-induced relaxations to the same extent as Linopirdine (Fig 4.A,B; green; P=<0.05). The structurally dissimilar K_V7.1 inhibitor Chromanol 293B also significantly attenuated MRE-269

mediated relaxation of male MAs (P=<0.05; Fig 4.C). In contrast, and consistent with previous reports (Stott *et al*., 2016), relaxations of MAs induced by the mixed β-adrenoceptor agonist isoprenaline were not affected by 10 μmol-L^{-1} HMR-1556 pre-incubation (Fig 4.D).

To corroborate the contribution of K_V7.1 to IP-receptor mediated vasorelaxation we transfected MA with morpholinos that prevent translation of Kv7.1 or a scrambled control. Immunocytochemsitry with an antibody for K_V7.1 validated by over-expression studies (Fig S1) showed a significant reduction in total cell fluorescence (A.U) in *Kcnq1* morpholino transfected arteries when compared to mismatch control (P=>0.05; Fig 5.A-C). Functionally, arteries incubated with mismatch control produced a greater relaxant response to 300 nmol-L^{-1} K_V7.1 activator ML277 (Yu, 2013; Baldwin *et al.*, 2020) compared to *Kcnq1* morpholino-transfected arteries (P=>0.05; Fig 5.D). Similarly, relaxation produced by 1 μmol-L^{-1} MRE-269 was greater in arteries transfected with mismatch control morpholino when compared *Kcnq1* morpholino transfected arteries (P=>0.05; Fig 5.E). Thus, a reduction in K_V7.1 protein was observed in conjunction with an attenuated relaxation by MRE-269.

Subsequently, we aimed to determine the signalling cascade activated in response to IP-receptor stimulation. Previously, Schubert *et al* (1996) demonstrated that Iloprost evoked hyperpolarization of rat tail artery VSMCs is mediated via G_s - cyclic adenosine 3'-5'-monophosphate (cAMP) - protein kinase A (PKA) stimulation of potassium currents (Schubert *et al.*, 1996) in a process attributed to IP receptors although not fully defined. Here we similarly demonstrate that adenylate cyclase inhibitor SQ22,562 (10 μmol-L^{-1}) and PKA inhibitors Rp8 (1 μmol-L^{-1}) and KT5720 (1 μmol-L^{-1}) significantly attenuated MRE-269 mediated relaxation (1 μmol-L^{-1}; P=>0.05; Fig 6.A-C). In addition, inhibition of an alternative secondary signalling molecule activated by G_s -cAMP, exchange protein directly activated by cAMP (EPAC), via ESI-09 had no effect (100nmol-L^{-1}; Fig 6.D). Finally, $G_{\beta\gamma}$ inhibition by M119K (1 μmol-L^{-1}) also significantly attenuated MRE-269 mediated relaxation (P=>0.05; Fig 6.E)

BK_{Ca} and K_{ATP} channels have also been identified as down-stream targets of cAMP-PKA dependent relaxations evoked by Iloprost (Schubert *et al.*, 1997). Here, we demonstrate that BK_{Ca} inhibitor

iberiotoxin (100 nmol-L^{-1}) but not K_{ATP} inhibitor glibenclamide (1 μmol-L^{-1}) partially inhibited MRE-269 mediated relaxation in male MAs (Fig S4.A,B), though this failed to reach statistical significance.

Oestrus cycle-dependent shifts in the sensitivity of MRE-269 mediated vasorelaxation to K_V7 channel modulators

The pan K_V7 channel inhibitor Linopirdine and $K_V7.1$ channel inhibitor HMR-1556 also attenuated MRE-269 mediated relaxation in MAs from female rats when compared to DMSO solvent control (Fig 8.A; P=<0.05), though the latter to a smaller degree when compared to Linopirdine. However, we observed two-distinct populations of possible responses to MRE-269 mediated relaxation and its subsequent sensitivity to K_V7 channel modulators, categorized into rats in Diestrus/Metestrus (Di/Met) or Pro-estrus/Oestrus (Pro/Est). Oestrus cycle stage was identified by defined histological changes in cells lifted from the cervix post-euthanasia (as per methods; (Cora *et al*., 2015)). Both the pan-K_V7 channel inhibitor Linopirdine and $K_V7.1$ specific inhibitor HMR-1556 impaired MRE-269-mediated relaxation to the same degree in arteries from female in Di/Met (Fig 8.B, P=<0.05) but had no effect in arteries from rats in Pro/Est (Fig 8.C). Moreover, MRE-269 mediated relaxation in arteries from female Pro/Est rats was significantly less sensitive to the IP-specific agonist than arteries from rats in Di/Met (Fig 8.D; P=<0.05). However, we observed no differences in $K_V7.1$ activator ML277 (0.1-0.3 μmol-L^{-1}) mediated relaxation on pre-contracted tone between the separated groups (Fig 7.E). These data reveal an oestrus cycle stage dependent regulation of the contribution of $K_V7.1$ to IP-receptor mediated relaxation which underpins diminished sensitivity to MRE-269 mediated relaxation during Pro/Est.

Discussion

To our knowledge, the present study is the first to highlight sex as a factor in the arterial response to prostacyclin mimetics. The study shows that application of Iloprost to pre-contacted MAs from male rats produced bimodal responses, relaxation at low concentrations, followed by contraction at higher concentrations, whereas MAs from female arteries presented with mono-phasic relaxation only. In arteries from both sexes the relaxant effect of Iloprost was enhanced by EP_3 receptor antagonist L-798106 and was ablated by IP receptor antagonist CAY-10441. Our data demonstrated Iloprost-mediated contractions in MAs from male rats were more efficacious when compared to vessels from females, correlating with a higher level of *Ptger*$_3$ expression. Finally, our data shows that the selective IP receptor agonist MRE-269 was a potent relaxant of pre-contracted MAs from both sexes. This relaxation was inhibited by both the pan-K_V7 blocker Linopirdine and $K_V7.1$ specific blocker HMR-1556 in arteries from males and Di/Met females but strikingly, not Pro/Est rats. These findings are the first observation of $K_V7.1$ as a downstream target of an endogenous vasoactive signalling cascade and reveal Oestrus cycle dependent regulation of K_V7 channels within the vasculature.

Iloprost evoked vasoconstriction

Whilst principally regarded as a vasodilator, PGI_2 mediates both relaxation and contraction of smooth muscle (Moncada *et al.*, 1976; Dusting, Moncada & Vane, 1977; Liu *et al.*, 2017). PGI_2 has subsequently been identified as an endothelial derived contracting factor produced in response to acetylcholine within rat aorta (Gluais *et al.*, 2005), mesenteric (Liu *et al.*, 2017), iliac (Zhang *et al.*, 2021) and renal arteries (Zhang *et al.*, 2021) in a process attributed to the activation of both EP and TP prostanoid receptors. Consistent with Liu *et al* (2017), we show that high concentrations of Iloprost evoked contractions that were ablated by the EP_3 receptor antagonist L-798,106. As Iloprost has a low affinity for TP receptors (Whittle *et al.*, 2012), TP receptor knockout has no effect on PGI_2 mediated contraction in MAs (Li et al., 2017) and all vessels in this study were precontracted with U46619, a TP receptor agonist, TP receptors were not considered for the scope of this investigation. Additionally, EP_1 receptor agonists do not elicit contractions in male MAs (Kobayashi *et al.*, 2011) and in agreement with

previous findings (Kobayashi *et al.*, 2011), a reduced expression of *Ptger1* was observed when compared to *Ptger3*. Furthermore, Iloprost had negligible contractile effect in MAs from female rats, which was associated with a lower expression level of *Ptger3* in these arteries.

K_V7.1 underpins IP receptor mediated relaxation

Our data shows that relaxations of MAs mediated by low concentrations of Iloprost were driven primarily through CAY-10441-sensitive IP receptor activation. We subsequently showed that CAY-10441-sensititve relaxations produced by the selective IP receptor agonist MRE-269 were impaired by the selective K_V7 channel blocker, linopirdine. Within the vasculature, of the five subtypes *Kcnq4* > *Kcqn5* > *Kcnq1* are the principally expressed transcripts with little to no expression of *Kcnq2/3* (Yeung *et al.*, 2007; Jepps *et al.*, 2011; Chadha *et al.*, 2012b). K_V7.4/K_V7.5 alone however are implicated in the regulation of the resting membrane potential (Mackie *et al.*, 2008) and basal tone (Mackie *et al.*, 2008; Ng *et al.*, 2011). In addition, pharmacological inhibition or molecular knockdown of K_V7.4/7.5 impairs relaxations to many different relaxants including isoprenaline, CGRP, adenosine (G_s linked), atrial natriuretic peptide (cGMP linked) and adipose derived relaxant factors in several arteries (Stott, Barrese & Greenwood, 2016; Chadha *et al.*, 2014; Stott *et al.*, 2015a; Gollasch, 2017; Khanamiri *et al.*, 2013; Byron & Brueggemann, 2018; Morales-Cano *et al.*, 2015). Our data suggests that IP receptor activation in male MAs is another GPCR that also relies on K_V7 channels for functional responses. In contrast to previous reports (Lombard *et al.*, 1999; Schubert *et al.*, 1997) IP-receptor mediated relaxation was not affected by K_{ATP} nor BK_{Ca} blockade, however, this discrepancy is potentially accounted for by a difference in vascular model used as Lombard *et al* (1999),. investigated rat middle cerebral artery and Schubert *et al* (1997)., used rat tail artery.

Surprisingly, MRE-269 evoked CAY-10441-sensitive relaxations in MAs from male rats were also inhibited considerably by two structurally dissimilar K_V7.1 specific inhibitors (HMR-1556, Chromanol 293B) and molecular knockdown of the channel. In contrast to K_V7.4/ K_V7.5, the role of K_V7.1 within the vasculature remains enigmatic. Though K_V7.1 is expressed within VSMCs (Chadha *et al.*, 2012b;

Baldwin *et al.*, 2020; Tsvetkov *et al.*, 2017) and $K_V7.1$ specific activators RL-1 and ML277 are effective relaxants of pre-contracted arterial tone (Chadha *et al.*, 2012a; Baldwin *et al.*, 2020), $K_V7.1$ has not been identified as the downstream target of any endogenous vasoactive signalling cascades (Stott *et al.*, 2015a; Chadha *et al.*, 2014; Stott, Barrese & Greenwood, 2016). Yet in the present study HMR-1556 produced as full an inhibition as linopirdine, which suggests $K_V7.1$, and not $K_V7.4/7.5$ contribute to MRE-269 mediated relaxations. Under the same conditions the mixed β-adrenoceptor agonist isoprenaline produced relaxations that were not HMR1556 sensitive. Thus, our findings appear not to be an off-target effect of HMR-1556. Moreover, a role for $K_V7.1$ in MRE-269 mediated relaxation was substantiated by morpholino-induced reduction in $K_V7.1$ protein levels. Whilst further work is required to validate these findings, to our knowledge our data is the first to describe an effect on vascular reactivity by $K_V7.1$ inhibition. Additionally, our data supports the notion that IP-receptor mediated responses are cAMP-PKA mediated. In agreement with previous work done by our lab (Stott *et al.*, 2015b, 2018; Stott, Barrese & Greenwood, 2016), we demonstrate that relaxations that are mediated PKA, but not EPAC, are also sensitive to $G_{\beta\gamma}$ inhibition. The identification of $G_{\beta\gamma}$ contribution to IP-receptor mediated relaxation adds new complexity to the vascular response and gives credence to the novel role of $G_{\beta\gamma}$ in the functional relationship between GPCRs and K_V7s.

To date, comparably little is known as to how K_V7 channels operate within the female. However, K_V7 has been shown to regulate both human and murine myometrium (McCallum, Greenwood & Tribe, 2009) and human chorionic plate artery (Mills *et al.*, 2015) contractility. Tissues where prostanoids are known key regulators of smooth muscle. Intriguingly, whilst MRE-269 mediated relaxation was attenuated by Linopirdine and HMR-1556 in arteries from female rats, the effect of the latter was far smaller than in the male. When separated into oestrus cycle stages, arteries from Females in Di/Met expressed sensitivities to HMR-1556 and Linopirdine equivalent to the male, whereas arteries from Females in Pro/Est were entirely insensitive to either. However, $K_V7.1$ activator-ML277 mediated relaxation was insensitive to changes in the oestrus cycle. As the functional output of pharmacological activation of the channel remains the same, the data indicates an Oestrus cycle dependent impairment

of $K_V7.1$ channel coupling to IP-receptor mediated relaxation. Oestrus cycle dependent regulation of vascular reactivity is a known, but incompletely understood phenomenon (Jaimes *et al.*, 2019) largely attributed to endogenous sex-hormones, primarily, Oestradiol. 17-β Oestradiol negatively regulates $K_V7.1$ in distal colic crypt cells and cardiac myocytes (O'Mahony *et al.*, 2007; Alzamora *et al.*, 2011; Rapetti-Mauss *et al.*, 2013; Waldegger *et al.*, 1996). As previous work demonstrates that within the Wistar rat, Oestradiol peaks within Pro-oestrus rats followed by comparably little to none in oestrus, metoestrus and dioestrus (Nilsson *et al.*, 2015), we propose that during Pro-oestrus, Oestradiol levels rise, impairing $K_V7.1$ coupling to IP-receptor during Pro/Est phase, thus reducing the potency of MRE-269 mediated relaxation and its HMR-1556 / Linopirdine sensitivity, which does not recover until Di/Met. our data implies an Oestrus cycle.

Perspectives

Sexual-dimorphism's in cardiovascular physiology and pathophysiology are known (Pabbidi *et al.*, 2018), whereby women express a cardioprotective factor or factors that differentiates the aetiology of vascular disease between age-matched men and women. Chronic aldosterone treatment in male Wistar-Kyoto rats induces hypertension through endothelial dysfunction attributed to upregulated COX-2 production of PGI_2 (Blanco-Rivero *et al.*, 2005). Diminished EP_3 mediated contraction/expression in female rats may potentially negate the pathophysiological levels of PGI_2 production observed by Blanco-Rivero *et al* (2005), contributing to the known cardioprotective phenotype expressed by females, though further work is required to validate this hypothesis.

Conclusion

The data of the present study demonstrates a remarkable sexual dimorphism in the vascular response to synthetic prostacyclin analogues and highlight the importance of considering sex as a determinant in vascular physiology. Strikingly, the potent relaxations to the selective IP receptor agonist MRE-269 were sensitive to the Kv7.1-specific blocker HMR1556 in MAs from males and females in Di/Met but

not at all in Pro/Est. A novel demonstration of both a functional role for $K_V7.1$ and cyclical changes in K_V7 channel activity across the oestrus cycle. Both of which will form the basis of future investigations.

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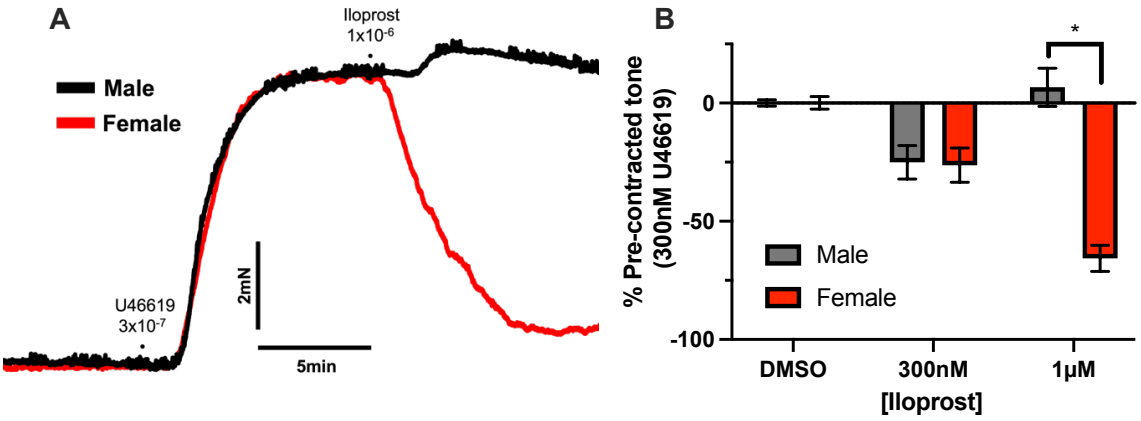
1

Tables

Gene name	(+) Forward primer sequence 3'-5' (-) Reverse primer sequence 5'-3'	Gene accession number	Amplicon	Concentration
EP_1 (*Ptger1*)	(+) AGTTCGAACGTTGGTCACGA (-) TAAGGTTGCAGCATTGTGCG	NM_001278475.1	112	300 nmol-L^{-1}
EP_2 (*Ptger2*)	(+) TATGCTCCCTGCCTTTCACAA (-) GGAGGTCCCACTTTTCCTTT	NM_031088.2	72	300 nmol-L^{-1}
EP_3 (*Ptger3*)	(+) GTGCAATTCCTTCCTAATCGCC (-) TCAGGTTGTTCATCATCTGGCA	NM_012704.1	122	300 nmol-L^{-1}
EP_4 (*Ptger4*)	(+) ATGAGCATTGAGCGCTACCT (-) AGATGCATAGACGGCGAAGA	NM_032076.3	102	300 nmol-L^{-1}
IP (*Ptgir*)	(+) TGACACTTTCGCCTTCGCTA (-) TAGATGGCAGGCAAAGCCAA	NP_001071112.1	156	300 nmol-L^{-1}
TXA2 (*Tbxa2r*)	(+) TTGACATTCCCAGGCCCAAA (-) ACGTGATAAGGGGGTCAACA	NM_017054.2	141	300 nmol-L^{-1}
Clnexin (*Canx)*	N/A (Primer design, Camberley, UK)			300 nmol-L^{-1}
Cytochrome C1 (*Cyc1*)	N/A (Primer design, Camberley, UK)			300 nmol-L^{-1}

Table 1 RT-qPCR primer sequences

1 Figures



2

Figure 1. Iloprost evokes bi-phasic vasoactive responses within male, but not female mesenteric arteries.

Representative traces of Iloprost (0.3-1 µmol-L^{-1}) mediated vasoactive responses on pre-contracted tone (300 nmol-L^{-1} U46619) within mesenteric arteries from male (A; black) or female (A; red) Wistar rats. Mean data for DMSO solvent control and Iloprost mediated vasoactive responses on pre-contracted (300nmol-L^{-1} U46619) mesenteric arteries (A; 0.3-1 µmol-L^{-1}; n=6-8). All values are expressed as mean ± SEM. A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significance values (*=P<0.05). (n=) number of animals used.

3

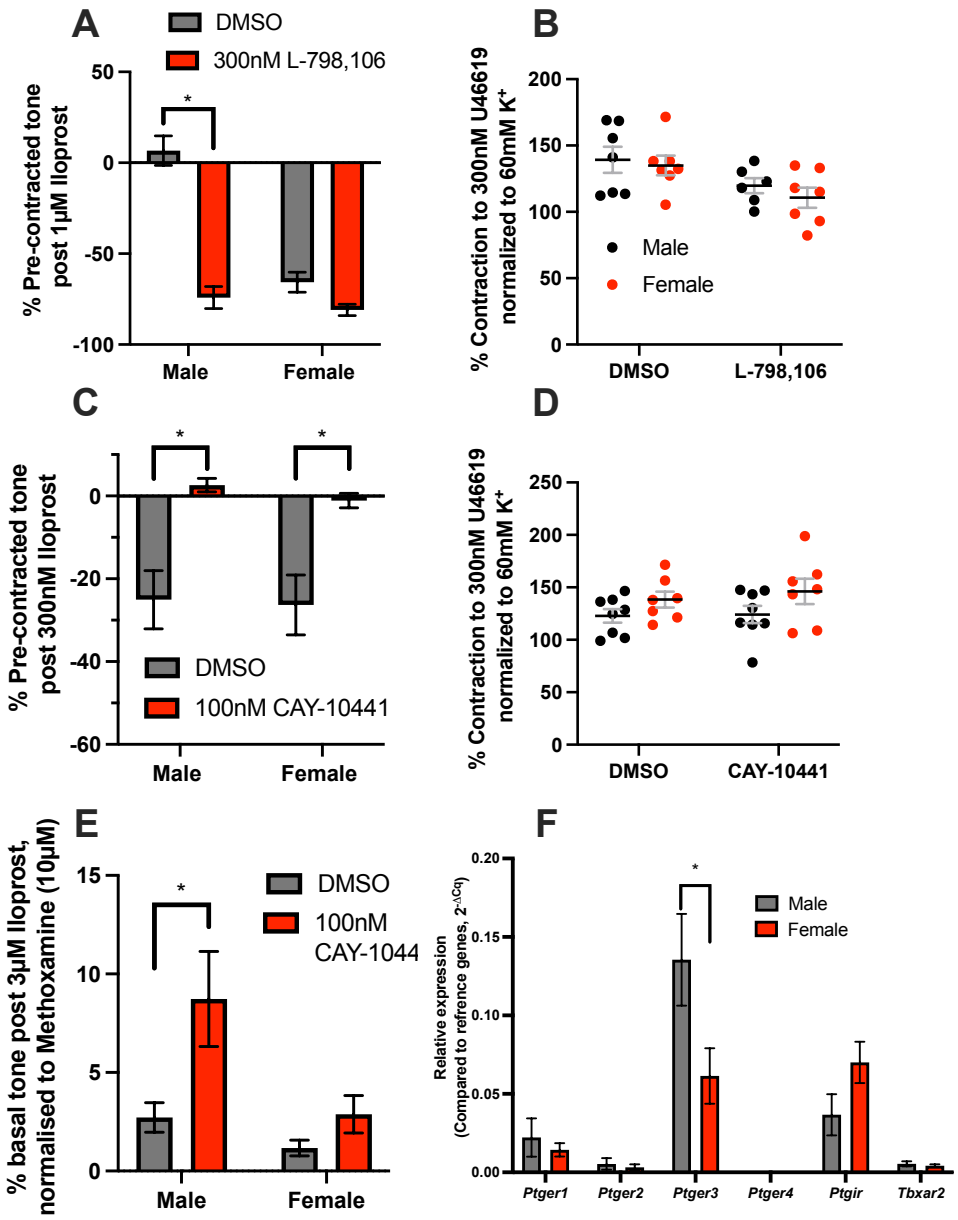


Figure 2. Iloprost mediated contraction and relaxation is ablated by EP_3 and IP-receptor mediated inhibition respectively.

Mean data for Iloprost mediated vasoactive responses (A; 1 µmol-L^{-1}; C 300 nmol-L^{-1}) within pre-contracted (300 nmol-L^{-1} U46619) mesenteric arteries from male and female rats pre-incubated in either solvent control (DMSO; A,C; *n*=6-8), 300 nmol-L^{-1} L-798,106 (A; *n*=6-8) or 100 nmol-L^{-1} CAY-10441 (C; *n*=6-8). Scatter graph showing individual and mean values of stable pre-contracted tone from male and female arteries prior to adding Iloprost to the chamber (B,D). Mean data for vasoconstriction from base line tension in response to 3 µmol-L^{-1} Iloprost in male (black; *n*=7) and female (red; *n*=10) mesenteric arteries in the presence of DMSO or 100nmol-L^{-1} CAY-10441 normalised to peak contraction in response to 10µmol-L^{-1} methoxamine (E). Relative gene expression of prostanoid receptors (*Ptger1-4* = $EP_{1\text{-}4}$, *Ptgi* = IP, *Tbxar2* = TXA2) normalised to stable housekeeper genes (*Canx, Cyc1*) expressed as $2^{-\Delta Cq}$ from male (black; *n*=5) and female (red; *n*=6-10) whole mesenteric artery lysates (F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

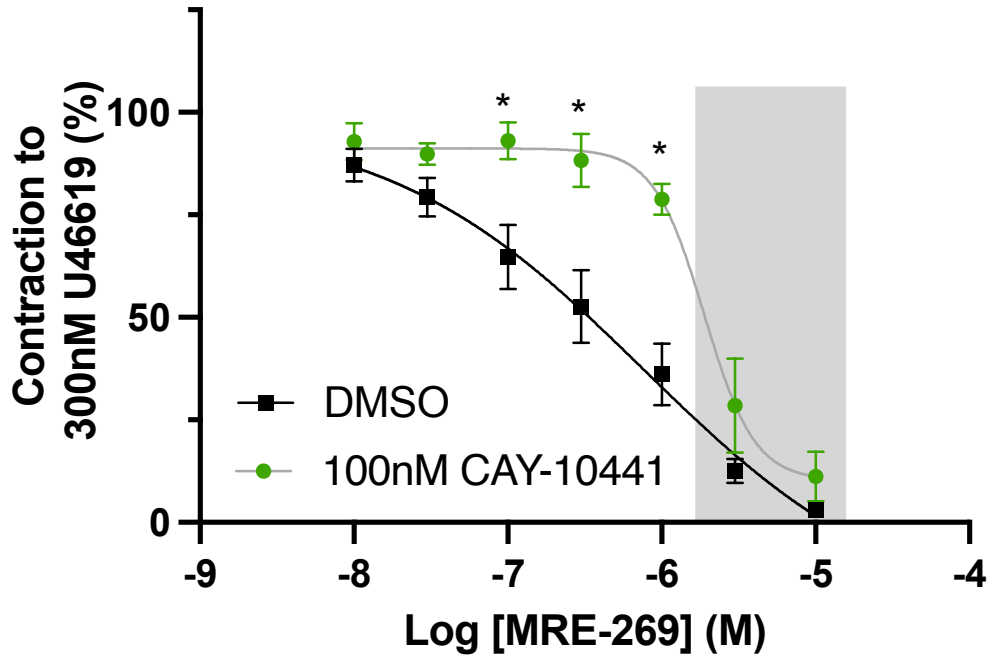


Figure 3. MRE-269 relaxation is inhibited by CAY-10441 to a threshold of 1µmol-L^{-1} in male mesenteric arteries.

Mean data of MRE-269 (0.01-10 µmol-L^{-1}) mediated relaxation of pre-contracted arterial tone (300nmol-L^{-1}) in vessels pre-incubated in DMSO solvent control (black; *n*=7) or 100 nmol-L^{-1} CAY-10441 (*n*=5) in male mesenteric arteries. Grey box demonstrates non-CAY-10441 sensitive MRE-269 mediated relaxation. All values are expressed as mean ± SEM. A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

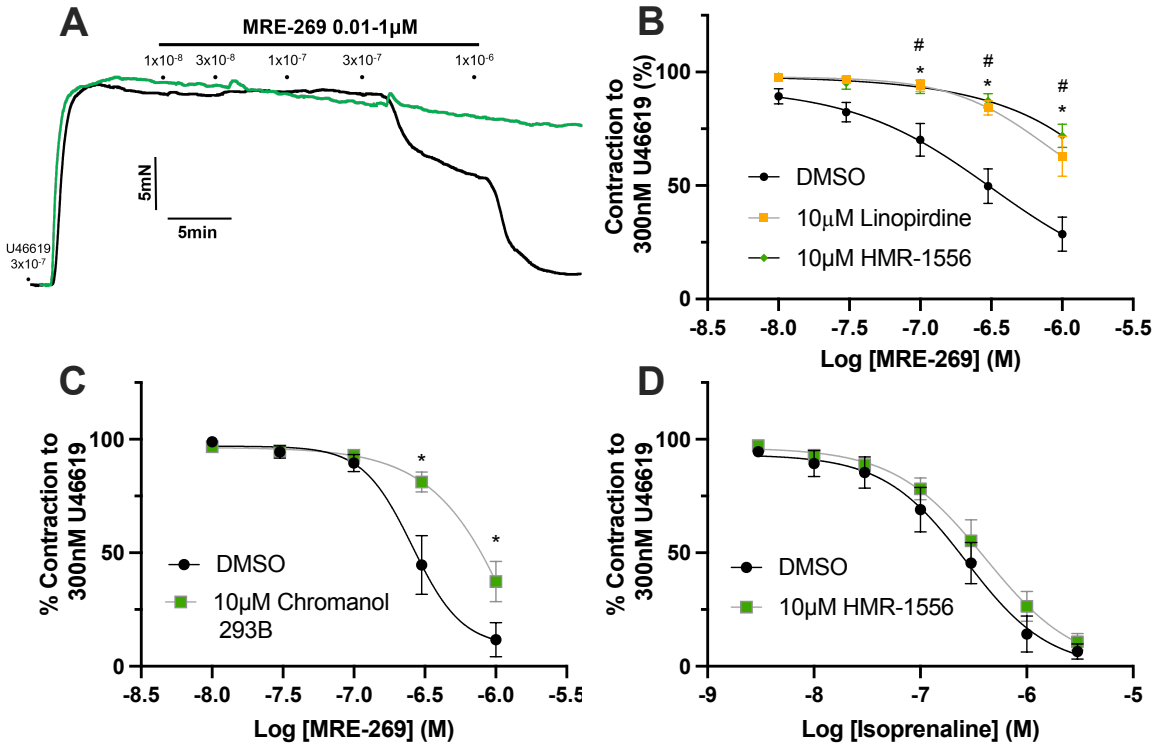


Figure 4. Linopirdine and HMR-1556 attenuate MRE-269 mediated vasorelaxation in mesenteric arteries from male and female rats.

Representative traces of MRE-269 mediated (0.01-1µmol-L^{-1}) relaxation of pre-contracted tone (300 nmol-L^{-1} U46619) within mesenteric arteries pre-incubated in either DMSO solvent control (A; black) or 10µmol-L^{-1} K_V7.1 specific blocker HMR-1556 (A; green) from male Wistar rats. Mean data for MRE-269 mediated vasorelaxation (0.01-1 µmol-L^{-1}) of pre-contracted tone (300 nmol-L^{-1} U46619) within mesenteric arteries pre-incubated in either DMSO (B; black) solvent control, 10 µmol-L^{-1} pan-K_V7 channel blocker linopirdine (B; yellow) or HMR-1556 (B; green) or 10µmol-L^{-1} K_V7.1 specific blocker Chromanol 293B (C; green; *n*=7-10). Mean data for Isoprenaline mediated relaxation in vessels pre-incubated in DMSO (black) or 10 µmol-L^{-1} HMR-1556 (green) in male mesenteric arteries (D; *n*=9). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Dunnets (B) or Bonferroni (C-D) test was used to generate significant values (*=P<0.05). (*n*=) number of animals used.

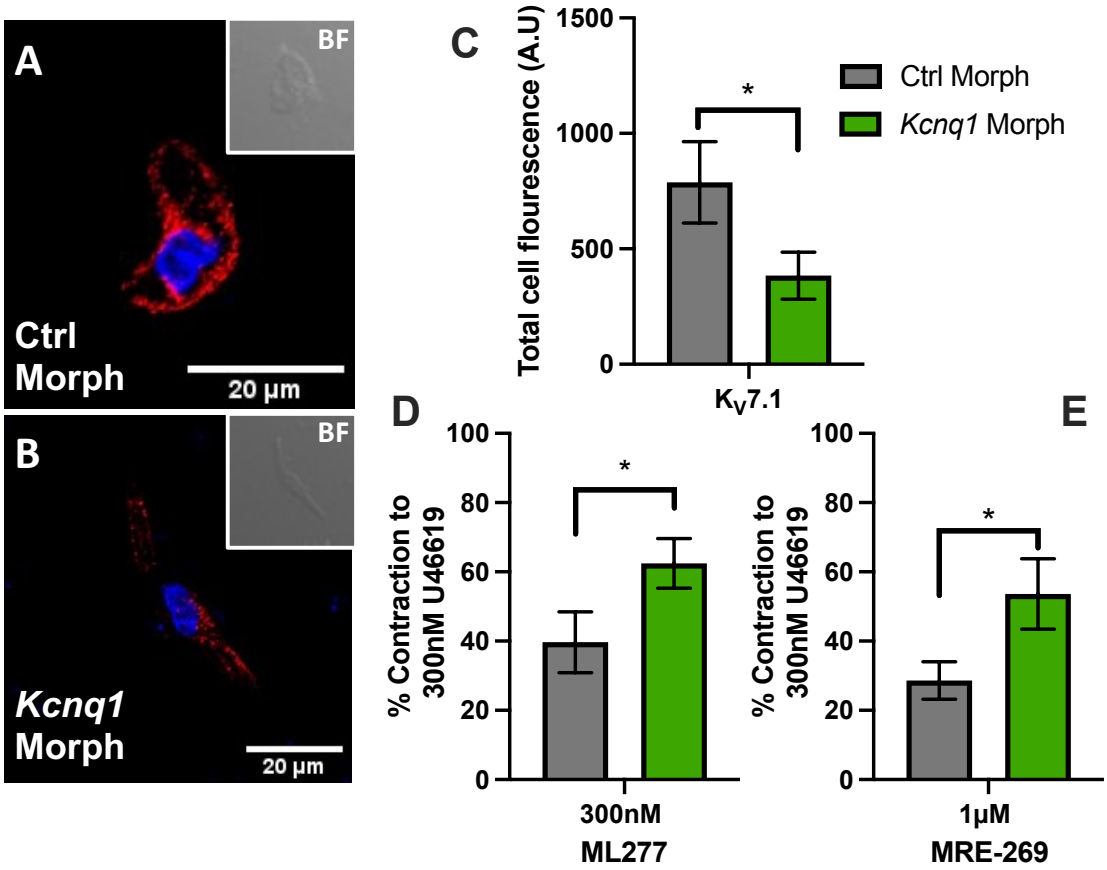


Figure 5. Molecular interference of *Kcnq1* via targeted morpholino knockdown impairs MRE-269 mediated relaxation.

Representative immunofluorescence showing $K_V7.1$ in isolated vascular smooth muscle cells from either scrambled control (Ctrl morph; A) or *Kcnq1 (Kcnq1* morph*;* B) morpholino transfected mesenteric arteries. $K_V7.1$ shown in red, nuclear staining in blue (DAPI [4',6-diamidino-2-phenylindole, dihydrochloride]). Insets show brightfield (BF) images of the cell. Mean data for total cell fluorescence measured in arbitrary units (A.U) for Ctrl morph (grey; *n*=3; N=10) and *Kcnq1* morph (green; *n*=3; N=10; D) transfected cells. Mean data for 300nmol-L^{-1} ML277 mediated relaxation (*n*=7; E). Mean data for 1µmol-L^{-1} MRE-269 mediated relaxation (*n*=7; F). All values are expressed as mean ± SEM (A-F). A paired Student's t test was used to generate significant values (*=P<0.05). (*n*=) number of animals used, (N=) number of cells per biological repeat.

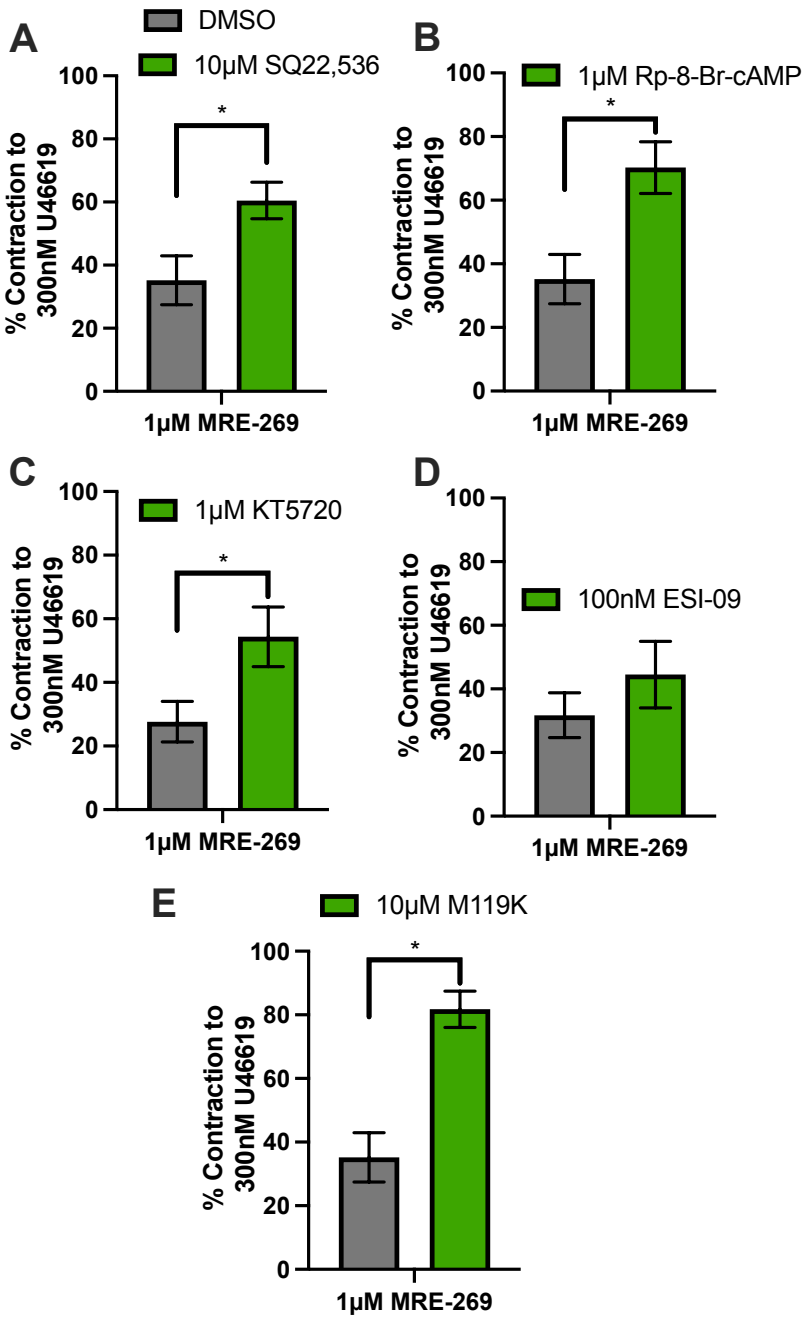


Figure 6. MRE-268 mediated relaxation is sensitive to adenylate cyclase, Gβγ and protein kinase A inhibition, but not effector protein activated by cAMP in male rat mesenteric arteries. Mean data for MRE-269 mediated vasorelaxation (1 μmol-L^{-1}) of pre-contracted tone (300 nmol-L^{-1} U46619) within mesenteric arteries pre-incubated in either DMSO (A-E; *n*= 9; grey) solvent control or inhibitors of the following (green); adenylate cyclase- SQ22,562 (10 μmol-L^{-1}; *n*=7; A), protein kinase A- Rp8 (1μmol-L^{-1}; *n*=5; B) / KT5720 (1μmol-L^{-1}; *n*=8; C), effector protein inhibited by cAMP- ES09 (100nmol-L^{-1}; *n*=8; D) and), Gβγ- ML119K (1μmol-L^{-1}; *n*=7; E). All values are expressed as mean ± SEM (A-E). An unpaired students T-test was used to generate significant values (*=P<0.05). (*n*=) number of animals used.

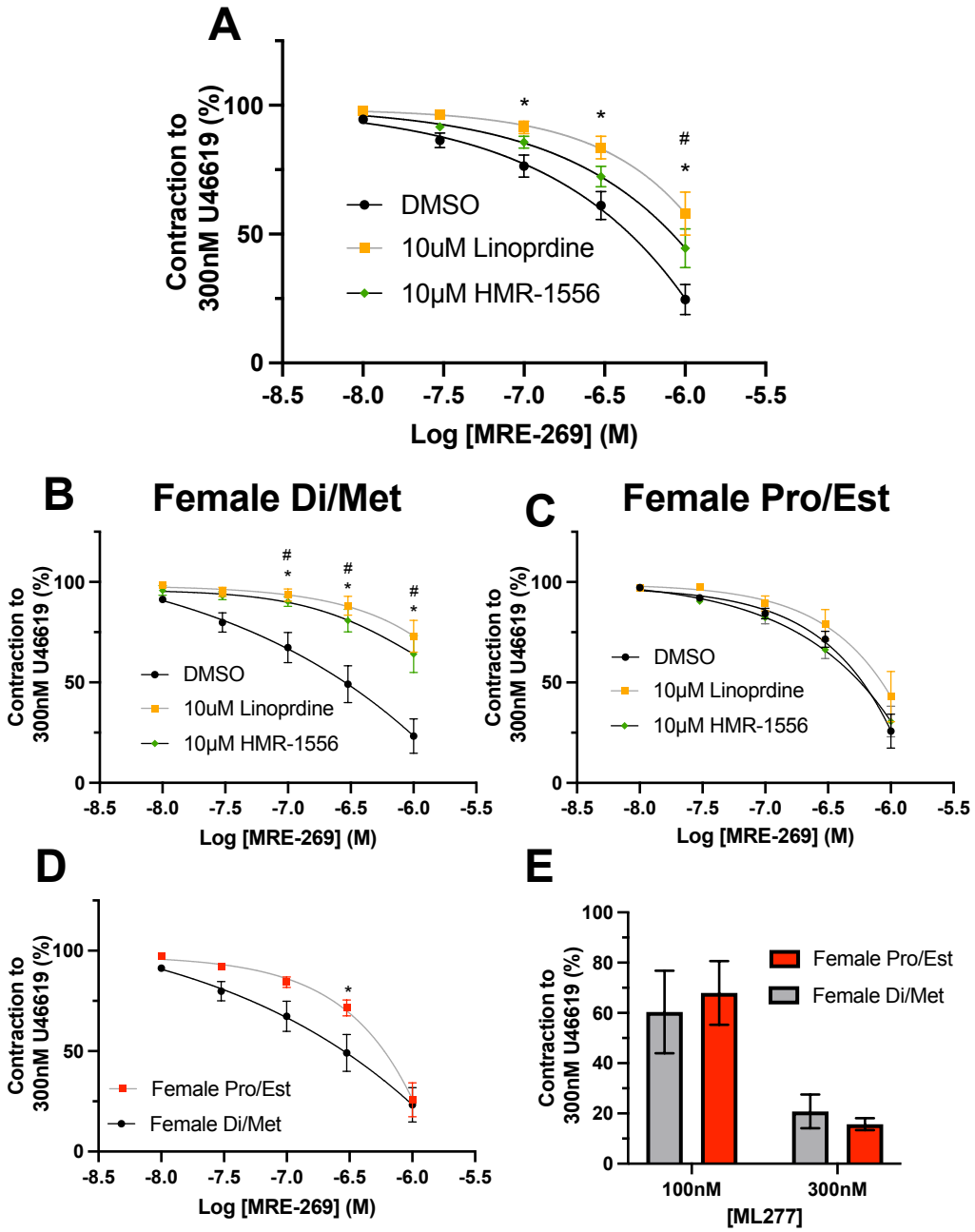
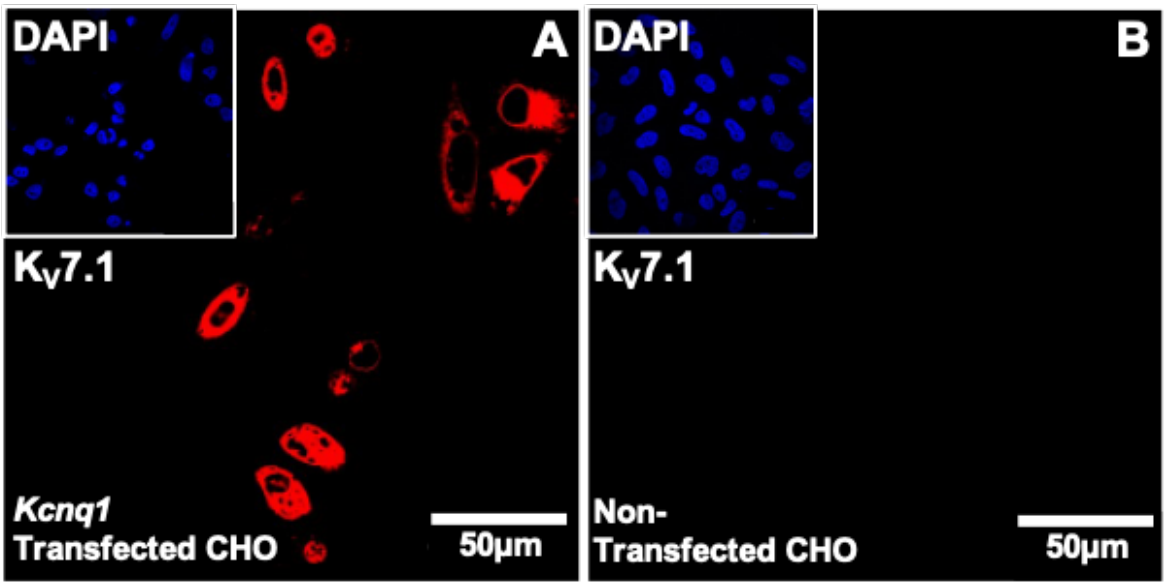


Figure 7. K_V7 channel inhibition attenuates MRE-269 mediated vasorelaxation in mesenteric arteries from Diestrus/Metestrus (Di/Met), but not Pro-estrus/Oestrus (Pro/Est) female rats. Mean data for MRE-269 mediated vasorelaxation (0.01-1 µmol-L^{-1}) within mesenteric arteries pre-incubated in DMSO solvent control (A-C; black), 10 µmol-L^{-1} pan K_V7 channel inhibitor linopirdine (A-C; yellow) or 10 µmol-L^{-1} $K_V7.1$ specific inhibitor HMR-1556 (A-C; green) from female Wistar rats. Data is expressed as either all female (A), female Di/Met (*n*=5-7, B) and female Pro/Est (*n*=6-8; C). MRE-269 (0.01-1 µmol-L^{-1}; D) and ML277 (0.01-0.03 µmol-L^{-1}) mediated relaxation in vessels from either Female Pro/Est (red; *n*=6-8) or Female Di/Met (black; *n*=5-6). All values are expressed as mean ± SEM (A-E). A two-way statistical ANOVA with a post-hoc Dunnet's (A-C) or Bonferroni (D,E) test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

1 Supplementary figures



2

Figure S1. Representative images of immunocytochemistry of CHO cells demonstrates antibody specificity.
Chinese hamster ovarian (CHO) cells transfected with *Kcnq1* containing plasmids (A), but not non-transfected CHO cells, present with diffuse labeling of K_V7.1, as seen in red (B). Insets contain 4′,6-diamidino-2-phenylindole (DAPI) staining only, blue. Bar = 50µm.

3

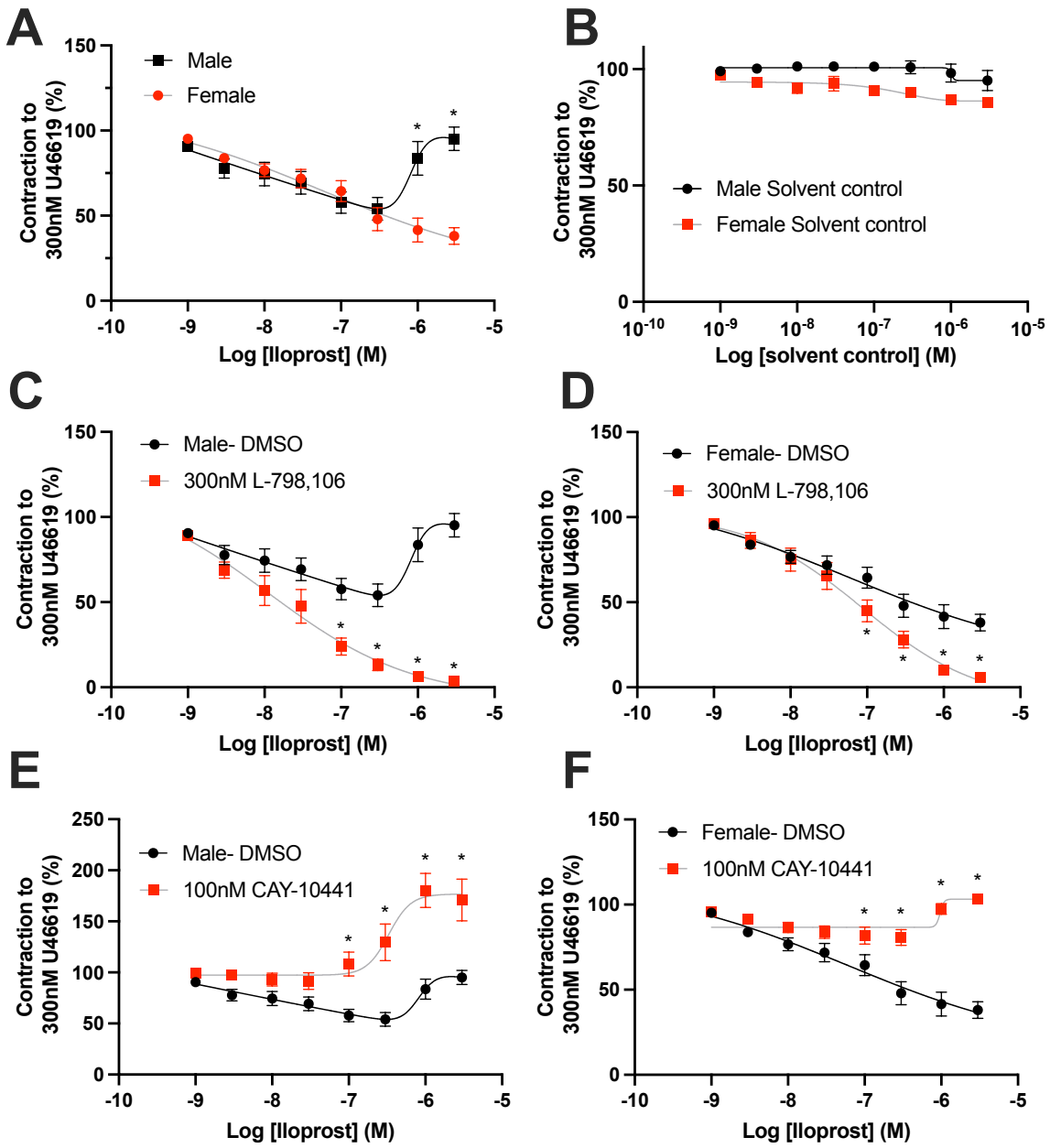
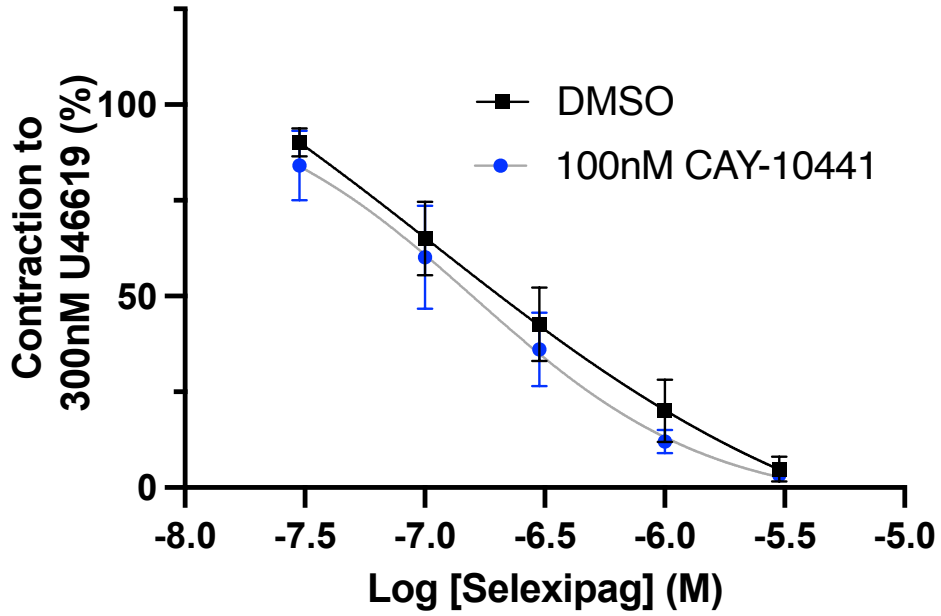


Figure S2. Cumulative concentration effect curves generated from Iloprost mediated vasoactive responses.

Mean data for Iloprost mediated vasoactive responses (A; 0.001-1 µmol-L^{-1}) within pre-contracted (300 nmol-L^{-1} U46619) mesenteric arteries arteries from male (black) and female (red) and female Wistar rats (A). The effect of equivalent cumulatively increasing volumes of DMSO solvent controls on pre-contracted vessels from male and female Wistars (B). Iloprost mediated responses in vessels from male and female Wistars pre-incubated in either solvent control (DMSO; C-F; *n*=6-8), 300 nmol-L^{-1} L-798,106 (C,D; *n*=6-8) or 100 nmol-L^{-1} CAY-10441 (E,F; *n*=6-8). All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.



1

Figure S3. Selexipag mediated relaxation was unaffected by CAY-10441 in male mesenteric arteries.

Mean data Selexipag (B; 0.03-3 µmol-L^{-1}) mediated relaxation in vessels pre-incubated in DMSO solvent control (black; *n*=6) or 100 nmol-L^{-1} CAY-10441 (*n*=5) in male mesenteric arteries. All values are expressed as mean ± SEM. A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.

2

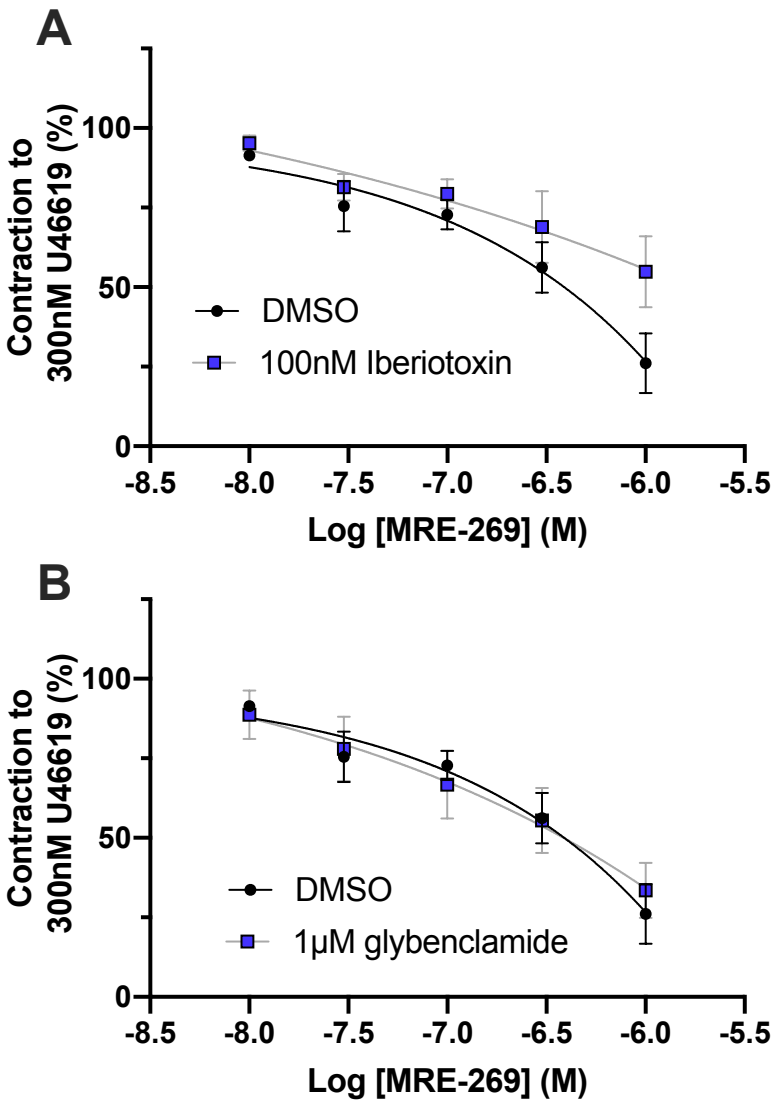


Figure S4. MRE-269 mediated relaxation is not attenuated K_{ATP} and BK_{Ca} inhibition.
Mean data for MRE-269 (A,B,C 0.01-10 μmol-L^{-1}) mediated relaxation in vessels pre-incubated in DMSO solvent control (A,B,C; black; *n*=4), 100 nmol-L^{-1} BK_{Ca} channel inhibitor Iberiotoxin (A; blue; *n*=5) or 1 μmol-L^{-1} K_{ATP} channel inhibitor glibenclamide (B; blue; *n*=5) in male mesenteric arteries. All values are expressed as mean ± SEM (A-F). A two-way statistical ANOVA with a post-hoc Bonferroni test was used to generate significant values (*=*P*<0.05). (*n*=) number of animals used.