Identification and characterization of KV7 channels within rat mesenteric endothelial cells

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Abstract

Background and purpose KCNQ-encoded KV7 channels are expressed within vascular smooth muscle cells (VSMCs) and are key regulators of vascular reactivity, regulating resting tone and as functional targets of endogenous responses. Endothelial cells (ECs) form a paracrine signaling platform that line all blood vessels and regulate tone, but little is known of KV7 channels in vascular ECs. This study aims to characterize the expression and function of KV7 channels within rat mesenteric artery ECs. Experimental approach In rat mesenteric artery, KCNQ transcript and KV7 channel protein expression were determined via RT-qPCR, immunocytochemistry, immunohistochemistry and immunoelectron microscopy. Wire myography was used to determine vascular reactivity. Key results KCNQ transcript was identified in EC marker expressing cells using a reductive approach. KV7.4 and KV7.5 protein expression was determined in both isolated EC and VSMC and in whole vessels. Removal of ECs attenuated vasorelaxation to two structurally different KV7.2-5 activators S-1 and ML213. KIR2 blockers ML133 and BaCl also attenuated S-1 or ML213-mediated vasorelaxation in an endothelium-dependent process. KV7 inhibition attenuated receptor-dependent nitric oxide (NO)-mediated vasorelaxation to carbachol, but had no impact on relaxation to the NO donor, SNP. Conclusions and implications In rat mesenteric artery ECs, KV7.4 and KV7.5 channels are expressed, functionally interact with endothelial KIR2.x channels and contribute to endogenous eNOS-mediated relaxation. This study identifies KV7 channels as novel functional channels within rat mesenteric ECs and suggests that these channels are involved in NO release from the endothelium.

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- SL Sandow contributed essential reagents or tools.
- SN Baldwin, SL Sandow and G Mondéjar-Parreño analysed the data.
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Conflict of interest

The authors declare no conflict of interest.

Abstract

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KCNQ-encoded K_V7 channels are expressed within vascular smooth muscle cells (VSMCs) and are key regulators of vascular reactivity, regulating resting tone and as functional targets of endogenous responses. Endothelial cells (ECs) form a paracrine signaling platform that line all blood vessels and regulate tone, but little is known of K_V7 channels in vascular ECs. This study aims to characterize the expression and function of K_V7 channels within rat mesenteric artery ECs.

Experimental approach

In rat mesenteric artery, KCNQ transcript and K_V7 channel protein expression were determined via RT-qPCR, immunocytochemistry, immunohistochemistry and immunoelectron microscopy. Wire myography was used to determine vascular reactivity.

Key results

KCNQ transcript was identified in EC marker expressing cells using a reductive approach. $K_V7.4$ and $K_V7.5$ protein expression was determined in both isolated EC and VSMC and in whole vessels. Removal of ECs attenuated vasorelaxation to two structurally different $K_V7.2$-5 activators S-1 and ML213. $K_{IR}2$ blockers ML133, and BaCl also attenuated S-1 or ML213-mediated vasorelaxation in an endothelium-dependent process. K_V7 inhibition attenuated receptor-dependent nitric oxide (NO)-mediated vasorelaxation to carbachol, but had no impact on relaxation to the NO donor, SNP.

Conclusions and implications

In rat mesenteric artery ECs, $K_V7.4$ and $K_V7.5$ channels are expressed, functionally interact with endothelial $K_{IR}2.x$ channels and contribute to endogenous eNOS-mediated relaxation. This study identifies K_V7 channels as novel functional channels within rat mesenteric ECs and suggests that these channels are involved in NO release from the endothelium.

Key words

- K_V7 channel
- Endothelial cell
- Vascular smooth muscle cell
- K_{IR} channel

What is already known

K_V7 channels and endothelial cells are key regulators of vascular tone.

What this study adds

- When stimulated, endothelial cell K_V7 channels which interact with $K_{IR}2.x$ channels.
- Endothelial K_V7 channels contribute to carbachol-mediated eNOS dependent relaxation.

Clinical significance

Endothelial K_V7 channels represent novel targets in endothelial dysfunction.

Abbreviations

4-AP 4-aminopyridine

Acta2 α-smooth muscle actin-2
B2M Beta-2-microglobulin
CCh Carbachol
cGMP Cyclic guanosine monophosphate
Cq Quantification cycle
CYC1 Cytochrome C1
EC Endothelial cell
EDH Endothelium derived hyperpolarization
eNOS endothelial nitric oxide synthase
IK_{Ca} Intermediate conductance calcium-activated potassium channel
IEL internal elastic lamina **K_{IR}** Inwardly rectifying potassium channel
K_V Voltage-gated potassium channel
L-NAME L-nitroarginine methyl ester
MA Mesenteric artery
MO Methoxamine
Myh11 Myosin heavy chain 11
NO Nitric oxide
PECAM-1 Platelet endothelial cell adhesion molecule-1
RT-qPCR Reverse-transcription quantitative polymerase chain reaction
SEM Standard error of the mean
SK_{Ca} Small conductance calcium-activated potassium channel
SNP S-nitroprusside
TEA Tetraethylammonium
UBC Ubiquitin C **VGCC** Voltage-gated calcium channels
VSMC Vascular smooth muscle cell
vWBF von-Willebrand factor

Introduction

KCNQ-encoded K_V7 channels are key regulators of arterial reactivity. Of the five KCNQ subtypes, KCNQ4 is the most predominantly expressed in the vasculature, followed by KCNQ5 then KCNQ1, with little to no contribution from KCNQ2/3 (Ng et al., 2011; Ohya, Sergeant, Greenwood, & Horowitz, 2003; Yeung et al., 2007). In human and rodent blood vessels K_V7 channels contribute to resting tone (Ohya *et al.* , 2003; Yeung *et al.* , 2007; Ng *et al.* , 2011, Mackie et al., 2008) whereby K_V7 blockers like linopirdine or XE991 produce contractions or enhance vasoconstrictor responses. In addition, a range of compounds like retigabine or S-1 that increase the activity of $K_V7.2$-7.5 are effective relaxants of pre-contracted arteries. K_V7 channels are also functional end targets for a myriad of endogenous vasoactive responses. Channel activity is impaired during PKC-mediated vasoconstriction (L. I. Brueggemann et al., 2006) and enhanced as a result of cGMP and cAMP dependent receptor-mediated vasodilatations (e.g. Chadha *et al.* , 2012; Khanamiri et al., 2013; Stott, Jepps and Greenwood, 2014; Stott *et al.* , 2015; Mani *et al.* , 2016; Brueggemann *et al.* , 2018). To date, vascular studies on K_V7 channels have focused predominantly on vascular smooth muscle cells (VSMCs), and as a result it is currently unclear whether endothelial cell (EC) K_V7 channels exist and if so, what their functional role may be.

The inner layer of blood vessels is comprised of ECs, which constitute a paracrine signaling platform that lines all blood vessels. These cells regulate VSMC contractility, vascular resistance and ultimately blood flow through the release of nitric oxide (NO), prostacyclin, epoxyeicosatrienoic acid and others as well as the generation and spread of endothelium-derived hyperpolarization (EDH) (McGuire, Ding, & Triggle, 2001). Myoendothelial projections within fenestrations (holes) of the internal elastic lamina (IEL) facilitate the presence of myoendothelial gap junctions (MEGJs) at a proportion of such sites (˜50% in adult rat 1^{st}-3^{rd} order 'large' mesenteric arteries; MA; (Sandow et al., 2009). Such junctions facilitate heterocellular electrochemical coupling via connexins at junction sites (Sandow, Senadheera, Bertrand, Murphy, & Tare, 2012). Ultimately, MEGJs enable EC-derived signaling pathways via the flow of both small molecules <˜1 kDa and selective currents between cell types. Within rat MA EC-derived vasorelaxant microdomain signaling cascades, previous data has implicated fundamental roles for small/intermediate conductance calcium-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively; (Sandow, Neylon, Chen, & Garland, 2006); Dora et al., 2008 Circ Res), transient receptor potential canonical type 3 channels (Senadheera et al., 2012), inositol-1,3,4 trisphosphate receptor/s (Sandow CEPP 2009), and inwardly rectifying potassium channels ($K_{IR}2$) (Goto, Rummery, Grayson, & Hill, 2004). More recently, within mouse MA, $K_{IR}2.1$ has been identified as a propagator of EC derived signals, acting as a hyperpolarization 'booster' (Sonkusare, Dalsgaard, Bonev, & Nelson, 2016).

Despite the contribution of K_V7 channels to baseline VSMC tension and receptor-mediated responses, their expression and function within ECs remains relatively unknown. This study shows that K_V7 channels are expressed in rat mesenteric ECs and contribute to both K_V7 activator mediated vasorelaxation via a novel functional interaction with $K_{IR}2$ channels and endothelial nitric oxide synthase (eNOS)-dependent carbachol (CCh)-mediated vasorelaxation.

Methods

Animal models

Experiments were performed on mesenteric arteries from Male Wistar rats (Charles River, Margate, UK) ages 11-14 weeks (200-350 g) from the Biological Research Facility, St George's, London, UK; and from the Animal Resources Center, Perth, Australia. Animals were housed in cages with free access to water and food (RM1; Dietex Inter-national, UK)*ad libitum* , 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, the guidelines of the National Health and Medical Research Council of Australia and the UNSW Animal Ethics and Experimentation Committee (AEEC #18/86B). Animals were kept in a bedding of LSB Aspen

woodchip. Animals were culled by either cervical dislocation with secondary confirmation via cessation of the circulation by femoral artery severance or were anaesthetized with sodium pentathol (intraperitoneal, 100 mg/kg) in accordance with Schedule 1 of the ASPA 1986.

Either whole mesenteric plexus or $2^{nd}/3^{rd}/4^{th}$ order MA were used with vessel order identified from the second bifurcation of the superior mesenteric artery. Arteries were dissected, cleaned of fat and adherent tissue and stored on ice within 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 $CaCl_2$.

Reverse transcription quantitative polymerase chain reaction

To investigate gene expression in fresh ECs we used a subtractive approach rather than generating pure native EC samples. Relative fold-changes in expression levels of VSMC/EC markers and *Kcnq*transcript was determined in denuded MA samples (an EC(-) population) compared to whole MA samples (an EC(+) population) via Reverse transcription quantitative polymerase chain reaction (RT-qPCR).

EC(-) MA samples were prepared as described previously (Askew Page et al., 2019); Briefly, vessels were cut open longitudinally and pinned on a Sylgard dish, the lumen of the vessel was then rubbed with human hair, and vessels washed in 0.1% (phosphate buffered saline) PBS-Triton X for 1 x 1 min, then 3 times in PBS (1 minute each) on ice. EC(+) MA samples were whole MA plexus that had not undergone EC removal as above.

mRNA from both whole MA EC(+) and MA EC(-) was extracted using Monarch Total RNA Miniprep Kit (New England BioLabs, Ipswich, Massachusetts, USA) with a LunaScript RT SuperMix Kit (New England BioLabs, Ipswich, Massachusetts, USA). Quantitative analysis of relative gene expression was assessed via CFX-96 Real-Time PCR Detection System (BioRad, Hertfordshire, UK). Samples were run in duplicate to account for variation. Samples were run in BrightWhite qPCR plate (Primer Design, Camberley, UK), with each well containing 20 µL of reaction solution containing: 10 µL of PrecisionPLUS qPCR Master Mix (Primer Design, Camberley, UK), 300 nmol-L^{-1} of gene specific target primer (Thermofisher scientific, Waltham, Massachusetts, USA) and 10 ng of cDNA sample made up to 20 µL total volume with nuclease free water. Run protocol: 1. activation step (15 min:95°C), 2. denaturation step (15 sec: 94°C), 3. annealing step (30 sec: 55°C) and 4. extension step (30 sec: 70°C). Steps 2- 4 were repeated x 40. Quantification cycle (Cq) was determined via Bio-Rad CFX96 Manager 3.0. Values are Cq values normalised to housekeeper genes expressed as a $2^{-\Delta\Delta^{\gamma}}$of MA EC(-) compared to MA EC(+) samples. Appropriate reference genes including ubiquitin C (UBC), cytochrome C1 (CYC1) and Beta-2-microglobulin (B2M; Primer design, UK) were determined using qbase+ PCR analysis system (Biogazelle, Ghent, Belgium) geNorm programme. See Table 1.1 for a list of the primers used in the following (Askew Page et al., 2019; Thomas A. Jepps et al., 2011); ThermoFisher Scientific).

Immunocytochemistry

2^{nd} and 3^{rd} order MA segments were enzymatically digested to obtain freshly isolated ECs as previous (Greenberg et al., 2016). Briefly, vessels were washed in Hanks' Balanced Salt Solution (HBSS; ThermoFisher Scientific, GIBCO, 14170-088) containing 50 µmol-L^{-1} $CaCl_2$ for 5 min at 37 °C and then placed in 1mg/mL collagenase IA (Sigma Aldrich, C9891, UK) in the same solution for 15 min at 37°C. Vessel were washed in HBSS containing 50 µmol-L^{-1} $CaCl_2$ for 10 min at 37°C. The supernatant was removed and the vessels cells suspended in fresh HBSS containing 0.75 mmol-$L^{-1}CaCl_2$. ECs were dissociated using a wide-bore smooth-tipped pipette. The cell-containing solution was plated onto coverslips and left at RT for 1 h before use.

Freshly dispersed ECs, together with residual VSMC, were fixed in 4% paraformaldehyde (*Sigma-Aldrich, UK*) in PBS for 20 min at RT as previously described (Barrese, Stott, Figueiredo, et al., 2018). Cells were treated with 0.1 mol-L^{-1} glycine for 5 min and incubated for 1 h with blocking solution (PBS-0.1% Triton X-100-10% bovine serum albumin) at RT. Following the incubation overnight at 4°C with primary antibodies (Table 1.2) diluted in blocking solution (anti-PECAM-1 for ECs, anti-α-actin for VSMCs and anti-K_V7.4 or K_V7.5 channel for ECs/VSMCs), cells were then washed for 20 min with PBS, incubated for 1 hr at RT with the secondary conjugated antibodies diluted in blocking solution. Excess secondary antibody was removed by washing with PBS and mounted using media containing DAPI for nuclei counterstaining. Using triple staining, ECs and VSMC were differentiated via the following: ECs were positive for anti-CD31 (endothelial cell-specific marker) and negative for anti-α-actin (data not showed); while VSMC was positive for anti-α-actin and negative for anti-PECAM-1 (data not showed). Cells were analysed using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope (Image Resource Unit St George's University of London).

Immunohistochemistry

Animals were anaesthetized with sodium pentathol (intraperitoneal, 100 mg/kg) and perfusion fixed (Sandow, Goto, Rummery, & Hill, 2004) in 2% paraformaldehyde in 0.1 mol-L^{-1} PBS. Third to 4^{th} order mesenteric artery segments were dissected, opened laterally and pinned as a sheet to a Sylgard dish. Segments were washed in PBS (3 x 5 min), incubated in blocking buffer (PBS with 1% BSA and 0.2% Triton) at room temperature (RT) for 2 h and then overnight with primary antibody (Table 1.2) in blocking buffer at 4°C, washed again (3 x 5 min with gentle agitation), and incubated in secondary antibody (Table 1.2; matched to the respective primary) in PBS with 0.1% Triton in PBS for 2 h at RT. Tissue was mounted on slides in anti-fade media containing propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI; Table 1.2) and imaged with uniform confocal settings. Incubation of tissue with secondary only was used as a 'zero' setting for confocal imaging. Controls involved substitution of primary with isotype control, with concentration (where provided by manufacturer) matched, or 10-fold higher than the respective antibody of interest (Table 1.2). Working Ab dilutions were prepared in accordance with previous work (Chadha et al., 2012; Thomas A. Jepps, Greenwood, Moffatt, Sanders, & Ohya, 2009). Confocal image stacks were collected at 0.2 μm intervals. The optimal rinsing protocol was determined by incubating in secondary only; and rinsing after successive 5 min incubations until fluorescence was reduced to background. Note that if this was not done secondary alone was specifically highly localized to IEL hole sites; as potential false positives at such sites; suggesting that such sites have an affinity for IgG-secondary label alone.

Immunoelectron microscopy

Animals were anaesthetized as above and perfusion fixed in 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol-L^{-1}PBS (pH 7.4). Mesenteric artery segments (˜2 mm in length) were washed (3 x 5 min) and processed in a Leica EMPACT 2 high-pressure freezer using 0.7% low melting agarose as a cryoprotectant. Samples were then freeze-substituted in a Leica AFS2 into 0.2% uranyl acetate in 95% acetone (from -85 to -50°C) and infiltrated with Lowicryl (at -50°C), before UV polymerization (2 d each at -50 and 20°C; (Zechariah et al., 2020).

Individual serial transverse sections (˜100 nm) were mounted on Formvar-coated slot grids and processed for antigen localization as for confocal immunohistochemistry (per above and Table 1.2). The secondary used was 5 or 10 nmol-L^{-1}colloidal gold-conjugated antibody (1:40; 2 h) in 0.01% Tween-20. Sections were imaged at x10-40,000 on a JEOL transmission electron microscope at 16 MP (Emsis, Morada G3). Background gold label density was determined from randomly selected (4 x) 1 x 1 μm regions per sample of lumen and IEL, compared to the same sized regions of interest in the endothelium.

Wire Myography

2nd order MA segments (˜2 mm in length) were mounted on 40 μm tungsten wire in a tension myograph chamber (Danish Myo Technology, Arhus, Denmark) containing 5 mL of PSS (composition, as 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), and recorded by LabChart software (ADInstruments, Oxford, UK). Vessels were then challenged with 60 mM [K^+] to determine viability. Vessels were then constricted with 10 μmol-L^{-1}methoxamine, an α-1 adrenoreceptor agonist, and EC integrity determined via addition of 10 μmol-L^{-1} CCh. Vessels displaying [?]90% vasorelaxation in response to CCh (10 μmol-L^{-1}) were considered EC positive (EC+). Vessels were denuded of ECs by gently passing a human hair through the lumen. Vessels expressing [?]10% vasorelaxation in response to CCh (10 μmol-L^{-1}) were considered EC negative (EC-). During functional investigations, all vessels were pre-constricted with the thromboxane A2 receptor agonist U46619 (300 nmol-L^{-1}) to elicit an EC_{80} contraction. Concentration-dependent relaxant responses to S-1 (0.1-10 μmol-L^{-1}), ML213 (0.1-10 μmol-L^{-1}), ML277 (0.03-1 μmol-L^{-1}), CCh (0.3-10 μmol-L^{-1}) and S-nitroprusside (SNP; 0.01-3 μmol-L^{-1}) were determined in the presence and absence of ECs, linopirdine (10 μmol-L^{-1}), HMR-1556 (10 μmol-L^{-1}), ML133 (20 μmol-L^{-1}), barium chloride (BaCl; 100 μmol-L^{-1}), L-nitroarginine methyl ester (L-NAME; 100 μmol-L^{-1}), TRAM34 (1 μmol-L^{-1}), Apamin (10 nmol-L^{-1}), 4-aminopyridine (4-AP; 1 mmol-L^{-1}) and tetraethylamonium (TEA; 1 mmol-L^{-1}).

Dara and statistical analysis

All functional figures express mean data from at least 5 animals ±standard error of the mean (SEM). For functional experiments involving cumulative concentrations, a transformed data set was generated using; X=Log(X), to reduce representative skew. A four parametric linear regression analysis was then performed using the following equation; (Log(Agonist) vs. response – variable slope (four parameters bottom/hillslope/top/EC_{50})) using GraphPad Prism (Version 8.2.0) to fit a CEC to the figure. For data comparing multiple groups, a two way-ANOVA followed by a *post hoc* Bonferonni test, to account for type 1 errors in multiple comparisons was performed for comparison of mean values. Significance values are represented as follows; P <0.05 (*). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018).

Results

Identification of K_V7 channels within MA ECs

Initial investigation sought to identify $K_V7.4$ and $K_V7.5$ channel transcript and protein within MA ECs, as these subtypes are implicated in vascular reactivity (Barrese *et al* , 2018). Transcript levels for EC and VSMC markers were determined in cell lysates from MA with and without EC removal by mechanical abrasion (see Methods). In cell suspensions from EC-denuded vessels there was a reduction in EC markers (*Pecam/Vwbf*) and an increase in VSMC markers (*Acta2/Myh11*) compared to lysates from unabraded MA (Figure 1). This was associated with a 1.8- and 2.1-fold decrease in relative transcript abundance of *Kcnq4* and *Kcnq5,* (Figure 1) showing that a considerable component of these transcripts were derived from EC cells. This data suggests expression of *Kcnq4* and*Kcnq5* within EC marker expressing cells (Figure 1).

$K_V7.4$ was $K_V7.5$ was detected in isolated ECs by immunodetection (Figure 2A,C). $K_V7.4$ had a punctate distribution in isolated ECs (Figure 2A) whereas $K_V7.5$ label appeared to be predominantly cytoplasmic with some diffuse label around the nucleus (Figure 2C). Similar to previous reports (Zhong *et al.* , 2010; Oliveras *et al.* , 2014; Mills *et al.* , 2015; Morales-Cano *et al.* , 2015, Barrese et al., 2018) $K_V7.4$ and 7.5 were identified in isolated MA VSMCs (Figure 2B,D). Notably, $K_V7.4$ and $K_V7.5$ were also detected in both ECs

and VSMCs in*en face* whole-mount tissue (Figure 2.E-H). $K_V7.4$ was also detected in EC by immunoelectron microscopy (supplemental figure 1). Further, both $K_V7.4$ and 7.5 were expressed at a proportion of IEL hole sites at an apparently higher level than the associated EC membrane label (Figure 3).

Removal of ECs modulates $K_V7.2$-5 activator efficacy

A comprehensive pharmacological analysis was undertaken to determine if K_V7 channels have a functional role in MA ECs. Effects of K_V7 channel modulators were examined in MAs where the endothelium was intact or had been removed. S-1 and ML213 are structurally dissimilar activators of $K_V7.2$-7.5 that interact with the same pharmacophore centered around a tryptophan in the S5 domain (Bentzen et al., 2006; Lyubov I. Brueggemann, Haick, Cribbs, & Byron, 2014; T. A. Jepps et al., 2014; Schenzer, 2005). ML277 is a potent activator of $K_V7.1$ (Yu, 2013) with a 100-fold increase in selectivity for $K_V7.1$ compared to $K_V7.2$-5 (Yu, 2013). Consistent with previous findings (Chadha et al., 2012; T. A. Jepps et al., 2014), S-1- and ML213-mediated vasorelaxation was ablated by pre-incubation with 10 μmol-L^{-1} pan-K_V7 channel inhibitor linopirdine (Schnee & Brown, 1998); Figure 4.A,B). Relaxations produced by 10 to 300 nmol-L^{-1} ML277 were also prevented by incubation with linopirdine (Figure 4C). However, relaxations produced by concentrations >1 μmol-L^{-1} ML277 were not attenuated by linopirdine and are therefore not mediated by Kv7.1 activation.

EC removal for the following experiments was confirmed by ablation of vasorelaxation in response to 10 μmol-L^{-1} CCh (Figure 5A). Removing the endothelium by mechanical abrasion has no impact on the peak contraction produced by 300 nmol-L^{-1} U46619 (Figure 4B), but significantly attenuated the potency of S-1 mediated vasorelaxation increasing EC_{50} from 2±0.2 μmol-L^{-1} to 3±0.7 μmol-L^{-1} (Figure 5C,D). The potency of ML213 was also impaired by endothelial removal (EC(+) EC_{50} = 1±0.2 μmol-L^{-1} vs EC(-) EC_{50} = 3±0.2 μmol-L^{-1}; Figure 5E). The linopirdine-sensitive relaxation produced by ML277 was not affected by endothelial removal (Figure 5F). The accumulated findings reveal that ECs express $K_V7.4$ and $K_V7.5$ and the presence of the endothelium enhances the sensitivity of two structurally different $K_V7.2$-7.5 activators.

IK_{Ca}/SK_{Ca} inhibitors have no impact on K_V7 activator mediated relaxation

Having identified that the endothelium modulates responses to Kv7 activators, experiments were performed to identify the mechanism/s involved. Endothelial IK_{Ca} and SK_{Ca}channels contribute to relaxation responses in rat MA (Crane, Gallagher, Dora, & Garland, 2003). Thus, it is feasible that K_V7 channels interact with other key endothelial potassium channels; particularly in microdomains (Sandow et al., 2009). However, in agreement with previous reports (T. A. Jepps, Olesen, Greenwood, & Dalsgaard, 2016), pre-incubation with a combination of IK_{Ca} inhibitor TRAM-34 (1 μmol-L^{-1}; (Wulff et al., 2000) and SK_{Ca} inhibitor apamin (100 nmol-L^{-1}; (Spoerri, Jentsch, & Glees, 1975) had no effect on K_V7 activator mediated vasorelaxation (Figure 6A-C).

EC K_{ir} channels modulate $K_V7.2$-5 activator sensitivity

Within MAs of a EC-$K_{IR}2.1$-/- *murine* model, endothelial KCNJ2-encoded $K_{IR}2.1$ channels have been identified as 'signal boosters' that enhance EC-derived relaxation (Sonkusare et al., 2016). Comparatively, the current literature regarding *rat* mesenteric $K_{IR}2$ channels is limited; with $K_{IR}2.1$ expression being demonstrated in EC of whole mount rat MA (Dora, Gallagher, McNeish, & Garland, 2008), where inwardly rectifying Ba^{2+} sensitive channels are apparently restricted to the endothelial layer (Crane, Walker, Dora, & Garland, 2003), and K_{IR} channels contribute to acetylcholine-mediated responses (Goto et al., 2004). We propose that like mice, rat mesenteric ECs express functional $K_{IR}2$ channels that propagate EC signals in a similar process. Therefore, we performed a series of studies investigating the effect of two well characterized $K_{IR}2$ blockers, $BaCl_2$(Hagiwara, Miyazaki, Moody, & Patlak, 1978) and ML133 (Wang et al., 2011; Wu et al., 2010) on K_V7 activator-mediated vasorelaxation.

In arteries with a functional endothelium, $K_{IR}2$ blockers, BaCl (100 μmol-L^{-1}) and ML133 (20 μmol-L^{-1}), significantly impaired vasorelaxation by S-1 (Figure 7A, EC_{50}= DMSO, 1.89±0.2 μmol-L^{-1} / BaCl, 2.3±0.31 μmol-L^{-1}; Figure 7B, EC_{50}= DMSO, 0.52±0.12 μmol-L^{-1} / ML133, 3.1±1.5 μmol-L^{-1}, and ML213, Figure 7C,D, EC_{50}= DMSO, 0.9±0.3 μmol/L^{-1} / BaCl, 2.2±0.5 μmol/L^{-1} / ML133, 2.5±0.25 μmol-L^{-1}) when

compared to DMSO solvent control (Figure 7A-D). No attenuation of the response to ML277 was observed consistent with EC removal data. In arteries where the endothelium had been removed neither ML133 nor BaCl had any effect on ML213 mediated vasorelaxation (Figure 7G,H). These data show that the endothelium-dependent increase in potency to the K_V7 activators involves endothelial K_{IR} channels, but not IK_{Ca} or SK_{Ca} channels.

K_V7 channels contribute to CCh evoked vasorelaxation

The expression of functional K_V7 channels within ECs begs the question - Do they contribute to EC-derived responses? Acetylcholine produces endothelium-dependent relaxations through NO-, EDH- and prostanoid-dependent mechanisms in rat MA (Parsons, Hill, Waldron, Plane, & Garland, 1994; Peredo, Feleder, & Adler-Graschinsky, 1997; Shimokawa et al., 1996).

A distinct rightward shift in the sensitivity to vasorelaxation in response to CCh, a synthetic acetylcholine analogue, was produced by 100 μmol-L^{-1} eNOS inhibitor L-NAME when compared to DMSO solvent control (EC_{50} DMSO = 0.59±0.1 μmol-L^{-1}; L-NAME = 0.94±0.1 μmol-L^{-1}; Figure 8A). A combination IK_{Ca} and SK_{Ca}inhibitors, 1 μmol-L^{-1} TRAM34 and 100 nmol-L^{-1} apamin, respectively produced greater attenuation (EC_{50} TRAM34/apamin=1.5±0.7 μmol-L^{-1}; Figure 8A). These results show that both NO- and EDH-dependent signaling contributes to CCh-mediated vasodilation, though the main contributor to endothelial-dependent vasodilation in 2nd order MA appears to be EDH, conferring with previously published data (Shimokawa et al., 1996). Pre-incubating vessels with 10 μmol-L^{-1} pan-K_V7 channel inhibitor linopirdine significantly attenuated CCh-mediated vasorelaxation when compared to DMSO control (EC_{50} DMSO = 0.2±0.08 μmol-L^{-1}; linopirdine = 0.7±0.3 μmol-L^{-1}; Figure 8B). In contrast, pre-incubating vessels with either the $K_V7.1$ specific inhibitor HMR-1556 (10 μmol-L^{-1}; Figure 8C) or a combination of non-specific K_V channel inhibitors TEA (1 mmol-L^{-1}; (Choi, Aldricht, Yellen, & Hughes, 1991) and 4-AP (1 mmol-L^{-1} ; (Kurata & Fedida, 2006) had no significant effect on CCh-evoked vasorelaxation. These results imply a specific contribution of $K_V7.4$ and $K_V7.5$ channels to CCh-evoked relaxation in 2nd order rat MA.

Subsequent experiments explored whether linopirdine affected CCh-induced relaxations in the presence of L-NAME compared to responses in apamin/TRAM34. CCh-evoked relaxations were significantly attenuated in vessels pre-incubated in TRAM34/apamin and linopirdine compared to vessels only pre-incubated in TRAM34/apamin alone (EC_{50}DMSO = 0.24±0.05 μmol-L^{-1}; TRAM34/Apamin = 0.27±0.03 μmol-L^{-1}; TRAM34/Apamin + linopirdine = 0.61±0.2 μmol-L^{-1}; Figure 8E). In contrast, linopirdine failed to attenuate CCh relaxations in arteries pre-incubated with L-NAME (Figure 8F). These data suggest that $K_V7.4$ and $K_V7.5$ channels contribute to the NO pathway of CCh-mediated vasorelaxation.

K_V7 channels contribute to NO-mediated vasorelaxation in a vascular bed specific manner (Jennifer B. Stott et al., 2015). The present data demonstrate that pre-incubation with the pan-K_V7 channel blocker linopirdine (10 μmol-L^{-1}) has no effect on vasorelaxation produced by the NO-donor s-nitroprusside (SNP; Figure 8G). However, in contrast with previous reports (T. A. Jepps et al., 2016), pre-incubation with L-NAME (100 μmol-L^{-1}) significantly attenuated $K_V7.2$-5 activator mediated vasorelaxation (Figure 8H). These data suggest that the NO axis of CCh-mediated vasorelaxation is linopirdine sensitive, upstream of NO signaling.

Discussion

The present study identified *Kcnq4* and *Kcnq5* transcripts within EC marker expressing cells as well as respective $K_V7.4$ and $K_V7.5$ protein in isolated and whole mount rat MA ECs/VSMCs, consistent with previous studies in the endothelium (Chen, Li, Hiett, & Obukhov, 2016) and vascular smooth muscle (e.g Zhong *et al.* , 2010; Oliveras *et al.* , 2014; Mills *et al.* , 2015, summarised in Barrese *et al* ., 2018). Functionally, the present study demonstrates that the relaxation produced by two structurally different $K_V7.2$-5 activators, but not a $K_V7.1$ activator, were modulated by the endothelium. These relaxations were also sensitive to

$K_{IR}2$ inhibition and suggest a novel functional interaction between K_V7 channels and endothelial $K_{IR}2.x$ channels. Furthermore, we have demonstrated that $K_V7.4/7.5$ channels contribute to the NO-mediated axis of CCh-evoked endothelium-dependent relaxations. Thus, K_V7 channels are expressed within ECs, contribute to endothelium-derived responses and when pharmacologically upregulated, are functionally coupled to other EC potassium channels.

K_V7 channel expression and function within ECs

Within smooth muscle, when active, K_V7 channels hyperpolarize the membrane potential decreasing voltage-gated calcium channel (VGCC) open probability, therefore decreasing the influx of extracellular calcium, with this decrease resulting in relaxation of the cell. Within rodent models, the pharmacopeia of K_V7 channel modulators has revealed $K_V7.4$ and $K_V7.5$ channels; 1. contribute to resting vascular tone via regulation of the resting membrane potential; 2. are upregulated during cGMP and cAMP/EPAC/PKA-mediated vasodilation, and; 3. are supressed via PKC-mediated vasoconstriction. Conjunctly, the use of molecular techniques has revealed that $K_V7.4/K_V7.5$ is the most predominant heterotetramer expressed within specific rodent arteries. Comparatively, no functional role for $K_V7.1$ in arteries has been identified (see (Byron & Brueggemann, 2018; Barrese *et al* , 2018), for review). A caveat of these observations is a lack of differentiation between VSMCs and ECs. However, K_V7 channels were recently identified in pig coronary artery ECs (Chen et al., 2016) where they modulate bradykinin-evoked endothelium-derived vasorelaxation (Chen et al., 2016). The data presented here demonstrate K_V7 channel expression in rat mesenteric endothelium and support their functional role.

In most arteries, the endothelium and smooth muscle are electrochemically linked via MEGJs formed from connexin proteins within heterocellular communicating microdomains (see (Sandow et al., 2012) for review). Via these connections, it has been purported that current injection into ECs passes into VSMCs (Sandow, Tare, Coleman, Hill, & Parkington, 2002). As K_V7 channels are expressed within rat mesenteric ECs, the present data suggest a potential role for this process in K_V7 activator-mediated vasorelaxation. This conjecture is supported by a significant attenuation of vasorelaxation of pre-constricted arterial tone by two structurally different $K_V7.2$-5 activators in the absence of ECs. In contrast, EC removal had no impact on $K_V7.1$ activator-mediated vasorelaxation, suggesting that only $K_V7.4$ and $K_V7.5$ channels are functionally expressed within MA ECs. However, Chen *et al,* (2016) suggest that endothelial removal had no impact on ML213-mediated relaxation in pig coronary artery segments. Consequently, these collective data suggest that this phenomenon is both animal and artery bed specific, potentially being dependent on the molecular architecture of the vessel, such as MEGJ and related microdomain properties.

A novel functional interaction with endothelial K_{IR}channels

$K_{IR}2.x$ channels have been characterized in a variety of rat vascular beds including cerebral and coronary arteries (Smith et al., 2008), where their selective inhibition by Ba^{2+}revealed K_{IR} channel amplification of a K^+ channel activator conductance (Smith et al., 2008). However, there is a degree of conflict regarding the role of $K_{IR}2.x$ channels within rat MAs. As above, a Ba^{2+} sensitive current and $K_{IR}2.x$ expression has been demonstrated in rat MA ECs (Crane, Walker, et al., 2003; Smith et al., 2008). Furthermore, within primary MA $K_{IR}2.x$ channels were purported to contribute to K_{Ca} mediated-hyperpolarization during ACh-derived EC-dependent responses (Goto et al., 2004). In contrast, Smith *et al.* , (2008) demonstrate that within 3rd order MA ACh-mediated K^+ conductance-dependent vasorelaxation was insensitive to Ba^{2+}, indicating that $K_{IR}2.x$ channels do not augment K^+conductance in these vessels during receptor-mediated vasodilation.

In the present study, significant attenuation of both S-1 and ML213 $K_V7.2$-5 activator-mediated vasorelaxation was found after pre-incubation in two structurally different $K_{IR}2.x$ blockers ML133 (Wu et al., 2010) and Ba^{2+} (Hagiwara et al., 1978). ML133 has been identified via high-throughput and mutagenesis investigation as a novel inhibitor of $K_{IR}2.1$ channels with an IC_{50} of 1.8 μmol-L^{-1} at pH 7.4, with little to no selectivity against the other members of the $K_{IR}2.x$ family (Wang et al., 2011; Wu et al., 2010) where it

exerts its activity via D172 and I176 within the M2 region of $K_{IR}2.1$ (Wang et al., 2011). Presently, ML133 is the most selective inhibitor of the $K_{IR}2$ family. In parallel to the endothelium denudation experiments, no effect was seen on the $K_V7.1$ activator ML277-mediated vasorelaxation; implying a specific interplay with $K_V7.4$ and $K_V7.5$ channels. Furthermore, $K_{IR}2$ blockers had no effect on $K_V7.2$-7.5 activator-mediated relaxation in EC-denuded arteries, supporting the notion that functional $K_{IR}2$ channels (in the rat MA bed) are restricted to the endothelium (Crane, Walker, et al., 2003), and the agents do not inhibit K_V7 channels *per se* . The present study suggests that pharmacological activation of endothelial $K_V7.4/7.5$ channels also activates an endothelial $K_{IR}2$ channel increased K^+ conductance, which in turn accounts for a significant degree of the EC augmentation of $K_V7.2$-5 activator-mediated vasorelaxation (Figure 11). However, based on the findings described by Goto *et al,.* (2004) and Smith *et al,.* (2008), the collective data suggest that this phenomenon is dependent on the branch order of MA. Furthermore, it remains unclear if this occurs during receptor mediated signaling, or that is only present during pharmacological activation of endothelial K_V7 channels.

A primary concern for identification of novel functional interactions between ion channels using pharmacological tools is off-target effects. However, K_V7 activator-mediated vasorelaxation in vessels pre-incubated in 10 μmol-L^{-1} linopirdine was abolished. If S-1 or ML213 were activating other channels, i.e. e.g. K_{IR} channels, a degree of vasorelaxation would still be observed in the presence of linopirdine. The present findings therefore suggest that both S-1 and ML213 work exclusively via K_V7 channels and that a novel functional coupling of $K_V7.4/7.5$ and $K_{IR}2$ occurs in rat MA ECs.

The contribution of K_V7 channels to CCh evoked relaxations within rat MA

K_V7 channels contribute to cGMP mediated vasorelaxation in a vascular bed specific manner (Stott et al., 2015). Within rat aortic, renal and pulmonary arteries, overexpression models, and native VSMCs $K_V7.4/K_V7.5$ have been identified as downstream targets of cGMP signaling during both isometric tension recording and whole cell current recording (Mondéjar-Parreño et al., 2019; Jennifer B. Stott et al., 2015). Conversely, K_V7 channels do not contribute to NO donor SNP-mediated vasorelaxation within rat renal arteries (Jennifer B. Stott et al., 2015).

In light of the significant impact of K_V7 inhibition on CCh-mediated vasorelaxation during the suppression of EDH, the present study suggests that K_V7 channels contribute to CCh-evoked NO-cGMP mediated relaxation within rat MA. Though, similar to renal arteries, K_V7 channels do not represent downstream targets of NO signaling in rat MA as K_V7 channel inhibition does not impair SNP-mediated relaxations. However, eNOS inhibition does impair relaxation to a K_V7 activator, implying that K_V7 channels are involved in the production or release of NO in response to CCh. Though this appears to be a vascular bed specific phenomenon, as L-NAME significantly impairs ML213 relaxations in pig coronary artery (Chen et al., 2016), but not rat penile artery (T. A. Jepps et al., 2016).

Conclusions

In conclusion, the present data identify a novel functional interaction between mesenteric endothelial $K_V7.4/7.5$ and $K_{IR}2$ channels and supports the proposition that endothelial K_V7 channels contribute to endogenous endothelial-derived responses. These findings highlight the complex nature of the vascular response to K_V7 channel upregulation and emphasize the importance of K_V7 channels to vascular signaling cascades. The present data are consistent with K_V7 channels representing a novel therapeutic target in endothelial dysfunction.

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Tables

Gene	(+) Forward primer sequence (-) Reverse primer sequence	Gene accession number	Amplicon (bp)
Myh11	CAGTTGGACACTATGTCAAGCCC ATGGAGACAAATGCTAATCAGCC	XM_017304998.1	78
Acta2	ATCCGATAGAACACGGCATC AGGCATAGAGGGACAGCACA	NM_031004.2	228
Vwbf	GTCGGAAGAGGAAGTGGAAC GGGCACACGCATGCGCTCTGTA	NM_011689.1	136
Pecam1	CTCCTAAGAGCAAAGAGAAAGC TACACTGGTATTCCATGTCTCTGG	NM_008816.1	100
Kcnq4	GAATGAGCAGCTCCCAGAAC AAGCTCCAGCTTTTCTGCAC	XM_006233477.8	133
Kcnq5	AACTGATGAGGAGGTCGATG GATG	XM_001071249.3	120

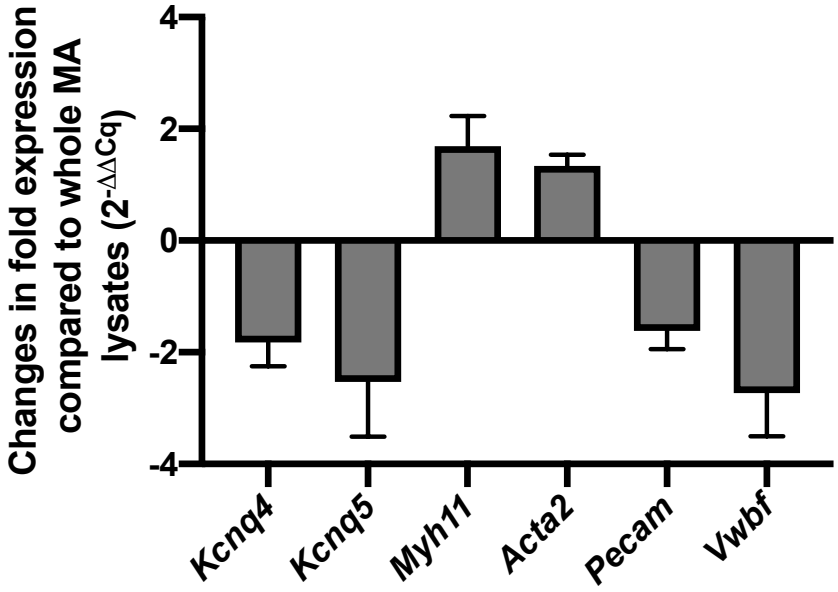
Reagent purpose	Detail	Source	Predicted MW, kDa	Epitope	[used]	Peptide availability	Peptide availability	Raised in
Primary antibodies	Kv7.4 / KCNQ4	NeuroMab, cat no 75-082, 1 mg/ml	77	Hu aa 2-77, clone N4 / 36 IgG	1:200 (5 µg/ml)	no	no	mouse
	Kv7.4 / KCNQ4*	Abcam, ab65797, lot GR94754, whole serum	77	N' domain	1:100 not available	no	no	rabbit
	Kv7.5 / KCNQ5*	Millipore ABN1372-q2476155; 1 ml/ ml	˜103	human IgG	1:100 (10 µg/ml)	no	no	rabbit
	PECAM-1 / CD31	Santa Cruz Biotechnol-ogy, Sc-1506, 200 µg/ml	130	699-727 aa at the C-terminus	1:100 (2 µg/ml)	-	-	goat
	SM-α-actin	Sigma Aldrich A2547	˜42	N-terminal	(1:100) not available	-	-	mouse
Nuclear labels / cell patency markers	DAPI, Vectasheild	Vectorlabs	-	nucleic acid		-	-	-
	propidium iodide (PI)	Sigma, P4170	-	nucleic acid	10 nM	-	-	-

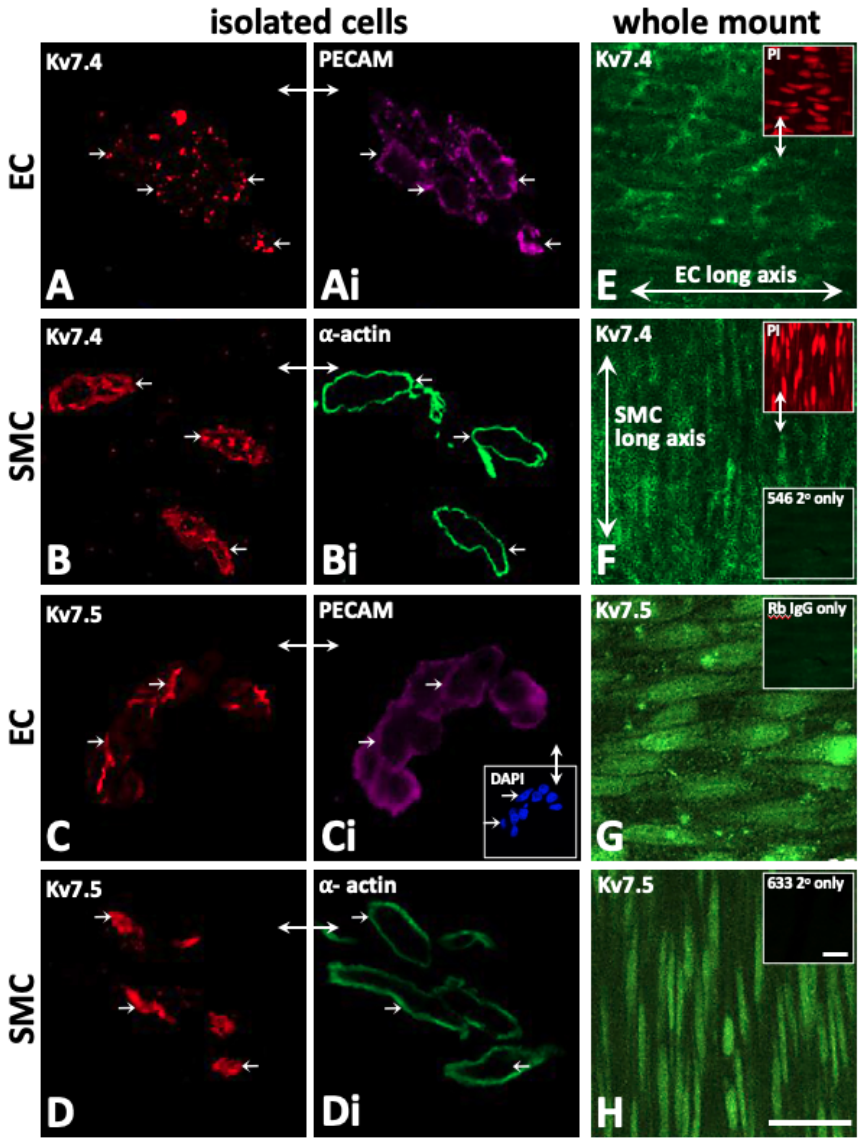
Reagent purpose	Detail	Source	Predicted MW, kDa	Epitope	[used]	Peptide availability	Peptide availability	Raised in
Immuno-histochemistry secondary antibodies	Mouse 568	Abcam, ab175700, lot GR320062-4, 2 mg/ml	-	IgG	1:100 (20 µg/ml)	-	-	donkey
	Rabbit 546	Thermofisher, A-11035	-	IgG	1:100 (20 µg/ml)	-	-	goat
	Rabbit 633	Merck, SAB4600132, lot 15C0423, 2 mg/ml	-	IgG	1:100 (20 µg/ml)	-	-	donkey
Immuno-cytochemistry secondary antibodies	Mouse 488	Thermofisher, A21202, 2 mg/mL	-	IgG	1:100 (0.02 mg/ml)	1:100 (0.02 mg/ml)	-	donkey
	Rabbit 568	Thermofisher, A10042, 2 mg/mL	-	IgG	1:100 (0.02 mg/ml)	1:100 (0.02 mg/ml)	-	donkey
	Goat 633	Thermofisher, A21082, 2 mg/mL	-	IgG	1:100 (0.02 mg/ml)	1:100 (0.02 mg/ml)	-	donkey
Isotype controls	Mouse IgG	ThermoFisher, 10400C	-	IgG	5 mg/ml	-	-	mouse
	Rabbit IgG	ThermoFisher, S31235	-	IgG	10 mg/ml	-	-	rabbit
Immunoelectron microscopy secondary antibodies	5 nm Au anti-rabbit	Merck, G7277, lot SLB3882V	-	IgG	1:100	-	-	goat
	10 nm Au anti-rabbit	Merck, G7402	-	IgG	1:100	-	-	goat

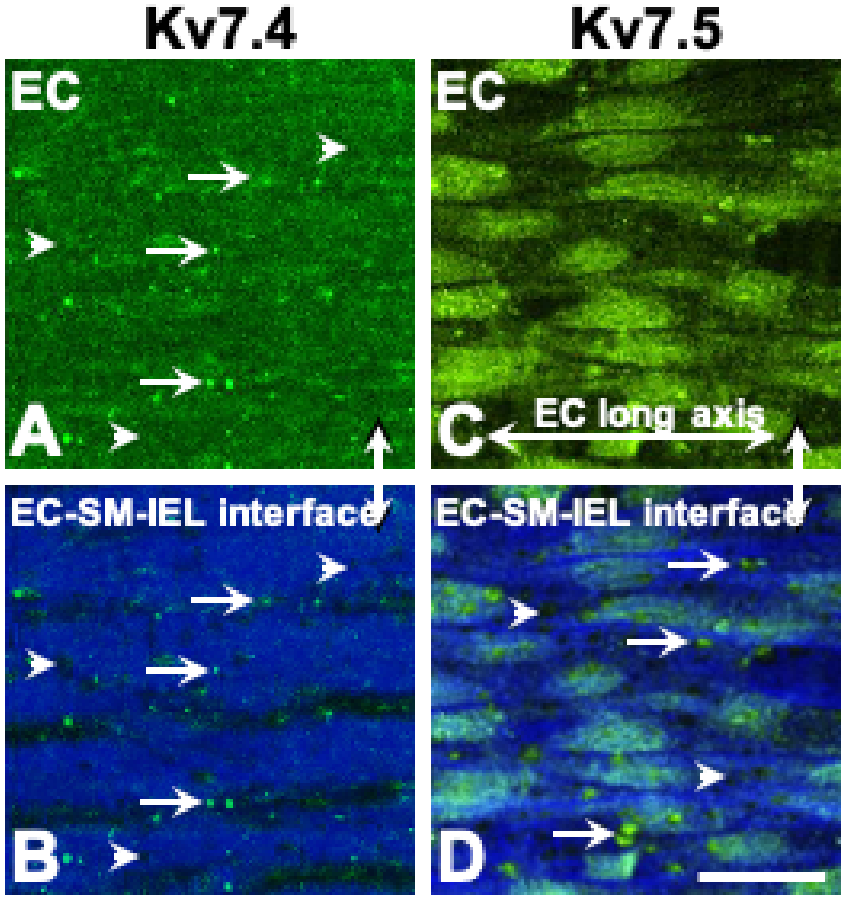
Table 1.2. Immunocyto/histochemistry reagents and use (Chadha et al., 2012; Thomas A. Jepps et al., 2009).

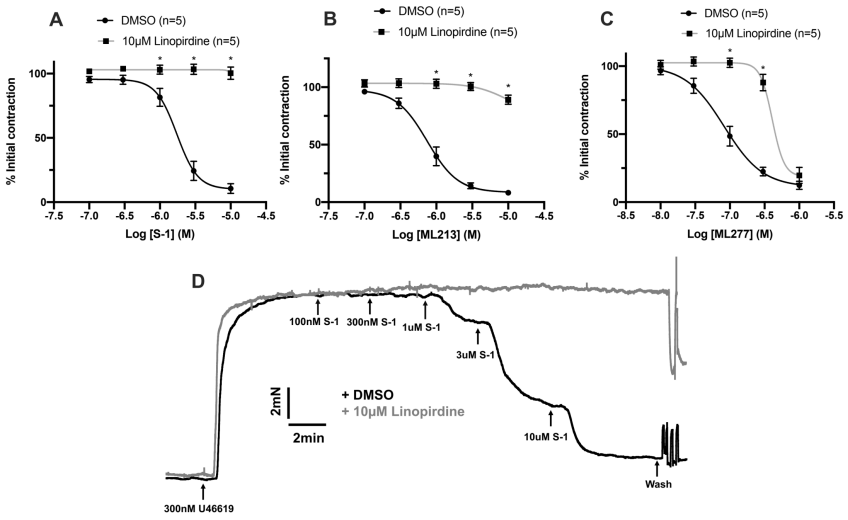
Abbreviations. CD31, cluster of differentiation 31; DAPI, 4',6-diamidino-2-phenylindole; PECAM, platelet endothelial cell adhesion molecule.

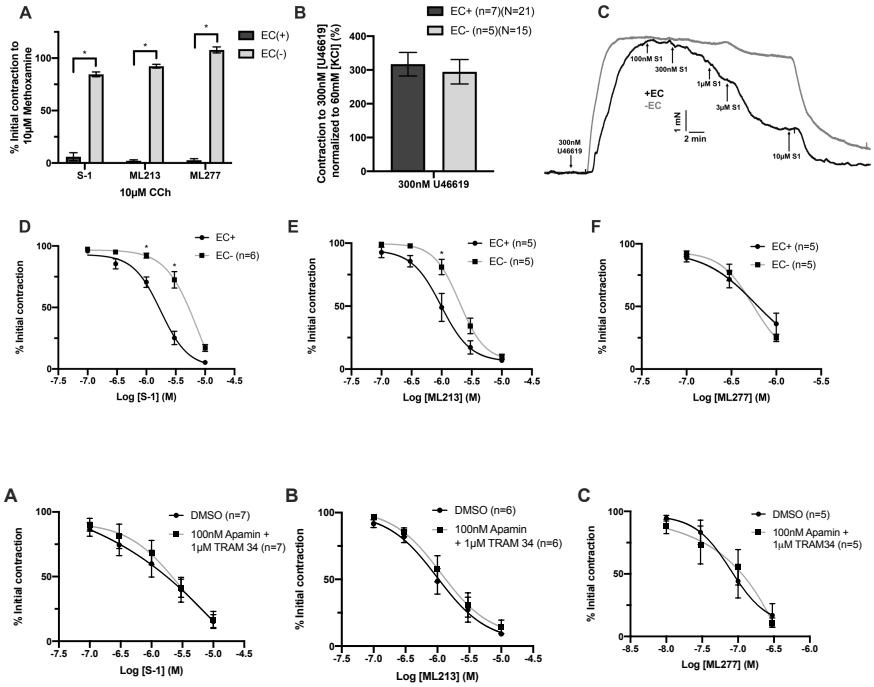
Figure legends

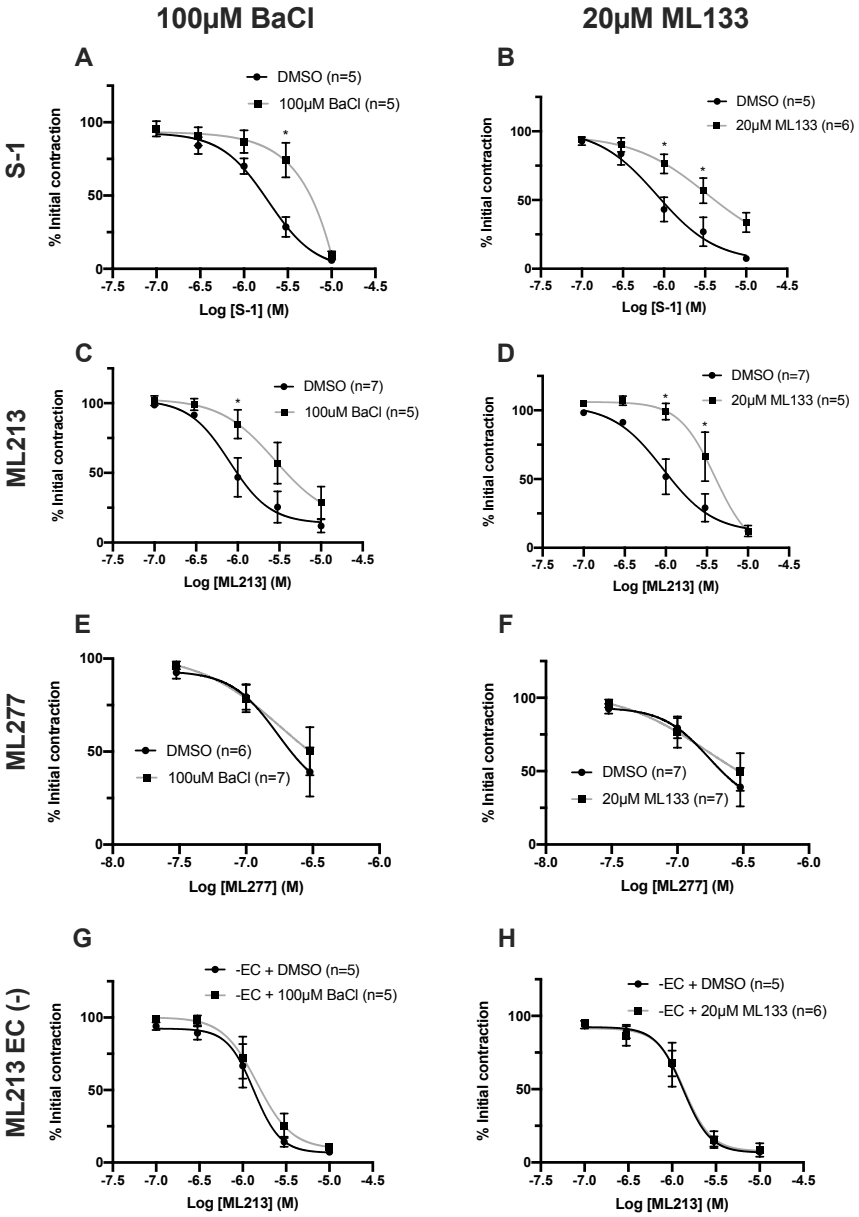


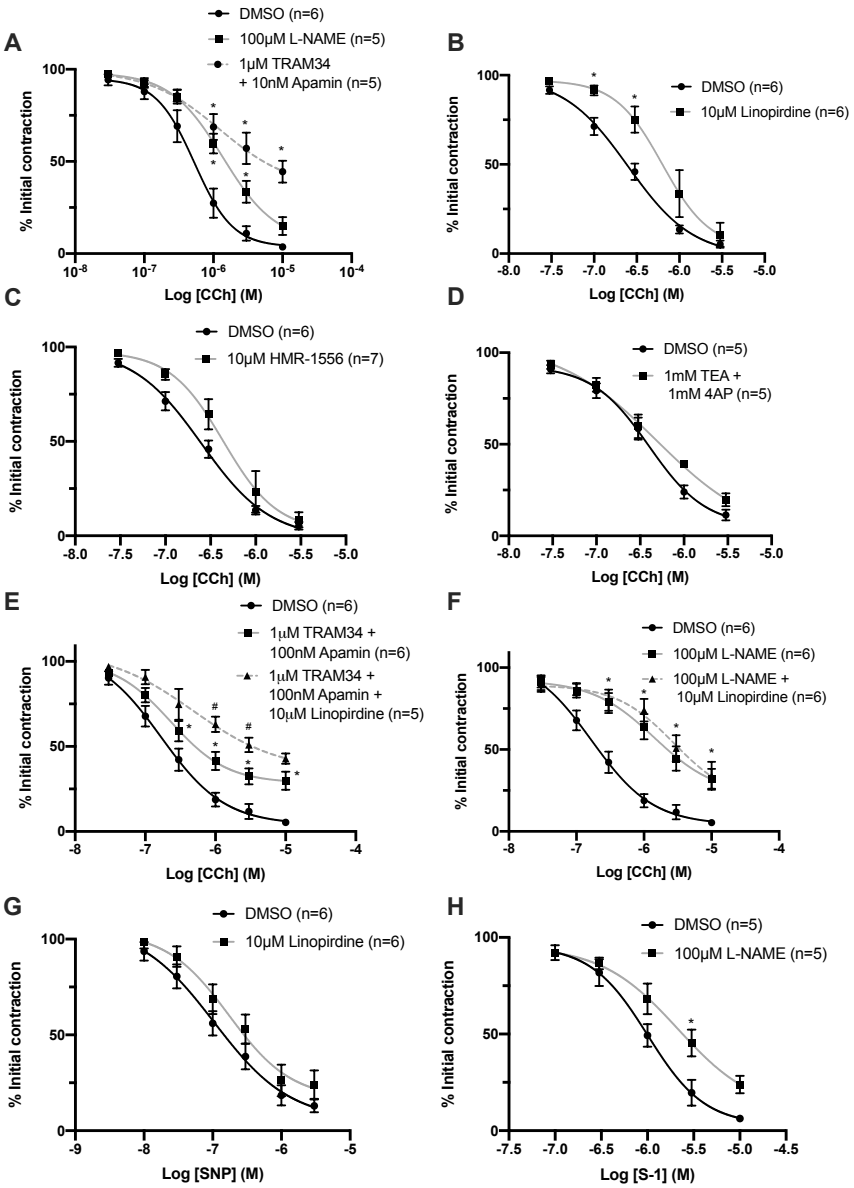


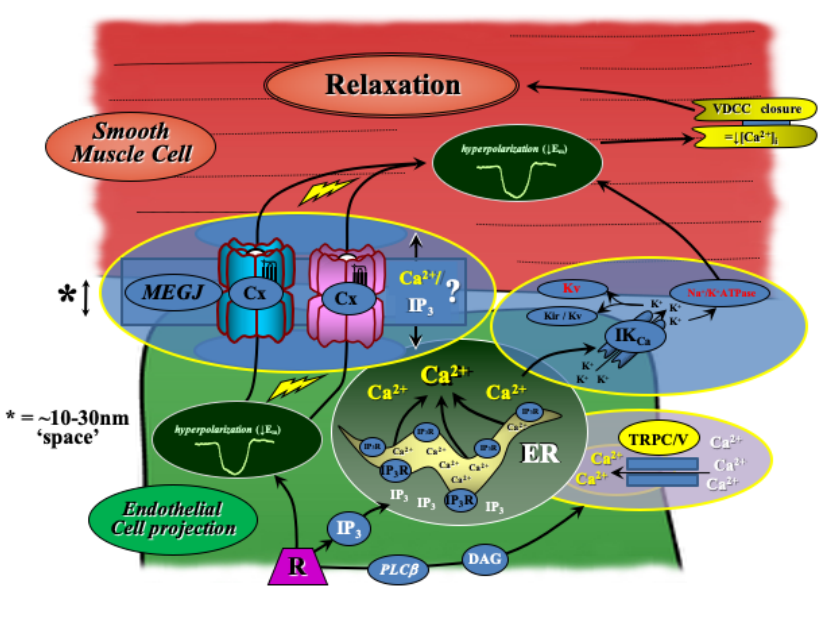












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