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RESEARCH PAPER

β_1-Adrenoceptor stimulation suppresses endothelial IK_{Ca}-channel hyperpolarization and associated dilatation in resistance arteries

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BACKGROUND AND PURPOSE

In small arteries, small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}) restricted to the vascular endothelium generate hyperpolarization that underpins the NO- and PGI_2-independent, endothelium-derived hyperpolarizing factor response that is the predominate endothelial mechanism for vasodilatation. As neuronal IK_{Ca} channels can be negatively regulated by PKA, we investigated whether β-adrenoceptor stimulation, which signals through cAMP/PKA, might influence endothelial cell hyperpolarization and as a result modify the associated vasodilatation.

EXPERIMENTAL APPROACH

Rat isolated small mesenteric arteries were pressurized to measure vasodilatation and endothelial cell $[Ca^{2+}]_i$, mounted in a wire myograph to measure smooth muscle membrane potential or dispersed into endothelial cell sheets for membrane potential recording.

KEY RESULTS

Intraluminal perfusion of β-adrenoceptor agonists inhibited endothelium-dependent dilatation to ACh (1 nM–10 μM) without modifying the associated changes in endothelial cell $[Ca^{2+}]_i$. The inhibitory effect of β-adrenoceptor agonists was mimicked by direct activation of adenylyl cyclase with forskolin, blocked by the β-adrenoceptor antagonists propranolol (non-selective), atenolol (β_1) or the PKA inhibitor KT-5720, but remained unaffected by ICI 118 551 (β_2) or glibenclamide (ATP-sensitive K^+ channels channel blocker). Endothelium-dependent hyperpolarization to ACh was also inhibited by β-adrenoceptor stimulation in both intact arteries and in endothelial cells sheets. Blocking IK_{Ca} {with 1 μM 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34)}, but not SK_{Ca} (50 nM apamin) channels prevented β-adrenoceptor agonists from suppressing either hyperpolarization or vasodilatation to ACh.

CONCLUSIONS AND IMPLICATIONS

In resistance arteries, endothelial cell β_1-adrenoceptors link to inhibit endothelium-dependent hyperpolarization and the resulting vasodilatation to ACh. This effect appears to reflect inhibition of endothelial IK_{Ca} channels and may be one consequence of raised circulating catecholamines.

Abbreviations

Ca^{2+}, free calcium ions; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; EDH, endothelium-derived hyperpolarization; (e)NOS, (endothelial) NOS; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channels; K_{ATP}, ATP-sensitive K^+ channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; MOPS, 3-[N-morpholino]propane-sulfonic acid; NA, noradrenaline; PE, phenylephrine; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channels

Introduction

Vascular endothelial cells contain two forms of K_{Ca} channel, the small and intermediate conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively; Edwards *et al.*, 1998). The SK_{Ca} and IK_{Ca} channels can be regulated independently to generate endothelial cell hyperpolarization (Crane *et al.*, 2003), reflecting a differential distribution of the two subtypes within the cell membrane (Dora *et al.*, 2008). Activated by increases in endothelial cell [Ca^{2+}], they generate hyperpolarization that spreads to relax the adjacent smooth muscle. Historically this effect was defined as the endothelium-derived hyperpolarizing factor (EDHF) response, because it was thought solely to reflect the action of a diffusible hyperpolarizing factor. It is now clear that hyperpolarization spreads to the muscle partly through myoendothelial gap junctions and partly via a diffusible factor that in the mesenteric artery is K^+ effluxing through the K_{Ca} channels. As such the term, endothelium-derived hyperpolarization (EDH) is now used to describe this complex NO/PGI_2-independent signalling pathway, that is a predominate endothelial influence on function in small arteries where the smooth muscle cells express voltage-dependent Ca^{2+}-channels in high density (reviewed by Garland *et al.*, 2011a).

We have previously shown that the activation of adenylyl cyclase (with forskolin) can selectively suppress the IK_{Ca} (not SK_{Ca}) channel component of EDH during endothelial cell stimulation with ACh in mesenteric resistance arteries (Dora *et al.*, 2008). This observation is consistent with data from neurons (Vogalis *et al.*, 2003; Neylon *et al.*, 2006) and Xenopus oocytes (Neylon *et al.*, 2004), where IK_{Ca} channels are suppressed by activation of PKA. Physiologically, vascular β-adrenoceptors couple through G_s to generate cAMP, raising the possibility that endogenous catecholamines might act in part to modulate endothelial cell IK_{Ca} hyperpolarization and thus influence vasodilatation.

β-Adrenoceptors have been visualized directly on endothelial cells in the rat mesenteric artery, but it is not clear what functional role, if any, these receptors serve (Briones *et al.*, 2005). They do not appear to exert any significant functional influence through NO, as dilatation to β-adrenoceptor agonists was not altered by blocking NOS activity (Briones *et al.*, 2005; Garland *et al.*, 2011b). We therefore investigated the possibility that the β-adrenoceptors might modulate endothelium-dependent responses to ACh in resistance arteries by targeting IK_{Ca} channels and influencing hyperpolarization. Our data indicate that adrenergic agonists can impair local endothelium-dependent dilatation by suppressing the EDH generated by IK_{Ca} channels through a cAMP-dependent mechanism. Furthermore, they show this action is mediated, at least in part, by β_1-adrenoceptors located on the endothelium.

Methods

Preparation of arteries for pressure or wire myography

Animal use complied with the University of Oxford local ethical guidelines and the Animals (Scientific Procedures) Act 1986. Male Wistar rats (225–250 g) were killed by cervical dislocation and exsanguination, as specified by Schedule 1 of the Animals (Scientific Procedures) Act 1986, UK. The mesenteric arcade was removed and placed in ice-cold 3-[N-morpholino]propane-sulfonic acid (MOPS) buffer containing (mM): 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4 \cdot 7H_2O$, 2.0 MOPS, 1.2 $NaH_2PO_4 \cdot H_2O$, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.75 NaOH with pH adjusted to 7.40 ± 0.02 (at 37°C). A third-order segment of mesenteric artery (external diameter between 250 and 350 μm at 70 mmHg) with no visible side branches was dissected free of adherent tissue. After the artery was mounted in either a pressure or wire myograph, reactivity was assessed by preconstriction with phenylephrine (PE, 0.5–3 μM) followed by endothelium-dependent relaxation to ACh (0.1 and 1 μM). Only vessels that relaxed by more than 95% to 1 μM ACh were used further.

Measurement of vascular responses

Arteries were cannulated with two glass pipettes in a temperature-regulated chamber (10 mL, 120CP, Danish Myo Technology, Aarhus, Denmark) placed on the stage of an inverted microscope (IX71, Olympus, Tokyo, Japan) as previously described (Yuill *et al.*, 2011). The preparations were then warmed to 37°C, and pressure, driven by a custom-built gravity-fed inflow and outflow system, was gradually increased to 70 mmHg. Arteries were visualized using a 10×/0.25 Olympus objective and video camera (KP-M1E/K-S10, Hitachi Kokusai Electric Inc., Tokyo, Japan) and vessel diameter changes tracked using Vedi View software (v.1.2, Danish Myo Technology). All experiments were performed in the presence of continuous luminal flow (5 $\mu L \cdot min^{-1}$; Bee Hive syringe pump system, Bioanalytical Systems, West Lafayette, IN, USA) that had no effect on tone. Arteries were preconstricted with PE (or other agonist, as indicated) to 70–80% of the minimum arterial diameter, and cumulative concentration response curves to ACh were obtained following addition to the bath solution. To rapidly introduce agonists to the lumen of arteries without disconnecting perfusion lines, an infusion manifold connected to multiple syringe pumps was positioned to the inflow line of one cannulating pipette.

Measurement of smooth muscle membrane potential

Segments of mesenteric artery (2 mm) were mounted in a Mulvany-Halpern wire myograph (model 400A, Danish Myo Technology) in Krebs solution containing (mM): 118 NaCl, 25 $NaHCO_3$, 3.6 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 11 glucose, and gassed with 21 % O_2, 5 % CO_2, balance N_2 at 37°C. During experiments, the concentration of $CaCl_2$ was either 1 mM or 2.5 mM, as stated. With 1 mM $[Ca^{2+}]_o$, there was no significant difference in EC_{50} and E_{Max} for hyperpolarization to ACh in arteries bathed in either MOPS buffered solutions or Krebs-buffered physiological solution (see Supporting Information Figure S1A). The temperature was increased to 37°C, and arteries normalized to a resting tension equivalent to that generated at 90% of the diameter of the vessel at 70 mmHg. The viability of the artery was assessed with PE and ACh, as described earlier.

The smooth muscle membrane potential was measured using sharp glass microelectrodes backfilled with 2 M KCl (tip

resistances *circa* 100 MΩ), as previously described (Garland and McPherson, 1992; Garland *et al.*, 2011b). Membrane potential was recorded through a pre-amplifier (Neurolog System, Digitimer Ltd., Welwyn, UK) linked to a MacLab data acquisition system (Model 4e, usually at 100 Hz; AD Instruments, Oxford, UK). All drugs were added directly to the bath.

Measurement of endothelial cell sheet membrane potential

For patch clamp studies, endothelial cells were isolated from mesenteric arteries that had been cut open and placed in nominally Ca^{2+}-free physiological saline solution (HEPES-PSS) containing (mM): 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 glucose, 10 HEPES (pH adjusted to 7.4 with NaOH) with the additional presence of 1 mg·mL^{-1} papain, 1 mg·mL^{-1} BSA (fraction V) and 1 mg·mL^{-1} dithiothreitol for 10 min at room temperature and then for 30 min at 36°C. The arteries were then washed in Ca^{2+}-free BSA-containing HEPES-PSS and gently triturated to release endothelial cells. Cell suspensions were stored on ice (the Ca^{2+} concentration was gradually increased to 0.5 mM) and used on the same day. All patch-clamp recordings were performed in HEPES-PSS containing 1 mM $CaCl_2$, 100 μM N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) and 10 μM indomethacin. The recording pipette solution contained (mM): 140 KCl, 2 MgATP, 0.1 Na_2GTP, 0.5 $MgCl_2$, 10 HEPES, 0.1 EGTA, pH adjusted to 7.2 with KOH.

Membrane potential was recorded from endothelial cell sheets (containing between 3 and >20 cells) using the current clamp mode of the whole-cell patch clamp technique at sampling rate 10 Hz (Axoclamp 200B amplifier; Axon Instruments, Union City, CA, USA). Pipette resistance, when filled with pipette solution, was 5–10 MΩ.

Measurement of endothelial $[Ca^{2+}]_i$ changes

In separate experiments, small mesenteric arteries were dissected, cannulated and reactivity assessed as described previously. Endothelial cells were then loaded with a combination of Ca^{2+} reporter dyes. Briefly, the intraluminal pressure was lowered to 4 mmHg and the artery perfused with buffer containing 0.02% Pluronic F-127 and the cell-permeable Ca^{2+} dye Fura Red AM (40 μM, 25 min) followed 30 min later with Oregon Green 488 BAPTA-1 AM (OGB-1, 10 μM, 30 min), selectively to load endothelial cells (Kansui *et al.*, 2008). After the dye was washed out of the chamber with MOPS buffer, the pressure was increased and the artery left for another 30 min to allow de-esterification. Arteries were then exited at 488 nm and emitted light simultaneously collected at 505–525 nm (OGB-1) and 655–755 nm (Fura Red) with a 40× water immersion objective (UApo N340, Olympus) mounted on an Olympus FluoView1000 microscope (Olympus). Endothelial cells were visualized in a clip box of 476 × 156 pixels allowing a scan frequency of ~3 Hz. Cells (6–10 cells) were selected and fluorescence intensity was determined off-line using MetaMorph software (v.7.7.4, Molecular Devices, Downingtown, PA, USA). Raw fluorescence values at each time point for each indicator dye were divided (to give a ratio $F_{OGB\text{-}1}/F_{Fura\ Red}$), and normalized to a 10 s period before the addition of ACh (F/F_0) to give values for ratio F/F_0. Each summary data value is the average of at least a 20 s period. Performing a ratio enabled more accurate comparisons of fluorescence changes to various concentrations of ACh before and after exposure to isoprenaline, each being paired. All agonists were added to the bath.

Data analysis

Data were analysed using Microsoft Excel 2011 and GraphPad Prism (v5.0, GraphPad Software, San Diego, CA, USA) software. Dilatation was expressed as a percentage reversal of tone induced by PE (100% corresponding to the maximal diameter). Results are summarized as mean ± SEM of n replicates, where n is the number of individual arteries, each obtained from a separate animal. Statistical analyses were performed using Student's unpaired t-test, one-way or two-way ANOVA analysis followed by Bonferroni post-test. A value of $P < 0.05$ was considered to be statistically significant.

Drugs and solutions

All drugs were obtained from Sigma (Poole, UK) with the exception of apamin (Latoxan, Valence, France), and forskolin (Biomol International, Exeter, UK). U46619, TRAM-34 {1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole} and forskolin were dissolved in dimethyl sulfoxide, while adrenaline and noradrenaline (NA) bitartrate salts were dissolved in 10^{-4} M ascorbic acid. All other stock solutions were prepared using purified (MilliQ, Billerica, MA, USA) water. All stock solutions were prepared at 10^{-2} M, except for L-NAME (10^{-1} M), and subsequently diluted in MOPS buffer (pressurized arteries), Krebs buffer (wire myograph) or HEPES-PSS (patch clamp). Inhibitors were pre-incubated with the arterial tissue for at least 20 min before agonist application.

Results

Intraluminal perfusion of β-adrenoceptor agonists inhibits endothelium-dependent dilatation to ACh

In pressurized small mesenteric arteries pre-contracted with PE, ACh (1 nM to 10 μM) stimulated concentration-dependent dilatation (pEC_{50} = 7.1 ± 0.02, n = 5; Figure 1A). When either adrenaline (0.5 μM) or NA (1 μM) were luminally perfused in PE pre-contracted arteries, neither stimulated contraction, even in the presence of propranolol (1 μM, n = 4–9). However, the luminal perfusion of β-adrenoceptor agonists reversibly inhibited ACh-mediated dilatation. Isoprenaline (1 μM), NA (1 μM) or adrenaline (0.5 μM) each right-shifted ACh concentration response curves (isoprenaline: from pEC_{50} = 7.2 ± 0.01 to pEC_{50} = 6.4 ± 0.03, n = 6, $P < 0.05$, Figure 1B; NA: to pEC_{50} = 6.4 ± 0.3, n = 6, $P < 0.05$, Figure 1C; adrenaline: to pEC_{50} = 6.6 ± 0.02, n = 6, $P < 0.05$, Figure 1D). Inhibition of dilatation to ACh was mimicked by activation of adenylyl cyclase by luminal perfusion of forskolin (0.5–1 μM; from pEC_{50} = 7.2 ± 0.02 to pEC_{50} = 6.5 ± 0.03, n = 5, $P < 0.05$; Figure 1E). In contrast to the action of β-adrenoceptor agonists, luminal perfusion of either MOPS buffer alone or the α_1-adrenoceptor agonist, PE (0.5 μM, n = 5), did not modify responses to ACh.

The ability of isoprenaline to inhibit endothelium-dependent dilatation to ACh was blocked by pretreatment of

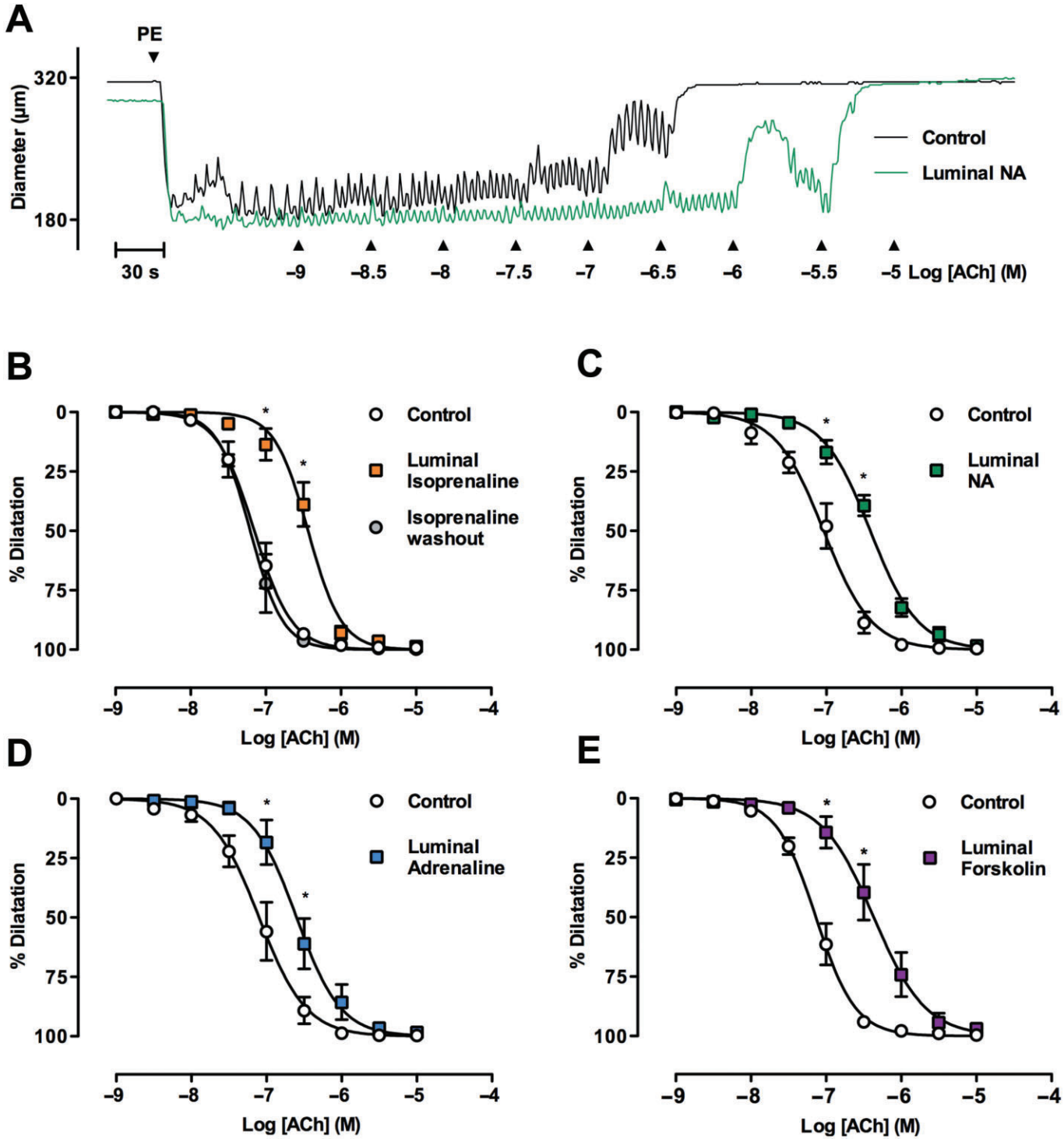

Figure 1

Luminal perfusion of adrenoceptor agonists inhibit endothelium-dependent dilatation to ACh in pressurized mesenteric arteries. Original traces illustrate dilatation to increasing ACh concentrations (1 nM – 10 μM) in an artery pre-constricted with PE. Luminal perfusion of 1 μM NA (black line: control buffer only; green line: luminal NA present) inhibits dilatation (A). Summarized data showing luminal perfusion of 1 μM isoprenaline inhibits dilatation to ACh and is restored on washout of isoprenaline (n = 4, B). Similar inhibition follows luminal perfusion of the adrenergic agonists 1 μM NA (n = 6, C) or 0.5 μM adrenaline (n = 6, D), or activation of adenylyl cyclase with 0.5 μM luminal forskolin (n = 5, E). Results shown are the mean ± SEM; *P < 0.05 versus control, paired data.

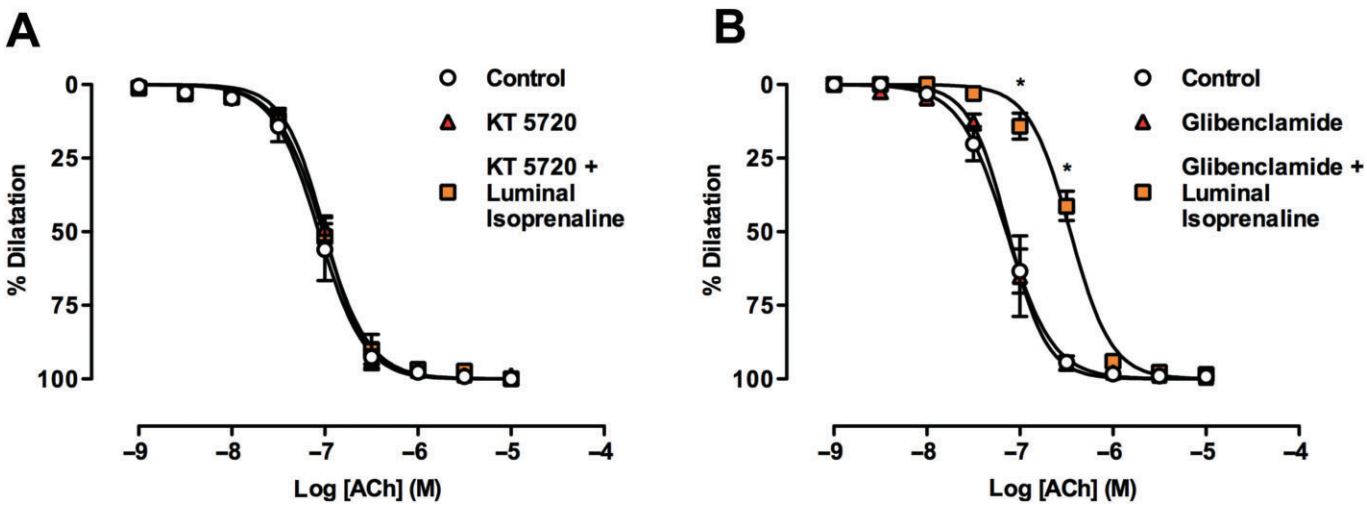

Figure 2

KT 5720, but not glibenclamide prevents β-adrenoceptor inhibition of endothelium-dependent dilatation to ACh. The PKA inhibitor KT 5720 (1 μM, A) did not modify endothelium-dependent vasodilatation to ACh ($n = 5$), but prevented the isoprenaline-mediated inhibition of dilatation to ACh ($n = 4$, A). The ability of isoprenaline to inhibit dilatation to ACh was not altered in the presence of glibenclamide (10 μM, $n = 4$; B). Data are the mean ± SEM; *$P < 0.05$ versus control, paired data.

arteries with the PKA inhibitor KT 5720 that alone had no effect (1 μM; Figure 2A), but not by the ATP-sensitive K^+ channels (K_{ATP}) channel blocker glibenclamide (10 μM, Figure 2B). Glibenclamide was used to prevent the hyperpolarization that is stimulated by isoprenaline through the opening of smooth muscle K_{ATP} channels in this artery, a change that could potentially interfere with EDH-mediated hyperpolarization to ACh by decreasing the driving force for K^+ efflux.

β-Adrenoceptor stimulation inhibits endothelium-dependent hyperpolarization to ACh

IK_{Ca} channels can be activated at resting membrane potentials in the mesenteric artery in Krebs buffered solution containing 1 mM, but not 2.5 mM, $[Ca^{2+}]_o$ (Dora *et al.*, 2008). The resting membrane potential in 1 mM $[Ca^{2+}]_o$ (–51.5 ± 0.7 mV, $n = 8$) was no different to cells in the same arteries in 2.5 mM $[Ca^{2+}]_o$ Krebs (–52.4 ± 0.8 mV, $n = 6$). However, in the presence of 50 nM apamin (to remove SK_{Ca} input) raising $[Ca^{2+}]_o$ from 1 to 2.5 mM evoked a transient hyperpolarization (peak of –7.1 ± 1.8 mV at 38 ± 10 s after addition, $n = 5$), which reversed completely over the next 20 min (to –0.6 ± 1.3 mV by 9 ± 4 min, $n = 5$). This acute hyperpolarization as $[Ca^{2+}]_o$ is raised above 1 mM has been shown previously, and ascribed to activation of IK_{Ca} channels through calcium-sensing receptors on the endothelium (see trace in Weston *et al.*, 2005).

In 1 mM $[Ca^{2+}]_o$ Krebs, ACh evoked concentration-dependent smooth muscle hyperpolarization (from a resting potential of –54.2 ± 1.8 mV, by a maximum of 20.9 ± 1.1 mV, $pEC_{50} = 7.2 \pm 0.11$, $n = 5$). In the presence of 10 μM glibenclamide, to block β–adrenoceptor-stimulated hyperpolarization, isoprenaline (1 μM) significantly suppressed the smooth muscle hyperpolarization to ACh [decreased to a maximum of 13.1 ± 1.3 mV, from a resting potential of –53.8 ± 0.9 mV, $n = 5$; Figure 3A top (i) and middle trace (ii) & 3B]. The ACh-hyperpolarization that persisted in the presence of isoprenaline was mediated by SK_{Ca}, as it was blocked by 50 nM apamin [Figure 3A bottom trace (iii), summary data Figure 3B]. Apamin alone depressed ACh-hyperpolarization to a similar extent to isoprenaline (Figure 3B). Atenolol (1 μM, selective β_1-adrenoceptor antagonist) prevented isoprenaline from blocking the ACh- IK_{Ca} hyperpolarization (that persisted in the presence of apamin, Figure 3B, $n = 6$). Atenolol did not alter the concentration-dependent hyperpolarization to ACh in the presence of apamin (maximum of 13.5 ± 1.9 mV, $n = 5$).

In freshly isolated endothelial cell sheets incubated with apamin, ACh (1 μM) applied for 30 s every 5 min evoked reproducible and reversible hyperpolarization that was blocked in a time-dependent manner with isoprenaline (1 μM, Figure 3C and summarized in Figure 3D). These data demonstrate the presence of functional β-adrenoceptors in endothelial cells of mesenteric arteries, and confirm their role in suppressing the ability of ACh to activate endothelial cell IK_{Ca} channels.

Endothelial IK_{Ca} channels underlie β-adrenoceptor inhibition of endothelium-dependent hyperpolarization

NA suppressed ACh-hyperpolarization to a similar extent as isoprenaline. In 1 mM $[Ca^{2+}]_o$ Krebs, ACh stimulated a maximum hyperpolarization of 20.5 ± 1.7 mV ($pEC_{50} = 7.0 \pm 0.03$, initial membrane potential –51.5 ± 0.7 mV, $n = 8$). In the presence of prazosin (1 μM, to block α_1-adrenoceptors) and glibenclamide, NA (1 μM) suppressed hyperpolarization (maximum now 11.1 ± 1.8 mV from a resting potential of –52.6 ± 0.7 mV, $n = 5$; Figure 4A & B). Apamin (50 nM) significantly reduced the remaining hyperpolarization (Figure 4B). TRAM-34 (1 μM) also supressed ACh-

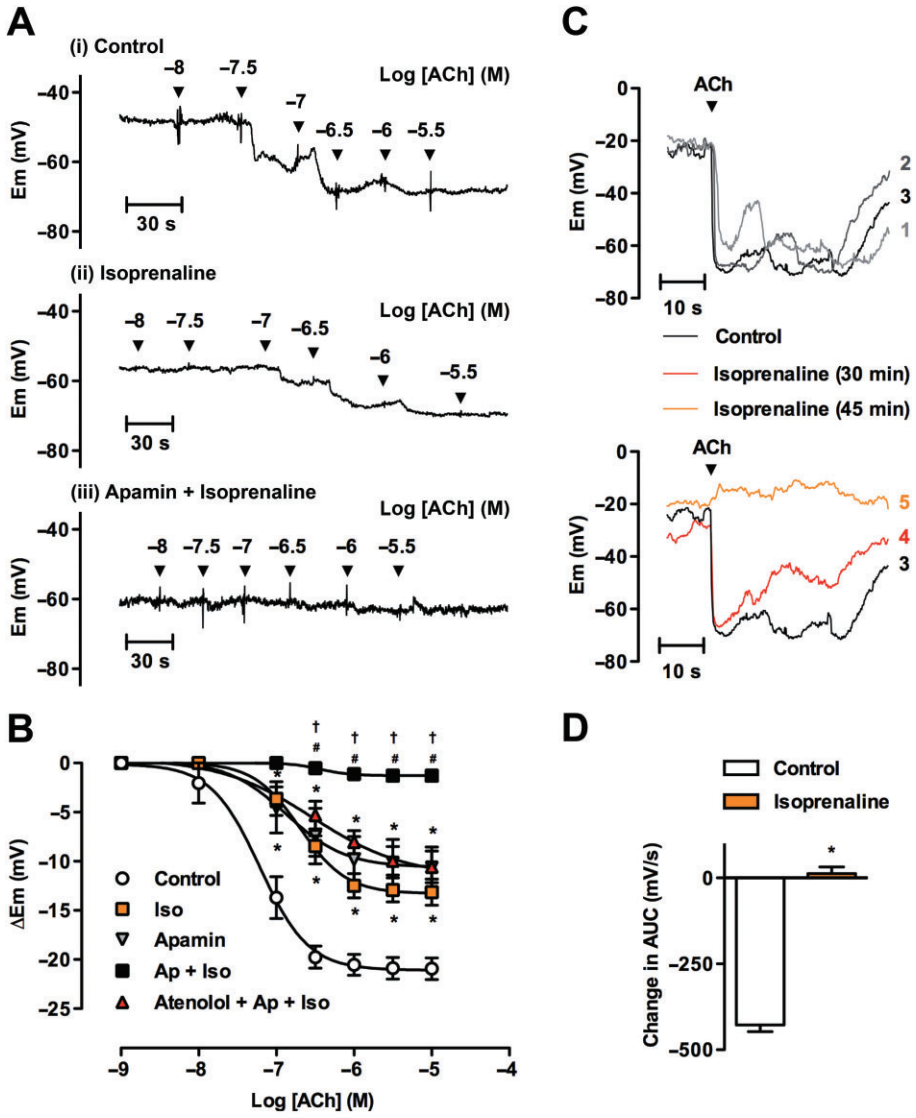

Figure 3

Isoprenaline inhibits IK_{Ca}-channel hyperpolarization evoked by Ach. Original trace of smooth muscle cell membrane potential recording showing ACh-evoked, concentration-dependent hyperpolarization (Ai) inhibited in the presence of 1 μM isoprenaline (Aii) and blocked in the additional presence of 50 nM apamin (Aiii). (B) Summary of intracellular recordings showing similar depression of ACh hyperpolarization with 1 μM isoprenaline or 50 nM apamin present; $^{*}P < 0.05$ versus control, paired data, $n = 5$. In combination, isoprenaline and apamin abolished hyperpolarization to ACh; $^{\#}P < 0.05$ versus isoprenaline, $^{\dagger}P < 0.05$ versus apamin, paired data, $n = 5$, and this block was prevented by the presence of 1 μM atenolol ($n = 6$). Note that TRAM-34 blocks the same component as isoprenaline (see Figure 4C). (C) Original traces of endothelial cell membrane potential recorded in endothelial cell sheets. Sequential exposure to 1 μM ACh (added at arrowhead, superimposed traces 1–5, each 30 s) stimulated endothelial cell hyperpolarization, which was blocked in a time-dependent manner by prior incubation with 1 μM isoprenaline (traces 4 and 5). (D) Summary of patch clamp data in endothelial cell sheets showing hyperpolarization to ACh (1 μM) in the presence of apamin (100 nM) was blocked in the presence of 1 μM isoprenaline; $^{*}P < 0.05$, $n = 4$. Data are expressed as the AUC and corrected for the basal membrane potential. Summary data are the mean ± SEM; 100 μM L-NAME present in all experiments, Krebs and HEPES buffers contained 1 mM Ca^{2+} to prevent inhibition of IK_{Ca} channels by $[Ca^{2+}]_o$ (Weston *et al.*, 2005; Dora *et al.*, 2008).

hyperpolarization, but in contrast to apamin it blocked the ability of 1 μM NA to cause further inhibition of hyperpolarization (Figure 4C), consistent with an effect of NA against IK_{Ca} channels. The combination of apamin and TRAM-34 abolished hyperpolarization to ACh (Figure 4C), but did not prevent hyperpolarization to the opener of K_{ATP} channels levcromakalim (5 μM, –22.3 ± 2.0 mV, $n = 3$). When $[Ca^{2+}]_o$ was increased to 2.5 mM, an inhibitory effect of NA against EDH-hyperpolarization was not observed (maximum hyperpolarization to ACh of –21.2 ± 1.8 mV was not reduced in the presence of NA, –19.3 ± 1.5 mV, $n = 6$ and 4, Figure 4D). In 2.5 mM $[Ca^{2+}]_o$, ACh-mediated hyperpolarization is entirely due to SK_{Ca} channel activation (Crane *et al.*, 2003; Dora *et al.*, 2008).

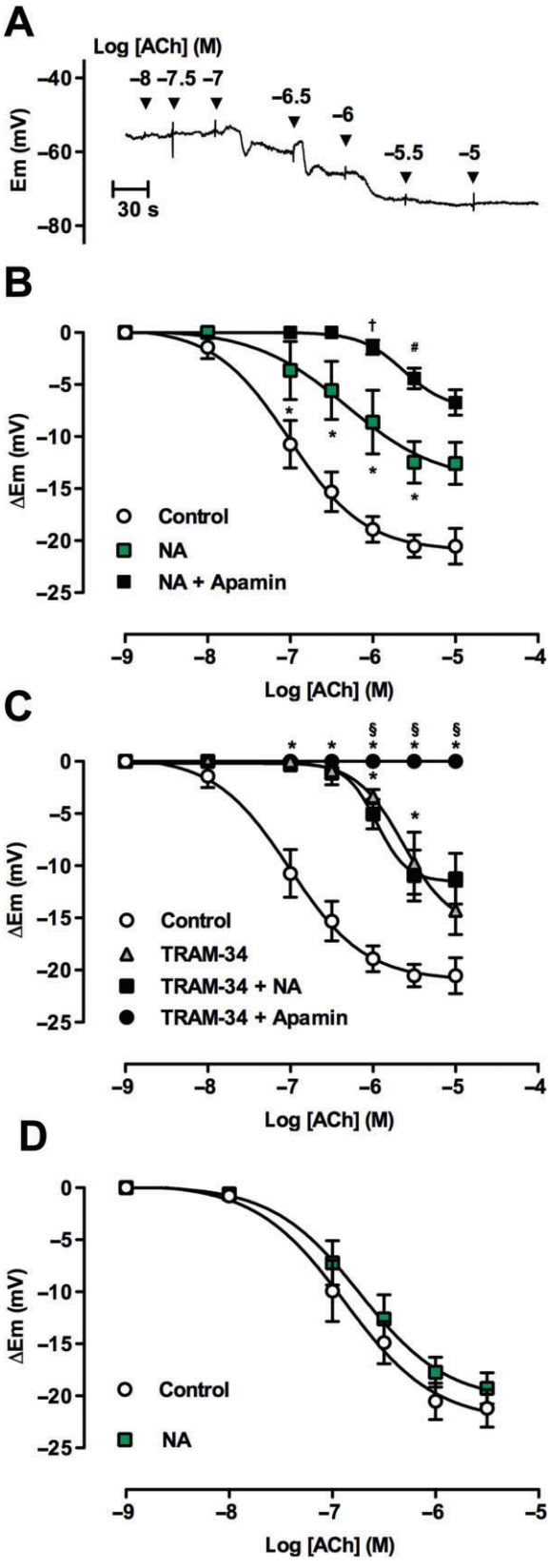

Figure 4

TRAM-34, but not apamin blocks β-adrenoceptor mediated inhibition of hyperpolarization evoked by ACh. (A) Original trace of smooth muscle membrane potential showing hyperpolarization to cumulative addition of ACh (1 nM – 3 μM) has been suppressed during incubation with NA (1 μM; see control trace in Figure 3Ai). (B) Summarized data showing ACh-mediated hyperpolarization is partially suppressed in the presence of 1 μM NA (n = 7, $^{*}P < 0.05$) and further by the additional presence of apamin (50 nM, n = 6, $^{\dagger}P < 0.05$ vs. apamin, see Figure 3C, and $^{\#}P < 0.05$ vs. NA). (C) TRAM-34 (1 μM) depressed ACh-mediated hyperpolarization, 1 μM NA had no further effect, $^{*}P < 0.05$ versus control, n = 6 in each case; whereas TRAM-34 together with apamin fully blocked hyperpolarization to ACh, $^{\S}P < 0.05$ versus TRAM-34, n = 3. (D) 1 μM NA did not modify endothelium-dependent hyperpolarization to ACh in mesenteric arteries bathed in Krebs buffer containing 2.5 mM Ca^{2+} (n = 4–6). All data shown are the paired mean ± SEM; 100 μM L-NAME, 1 μM prazosin and 10 μM glibenclamide were present in all experiments when NA was present. Krebs buffer contained 1 mM Ca^{2+} to prevent inhibition of IK_{Ca} channels by $[Ca^{2+}]_o$, unless otherwise stated.

Endothelial IK_{Ca} channels underlie β-adrenoceptor inhibition of endothelium-dependent dilatation to ACh

As shown previously, inhibition of NOS right-shifted the concentration–response curve to ACh without depressing the maximum response (100 μM L-NAME, pEC_{50} from 7.2 ± 0.04 to 6.8 ± 0.1, n = 6, $P < 0.05$). The residual dilatation is due to hyperpolarization (EDH dilatation, Crane *et al.*, 2003), and was reduced by apamin (50 nM; pEC_{50} = 6.2 ± 0.1, n = 6, $P <$ 0.05), then markedly suppressed by the subsequent addition of TRAM-34 (1 μM, n = 5, $P < 0.05$; Figure 5A).

EDH dilatation obtained in the presence of L-NAME was suppressed by luminal perfusion of NA (from pEC_{50} = 6.8 ± 0.02 to pEC_{50} = 6.2 ± 0.03, n = 4, $P < 0.05$; Figure 5B). The addition of TRAM-34 now failed to depress dilatation further (pEC_{50} = 6.2 ± 0.04, n = 4, $P > 0.05$), and NA together with apamin (50 nM) was equally effective at blocking ACh dilatation as TRAM-34 and apamin. If SK_{Ca} (rather than IK_{Ca}) channels were blocked (with apamin), each catecholamine (n = 4, $P < 0.05$) or forskolin (n = 5, $P < 0.05$) inhibited EDH-dilatation to ACh in a manner similar to apamin and TRAM-34 (Figures 5B & C). These data are consistent with the sharp micro electrode data, and further support an effect of both β-adrenoceptor agonists and activators of PKA against IK_{Ca} channels.

$β_1$-Adrenoceptors mediate inhibition of EDH-dilatation

The ability of β-adrenoceptor stimulation to suppress EDH-dilatation in the presence of apamin was blocked in the presence of either propranolol (1 μM, non-selective β-adrenoceptor antagonist, n = 4) or atenolol (1 μM, selective $β_1$-adrenoceptor antagonist, n = 3). In each case, the subsequent addition of TRAM-34 abolished the persistent dilatation (n = 3–4, Figures 6A & B). In contrast, the $β_2$-adrenoceptor antagonist ICI 18 551 (100 nM) did not prevent the NA-mediated inhibition of ACh responses in the presence of apamin (Figure 6C, n = 4, $P < 0.05$). Together these data support an inhibitory action of catecholamines via $β_1$-adrenoceptors.

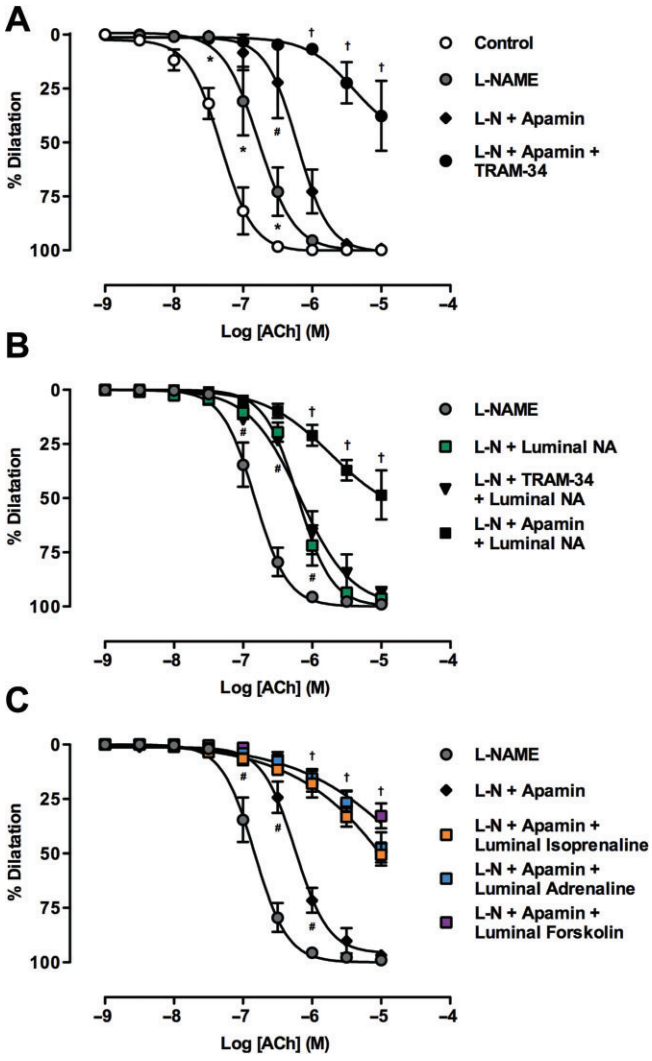

Figure 5

TRAM-34, but not apamin prevents the inhibition of EDH-dilatation to ACh by β-agonists in pressurized small mesenteric arteries. (A) Inhibition of NO synthesis with L-NAME (100 μM), and EDH with apamin (50 nM, to block SK_{Ca} channels) together with TRAM-34 (1 μM, to block IK_{Ca} channels) suppressed dilatation to ACh in arteries pre-contracted with PE (n = 5). (B) In the presence of L-NAME, luminal perfusion of 1 μM NA alone suppressed dilatation to ACh and prevented further inhibition with TRAM-34 (n = 4, $P > 0.05$), whereas apamin blocked a further component, similar to that observed with apamin and TRAM-34 (A). (C) Similarly, in the presence of apamin, luminal perfusion of other β-adrenoceptor agonists (1 μM isoprenaline or 0.5 μM adrenaline) or 0.5 μM forskolin, blocked the same TRAM-34-sensitive IK_{Ca}-channel component of ACh responses. All data are paired and represent the mean ± SEM, n = 4–7; $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus β-adrenoceptor agonist alone; $^{\dagger}P < 0.001$ versus K_{Ca} channel blocker alone.

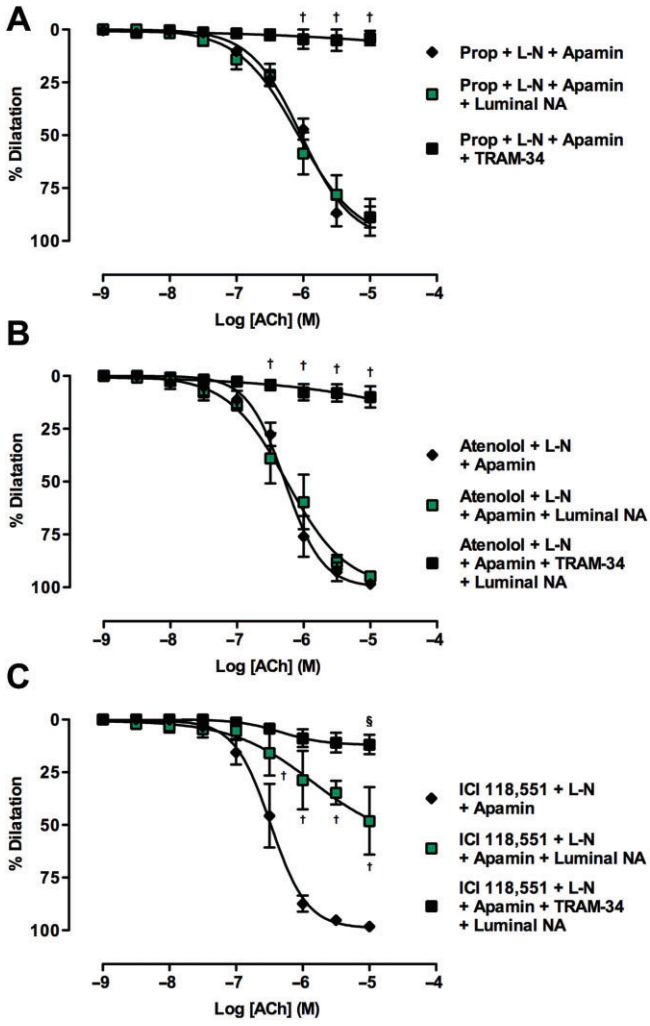

Figure 6

β_1-adrenoceptors underlie inhibition of EDH-dilatation to ACh in pressurized small mesenteric arteries. (A) In the presence of propranolol (1 μM), ACh-mediated dilatation associated with IK_{Ca}-channel activity (obtained in the presence of 100 μM L-NAME and 50 nM apamin) was not inhibited by luminal NA (1 μM), confirming a role for β-adrenoceptors in the actions of NA (see Figure 5B). IK_{Ca} channels alone underpinned the dilatation, as the subsequent addition of TRAM-34 (1 μM) abolished ACh-mediated dilatation (n = 4). (B) A similar profile of block was observed using the selective β_1-adrenoceptor antagonist atenolol (1 μM, n = 6); while the β_2-adrenoceptor antagonist ICI 118 551 (100 nM) did not prevent the inhibitory action of NA on ACh-mediated dilatation (n = 4; C). Data are mean ± SEM and paired; $^{\dagger}P < 0.05$ versus L-N and apamin in Figure 5A; $^{\S}P < 0.05$ versus ICI 118 551 + L-N + apamin + NA.

β_1-Adrenoceptors do not modify increases in endothelial $[Ca^{2+}]_i$ to ACh

Endothelial cells were imaged in pressurized arteries (Figure 7A). Concentration-dependent increases in cytoplasmic $[Ca^{2+}]_i$ were stimulated by ACh and detected ratiometrically (Figure 7B–D). Prior exposure to 1 μM isoprenaline did not alter increases evoked by ACh (n = 3–4, $P > 0.05$; Figure 7C, panel ii and D).

Discussion

We have shown that activation of vascular β-adrenoceptors can markedly reduce endothelium-dependent dilatation to

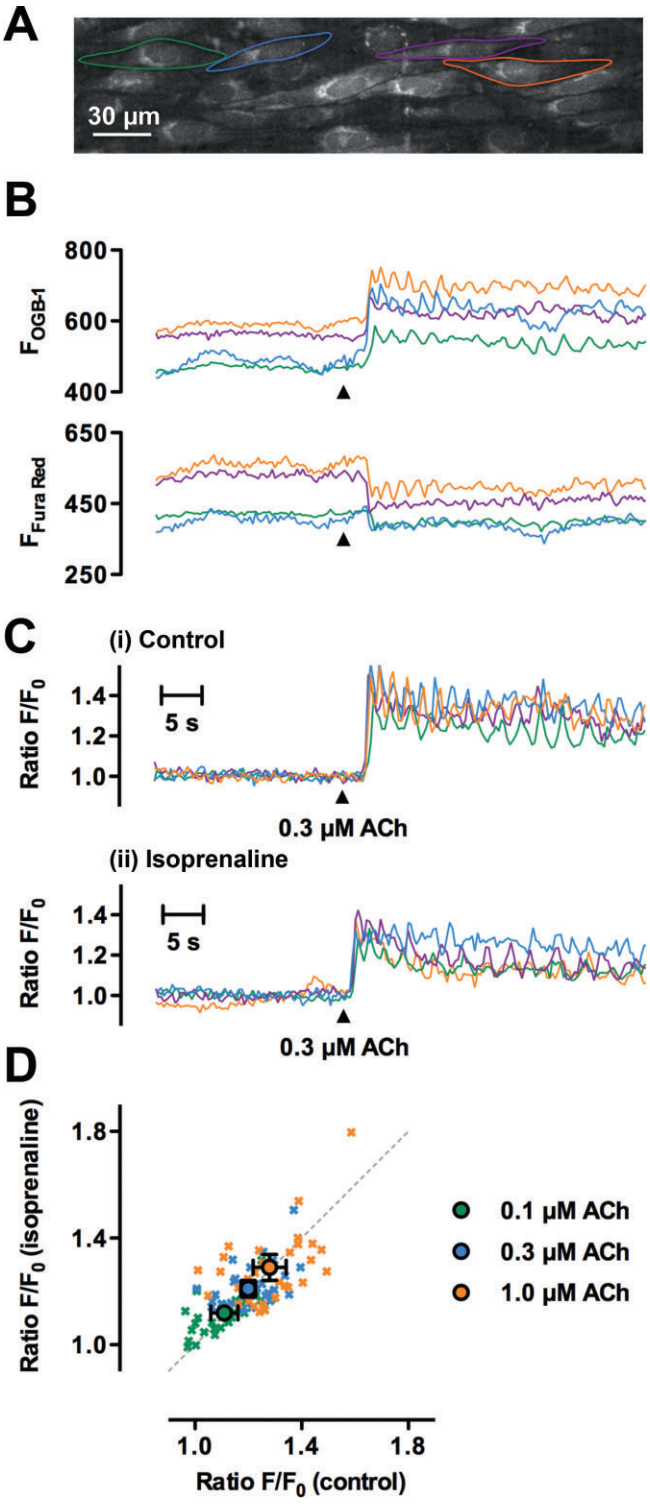

Figure 7

Isoprenaline did not alter ACh-evoked increases in endothelial cell $[Ca^{2+}]_i$ in pressurized mesenteric arteries. (A) Confocal fluorescent image of endothelial cells in a pressurized artery loaded with the Ca^{2+} indicators Oregon Green 488 BAPTA-1 (OGB-1) and Fura Red. (B) Original traces showing the time course of fluorescence intensity changes for each indicator dye ($F_{Fura\ Red}$ and F_{OGB-1}, arbitrary units), following application of 0.3 μM ACh to the bath at the point indicated by arrows. Note that $F_{Fura\ Red}$ decreases upon binding Ca^{2+}. (C) Top panel: raw values in B were divided to give a ratio ($F_{OGB-1}/F_{Fura\ Red}$), and normalized to a 10 s period before the addition of ACh (F/F_0) to give values for Ratio F/F_0. Bottom panel: repeated in the presence of 1 μM isoprenaline, paired data. (D) Paired values for each experiment and concentration of ACh show no trend for isoprenaline to alter the average increase in Ratio F/F_0. Summary data for each concentration of ACh (n = 3–4) show no deviation from equality (dashed line); data are the mean ± SEM; $P > 0.05$ versus control.

ACh. This effect appears due to the activation of endothelial cell β_1-adrenoceptors that couple via PKA to suppress hyperpolarization generated by endothelial cell IK_{Ca} channels. The observation that this inhibitory action was evoked by luminal perfusion of isoprenaline, and can be mimicked by either adrenaline or NA, suggests it is of physiological relevance in situations when circulating levels of catecholamines are raised.

Through the generation of NO, hyperpolarization (EDH) and in some arteries prostanoids, the endothelium relaxes the adjacent smooth muscle cells resulting in vasodilatation. In smaller resistance arteries, EDH is the predominant functional influence on the smooth muscle. EDH is activated by agonists, including ACh, that elevate endothelial cell $[Ca^{2+}]_i$ opening both SK_{Ca} and IK_{Ca} channels localized within the endothelium (Dora, 2010; Garland *et al.*, 2011a). These distinct K_{Ca} channels are differentially distributed within the endothelial cell membrane, and IK_{Ca} channels can be controlled independently of SK_{Ca} (Crane *et al.*, 2003; Dora, 2010; Garland *et al.*, 2011a). The SK_{Ca} channels reside within caveolae and appear to be particularly concentrated around the large homocellular gap junctions between endothelial cells. In contrast, IK_{Ca} channels are restricted to thin projections of the endothelial cell directed towards the adjacent smooth muscle (Sandow *et al.*, 2006; Dora *et al.*, 2008). Interestingly, the IK_{Ca} channels appear to reside outside the caveolae, and are found in close association with Ca^{2+}-sensing receptors, receptors that have been shown to modulate the activity of these K^+ channels (Weston *et al.*, 2005; Absi *et al.*, 2007). Endothelial cell projections therefore represent a complex signalling microdomain that seems to have a central role in the physiological control of artery diameter.

Hyperpolarization generated by IK_{Ca} channels can be regulated by the cAMP signalling pathway, as it is suppressed by forskolin (Dora *et al.*, 2008). Although there is very little data available regarding the regulation of IK_{Ca} channels by PKA, and only the one report in arteries (by Dora *et al.*, 2008), an inhibitory influence on channel activity mediated through cAMP signalling has been reported in other cell types. For example, PKA activation inhibited IK_{Ca} currents in ganglia within guinea pig duodenum (Vogalis *et al.*, 2003) and through channels expressed in Xenopus oocytes (Neylon *et al.*, 2004). Furthermore, in mouse jejunum, forskolin suppressed the Cl^- secretion evoked by the IK_{Ca}-channel opener 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO) (Hamilton and Kiessling, 2006). Therefore, our data now put the responses to forskolin into a more physiological context, by suggesting PKA-mediated inhibition of IK_{Ca} as a mechanism for suppression of EDH-dilatation during vascular β-adrenoceptor stimulation. It is likely the effect may be

explained by PKA phosphorylation of IK_{Ca} channels, analogous to the effect of PKA in other cell types. PKA phosphorylates sites located within the calmodulin-binding domain of the IK_{Ca} channel in Xenopus oocytes (Neylon *et al.*, 2004), and a similar mechanism has been suggested to explain the suppression of IK_{Ca} current within enteric neurons (Vogalis *et al.*, 2003; Neylon *et al.*, 2006). Alternatively, a pathway independent of the phosphorylation of IK_{Ca} channels could occur as a result of reduced increases in cytoplasmic $[Ca^{2+}]_i$ to ACh. While isoprenaline stimulation associated with increases in cAMP in cultured bovine aortic endothelial cells had no effect on $[Ca^{2+}]_i$ itself, it did reduce endothelial cell Ca^{2+} responses to ATP (Luckhoff *et al.*, 1990). To address this possibility in the present study, we showed that ACh-evoked increases in endothelial cell Ca^{2+} were not affected by isoprenaline in intact arteries. However, we cannot rule out the possibility that very discrete changes in $[Ca^{2+}]_i$ do occur, but are restricted to the immediate vicinity of the IK_{Ca} channels. We would not be able to resolve such changes with our imaging approach. Finally, by using glibenclamide, we ruled out the possibility that K_{ATP} channel opening might interfere with EDH-mediated hyperpolarization to ACh simply by decreasing the driving force for K^+ efflux. K_{ATP} channels are known to underlie the hyperpolarization to isoprenaline (and other catecholamines) in rat mesenteric arteries (Fujii *et al.*, 1999; White and Hiley, 2000b; Takano *et al.*, 2004).

The observation that β_1-adrenoceptors appear to account for the inhibition of IK_{Ca} channels by catecholamines ties these receptors to endothelial cells, as IK_{Ca} channels are not expressed in the smooth muscle cells of this artery (Walker *et al.*, 2001). This point is supported directly by the observation that atenolol blocked isoprenaline's ability to inhibit ACh-evoked (IK_{Ca}) hyperpolarization, and by our patch clamp data from isolated endothelial cells where isoprenaline was fully able to block IK_{Ca}-channel current. Previous evidence to suggest that β-adrenoceptors are located on endothelial cells in the mesenteric artery comes from the specific binding of a fluorescent β-adrenoceptor ligand, BIODIPY TMR-CGP 12177, within the intima of these arteries (as well as in both the media and adventitia; Briones *et al.*, 2005; Daly *et al.*, 2010). Previous functional studies have also been interpreted to indicate β_1-adrenoceptors are present on mesenteric artery endothelial cells, as dobutamine-mediated relaxation (against high KCl pre-contraction) was attenuated either by block of NOS or removal of the endothelium, whereas relaxation to salbutamol was not sensitive to L-NAME (Graves and Poston, 1993).

β_1 rather than β_2-adrenoceptors predominate in the mesenteric artery (Briones *et al.*, 2005; Garland *et al.*, 2011b). This is in itself interesting, because although both receptor subtypes associate with caveolae, the β_1-adrenoceptor is more widely distributed through the cell membrane and known to associate with extra-caveolar cell fractions, at least in cardiac myocytes (Rybin *et al.*, 2000). β_1-Adrenoceptors may therefore reside in membrane regions that also contain IK_{Ca} channels. If β_1-adrenoceptors do align in close proximity to the IK_{Ca} channels in endothelial cell projections, they will be ideally placed to influence the activity of these strategically positioned K-channels.

The fact that β_1-adrenoceptor stimulation can inhibit endothelial cell IK_{Ca} channels, and as a result depress EDH-mediated vasodilatation, seems strange as β_1-adrenoceptors also evoke potent smooth muscle relaxation that is associated with and in part reflects hyperpolarization (Garland *et al.*, 2011c). However, the hyperpolarization caused by β_1-adrenoceptors is entirely due to K_{ATP} channel activation, and at least in the mesenteric artery these channels are only present on the smooth muscle, not on endothelial cells (White and Hiley, 2000a; Takano *et al.*, 2004). One possibility, is that *in vivo* endothelial IK_{Ca} channels are influenced primarily by circulating β-adrenoceptor agonists, so at least in mesenteric vessels, the depression of endothelial function enhances vasoconstriction.

As EDH is activated by any agent that increases endothelial cell $[Ca^{2+}]_i$, β_1-adrenoceptor stimulation would be predicted to depress vasodilatation to a range of physiologically active autacoids. This may then explain the known ability of raised plasma catecholamine concentrations to impair endothelial function in humans (Higashi *et al.*, 2002; Kuklinska *et al.*, 2010). In the coronary microvasculature, dysfunction associated with elevated levels of catecholmaines is thought to play an important part in the development of *tako-tsubo* syndrome, and depressed endothelial cell activity has also been associated with raised sympathetic nerve activity and suggested to involve β-adrenoceptors (Pettersson *et al.*, 1990; De Caterina *et al.*, 2011). So reducing the endogenous stimulation of vascular β-adrenoceptors could explain in part the beneficial effects of β-blockers (Broeders *et al.*, 2000; Tzemos *et al.*, 2001; Reiter, 2004; Priviero *et al.*, 2007; Gupta and Wright, 2008; Wenzel *et al.*, 2009).

In summary, luminal perfusion of β-adrenoceptor agonists causes a significant suppression in endothelium-dependent vasodilation. This action is mediated through β_1-adrenoceptors, most probably located on the endothelium, and reflects inhibition of endothelial cell IK_{Ca} channels to depress endothelial cell hyperpolarization.

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Conflict of interest

The authors have no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Different buffer compositions do not affect hyperpolarization to Ach.

Summary data showing smooth muscle membrane potential increases in response to endothelial cell stimulation with ACh in mesenteric arteries bathed either in Krebs (1 mM Ca^{2+}, $n = 5$; 2.5 mM Ca^{2+}, $n = 6$) or MOPS buffer (1 mM Ca^{2+}, $n = 3$–4; 2 mM Ca^{2+}, $n = 3$) in the presence of 100 μM L-NAME. Concentration-dependent hyperpolarization to ACh was the same in each case. Data are the mean ± SEM.