

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 December ; 36(12): 2404–2411. doi:10.1161/ATVBAHA.116.308517.

Kv7 channel activation underpins EPAC-dependent relaxations of rat arteries

Jennifer B Stott, PhD, Vincenzo Barrese, PhD, and Iain A Greenwood, PhD

Vascular Biology Research Group, Institute for Cardiovascular and Cell Sciences, St George's University of London, London, UK

Abstract

Objective—To establish the role of Kv7 channels in EPAC dependent relaxations of the rat vasculature, and investigate whether this contributes to β -adrenoceptor mediated vasorelaxations

Approach—Isolated rat renal and mesenteric arteries (RA and MA respectively) were used for isometric tension recording to study the relaxant effects of a specific EPAC activator and the β -adrenoceptor agonist isoproterenol in the presence of potassium channel inhibitors and cell signalling modulators. Isolated myocytes were used in proximity ligation assay studies to detect localisation of signalling intermediaries with Kv7.4 before and after cell stimulation.

Results—Our studies showed that the EPAC activator (8-pCPT-2Me-cAMP-AM) produced relaxations and enhanced currents of MA and RA that were sensitive to linopirdine (Kv7 inhibitor). Linopirdine also inhibited isoproterenol mediated relaxations in both RA and MA. In the MA isoproterenol relaxations were sensitive to EPAC inhibition, but not protein kinase A inhibition. In contrast, isoproterenol relaxations in RA were attenuated by protein kinase A but not by EPAC inhibition. Proximity ligation assay showed a localisation of Kv7.4 with A-Kinase anchoring protein in both vessels in the basal state which increased only in the RA with isoproterenol stimulation. In the MA, but not the RA, a localisation of Kv7.4 with both Rap1a and Rap2 (downstream of EPAC) increased with isoproterenol stimulation.

Conclusions—EPAC dependent vasorelaxations occur in part via activation of Kv7 channels. This contributes to the isoproterenol mediated relaxation in mesenteric, but not renal, arteries.

Keywords

K Channel; Cyclic Nucleotide; Isoproterenol; Signalling Pathways; Vascular Smooth Muscle

Subject Codes

Vascular Biology; Cell Signalling/Signal Transduction; Ion Channels/Membrane Transport

Address Correspondence to: Iain A. Greenwood, PhD, Vascular Research Group, St George's, Cranmer Terrace, London, SW17 0RE, UK, Tel: +44 (0) 208 725 2857, greenwood@sgul.ac.uk.

Disclosures
None

Introduction

The first account of Kv7 channels contributing to physiologically relevant receptor-mediated vasorelaxations showed that pharmacological blockade of Kv7 channels or Kv7.4 knockdown resulted in impaired responses to the mixed β -adrenoceptor agonist isoproterenol in the rat renal artery¹. Subsequently, studies have shown that other vasodilatory agents which also work through increasing intracellular cyclic AMP (cAMP) levels via Gs coupled receptor activation, also produce vasorelaxations which are Kv7 dependent (adenosine² and forskolin³ in coronary artery, CGRP⁴ and forskolin⁵ in cerebral artery). Now that cAMP signalling is well recognised as regulatory to vascular Kv7 channels, the downstream signalling events which are responsible for this regulation need to be established.

Cyclic AMP activity stimulates two main intracellular signalling molecules – protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC). In the vasculature PKA activity has been extensively researched and is involved in a myriad of regulatory processes which result in vasorelaxation⁶. One of the prime targets of PKA is the A Kinase Anchoring Protein (AKAP) which is involved in cardiac and neuronal Kv7 channel regulation^{7, 8, 9}. By contrast, EPAC is more recently discovered and its effects are only beginning to be characterised (see 10–12 for recent reviews). EPAC acts as a guanine nucleotide exchange factor (GEF) and activates a number of small proteins, most prominently Rap proteins, which have important vascular effects^{13–17}. EPAC stimulation has been shown to contribute to vasorelaxations in rat mesenteric arteries^{18, 19}, in part via activation of calcium activated K channels (BK_{Ca})¹⁶ but the role of other vascular K channels in this process is unclear.

Here we aim to establish the role of Kv7 channels in EPAC dependent relaxations, and whether this contributes to the isoproterenol mediated relaxation of vessels.

Materials and Methods

Materials and methods are available in the online data supplement

Results

EPAC activation produces Kv7 dependent vessel specific relaxation

To examine the possible role of Kv7 channels in EPAC dependent relaxations in MA, we used the EPAC specific activator 8-pCPT-2Me-cAMP-AM at a concentration selective for EPAC (5 μ mol/L). This produced relaxations of both the MA and RA (n=13 and n=8, respectively Figure 1B and C). As it has previously been shown that BK_{Ca} channels have a role in this process¹⁸, we inhibited this channel with 1 μ mol/L paxilline which produced an impairment of the EPAC dependent relaxation in both MA and RA (n=5) but not complete blockade. To investigate the role of Kv7 channels, we used the pan-Kv7 channel blocker linopirdine, which inhibited 8-pCPT-2Me-cAMP-AM -mediated relaxations in MA at both 1 and 10 μ mol/L (n=6). In combination paxilline and linopirdine produced an additive inhibition of EPAC relaxation in the MA (n=6). In the RA linopirdine reduced relaxation to

the EPAC activator at both 1 μ mol/L (n=6) and 10 μ mol/L (n=5), but an additive effect with 1 μ mol/L paxilline was not seen (n=4).

Relaxations to 5 μ mol/L 8-pCPT-2Me-cAMP-AM were also tested in the presence of the Kv7.1 inhibitor HMR1556 (10 μ mol/L) and the EPAC inhibitor ESI-09 (300nmol/L). HMR1556 had no effect on relaxations in either MA or RA (n=3-6). 300nmol/L ESI-09 significantly inhibited the relaxation in both beds (n=3-5, Supplementary Figure 1) without any effect on basal tone. Previous reports have concluded that EPAC relaxations are endothelium dependent^{18, 19}, so we tested the effect of 5 μ mol/L 8-pCPT-2Me-cAMP-AM in MA endothelium denuded segments, which was assessed by the vasorelaxant response to 10 μ mol/L carbachol. Vessels with <20% relaxation to 10 μ mol/L carbachol were used for these experiments and we saw no effect of endothelium denudation on responses to 8-pCPT-2Me-cAMP-AM (n=6, Supplementary Figure 2).

To test the effect of EPAC stimulation directly on Kv7 channels, we used myocytes isolated from renal and mesenteric arteries and recorded whole cell K⁺ currents which were sensitive to 10 μ mol/L linopirdine (in the presence of 1 μ mol/L paxilline) before and after application of 1 μ mol/L 8-pCPT-2Me-cAMP-AM. In both RA and MA arterial myocytes we recorded a significant increase in the linopirdine sensitive current in the presence of the EPAC activator (Figure 1 D and E). We also utilised HEK293 cells which stably express Kv7.4 – the most abundant Kv7 isoform in the vasculature shown to be enhanced by cAMP^{20–22} and the isoform which has been most commonly implicated in mediating vasorelaxations^{1–5, 23–30}. End point PCR showed that these cells express both the EPAC1 and EPAC2 isoforms (Supplementary Figure 3). Kv7.4 channels produce voltage dependent currents when expressed in HEK293 cells, which increased significantly after addition of 1 μ mol/L 8-pCPT-2Me-cAMP-AM (1.6 \pm 0.3 times increase maximal current at -20mV in control, n=7, Figure 1F). This was associated with a leftward shift of the activation curve, with a change in V_{1/2} from -7.2mV in control to -17.5mV after addition of 1 μ mol/L 8-pCPT-2Me-cAMP (n=7, Figure 1G).

Signalling pathways involved in isoproterenol relaxations

We next sought to establish if EPAC dependent signalling via Kv7 channels contributes to isoproterenol mediated vasorelaxations. Isoproterenol produced dose dependent relaxations of MA which were significantly attenuated in the presence of 10 μ mol/L linopirdine (Figure 2A, n=9) or 1 μ mol/L paxilline and an additive inhibitory effect was seen when both agents were used (Figure 2B, n=5). This same pattern was seen in the RA (Figure 2C and 2D, n=5-7) where the role of Kv7 and other K⁺ channels in isoproterenol relaxations has previously been fully characterised¹. In MA, blockade of K_{ATP} channels (10 μ mol/L glibenclamide) had no effect on relaxations whilst non-specific Kv blockade (1mM 4-aminopyridine) enhanced vasorelaxations (n=4-6, Supplementary Figure 4).

In the MA inhibition of EPAC with 100nmol/L ESI-09 produced a significant impairment of isoproterenol relaxations (Figure 3A, n=9). In contrast, PKA inhibition by 1 μ mol/L KT 5720 (Figure 3C, n=10) or 1 μ mol/L PKI (Supplementary Figure 5), had no effect on isoproterenol relaxations. Linopirdine (10 μ mol/L) inhibited the isoproterenol relaxation in the presence of 1 μ mol/L KT5720 (n=7), but not 100nmol/L ESI-09 (n=6) (Figure 3 B and D). To investigate

whether there was any isoform specificity in the EPAC mediated relaxations we tested the relaxations to isoproterenol in the presence of 1 μ mol/L CE3F4 (EPAC1 inhibitor) and 1 μ mol/L HJC0350 (EPAC2 inhibitor). Individually neither had any effect on isoproterenol relaxations (Figure 3E, n=6), but in combination they produced a significant impairment (Figure 3F, n=5).

Strikingly, EPAC inhibition with 300nmol/L ESI-09 in the RA had no effect on isoproterenol relaxations (Figure 4A, n=5) whilst PKA inhibition with 1 μ mol/L KT5720 (n=9) or 1 μ mol/L PKI (n=7) produced a significant inhibition (Figure 4B and 4C). Consistent with a role for PKA in this vessel, an inhibitor of PKA anchoring (Ht31, 10 μ mol/L) produced significant inhibition of the isoproterenol relaxation in RA (Figure 4D, n=7).

Using the information obtained in the myograph experiments, we performed proximity ligation assays (PLA) on both MA and RA myocytes stimulated with 1 μ mol/L isoproterenol to detect the localisation of several signalling intermediaries with the Kv7.4 subunit. We investigated both AKAP (as a downstream modulator of PKA) and Rap proteins (downstream of EPAC). In MA there was an increase in Kv7.4-Rap1a (Figure 5A, N=3, n=16) and Kv7.4-Rap2 after isoproterenol stimulation (Figure 5B, N=3, n=15). High basal levels of Kv7.4-AKAP were detected, but surprisingly these decreased significantly in stimulated cells (Figure 5C, N=4, n=19). Conversely in RA, Kv7.4-AKAP levels increased after isoproterenol treatment (Figure 6C, N=3, n=15) but no increase in Kv7.4-Rap1a (Figure 6A, N=3, n=13) or Kv7.4-Rap2 was seen (Figure 6B, N=2, n=10). There was no change in Kv7.4-Rap1b levels in isoproterenol treated MA or RA myocytes (Supplementary Figure 6A and 6B). All antibody combinations were tested in untransfected HEK293 cells and produced low numbers of puncta (<5/cell) in these conditions (Supplementary Figure 6C).

Discussion

Here we provide the first evidence that EPAC dependent relaxations involve Kv7 channels and that EPAC signalling contributes to an endogenous vasodilatory response in the rat mesenteric artery. To our knowledge this is the first account of an activation of an ion channel by the same endogenous vasodilator via different intracellular signalling pathways. Moreover, we show that the signalling intermediate linking β -adrenoceptors to Kv7 channel differs in RA compared to MA

Since the discovery of EPAC as a downstream mediator of cAMP signalling, its' role in vascular biology has been under scrutiny. EPAC was first shown to be involved in vascular relaxations when a role in the downregulation of RhoA activity resulting in Ca²⁺ desensitisation was identified¹³. Subsequently, EPAC dependent relaxation of rat mesenteric arteries was shown to involve BK_{Ca} channel activation¹⁸. Whilst EPAC had previously been shown to negatively regulate vascular K_{ATP} channels³¹, this enhancement of BK_{Ca} was the first account of the positive modulation of a K⁺ channel by EPAC. Our data shows that Kv7 channels underlie, in part, the EPAC dependent vasorelaxation in rat MA and RA. We therefore propose that Kv7 channels are significant players in mediating EPAC dependent vasorelaxations in the rat vasculature.

Having established that EPAC stimulates Kv7 channels and produces vasorelaxations in a linopirdine sensitive manner, we investigated the role of EPAC signalling in a receptor mediated vasorelaxant pathway. Isoproterenol is a well characterised cAMP generator and vasorelaxant. In the mesenteric artery the potassium channel(s) underlying this has been debated for some time. Isoproterenol and cAMP dependent relaxations were initially believed to involve K_{ATP} channels³², however it has since been shown that although this results in membrane hyperpolarisation^{33–35} these channels do not contribute directly to vasorelaxation as glibenclamide has no effect on these relaxations ^{36–38}, present study. The BK_{Ca} channel has also been implicated in the vasoactive properties of isoproterenol ^{37–40}, present study and we report that like the EPAC dependent relaxation this is an effect which is additive to the role of Kv7 channels. Kv7 channels contribute to the EPAC dependent component, again an interesting parallel with BK_{Ca} channels which were reported to contribute to a PKA independent component ³⁷ (prior to the discovery of EPAC). Discovering the mechanisms which dually regulate Kv7 and BK_{Ca} channels in the mesenteric artery will be an interesting area of future study. Notably, our study did not show a dependence upon the endothelium for the EPAC dependent relaxation as shown previously^{18, 19}. Whilst we saw a wide range of relaxation responses to $5\mu\text{mol/L}$ 8-CPT-2Me-cAMP, this was not correlated to the responsiveness to $10\mu\text{mol/L}$ carbachol. A similar trend, or lack thereof, was seen with the responsiveness of the MA to $1\mu\text{mol/L}$ isoproterenol – this varied considerably between vessels, but no clear correlation was seen between this and the response to carbachol (Supplementary Figure 7). The role of the endothelium in isoproterenol dependent relaxation has been debated intensively for many years see refs ^{35, 41–43}. From our data with both isoproterenol and 8-CPT-2Me-cAMP we conclude that our data does not indicate that these are purely endothelial dependent responses, but this does not rule out a role for the endothelium completely. Therefore the reason for the variability is unclear, but could represent the inherent differences present in each animal.

We report that isoproterenol treated MA myocytes show an increase in PLA puncta between Kv7.4 and both Rap1a and Rap2 – small G proteins downstream of EPAC. Rap1 proteins have crucial effects within the vasculature¹⁵, with knockout of a singular isoform resulting in gross cardiovascular defects such as defective platelet function⁴⁴, angiogenesis^{45, 46} and hypertension¹⁴, whilst Rap2 proteins are involved in arteriogenesis¹⁷. Both Rap1a and Rap 2 are involved in membrane translocation of cellular components in the vasculature ^{16, 47, 48}, and we propose that this may be a possible mechanism that is involved in the response of Kv7.4 channels to EPAC stimulation, although it is not yet clear if this is via direct or indirect effects on the channel, and aim to investigate this further.

This work confirms previous findings from our lab that Kv7 channels mediate isoproterenol dependent relaxations in the renal artery¹. Similar to the MA, we now report that this is in combination with BK_{Ca} channel activity, as inhibitors of either channel attenuated the relaxation. However, we did not see an additive effect of BK_{Ca} and Kv7 channel inhibition as we had in the MA. The reason for this is unclear, but we speculate that it is due to reduced permeability in RA which is a much tougher vessel than the MA. We further show that unlike the MA this relaxation is dependent upon PKA, and we see an increase in Kv7.4-AKAP localisation in RA myocytes after isoproterenol stimulation. AKAP is known to form

multifunctional signalling complexes and has been shown to be regulatory to both cardiac (Kv7.1)⁷ and neuronal (Kv7.2, 7.3 and 7.5)^{8, 9, 49} Kv7 channels. Here we demonstrate that this could also be an important regulatory mechanism of Kv7 channels in the vasculature, a finding which warrants further study. We investigated the interactions with Kv7.4 due to its' crucial role in the regulation of the vasculature, as highlighted by the impact of KCNQ4 knockdown^{4, 27}, and the stimulating effect of EPAC on Kv7.4 dependent currents. An overexpression system was used to remove artery specific ion channel structure and these experiments represent a proof of concept that side steps the vagaries of individual arteries. However one caveat to this is that it is known that Kv7.5 channels form heterotetramers with Kv7.4 in the vasculature^{4, 50}, and Kv7.5 has been shown to be an endpoint for PKA dependent signalling in response to isoproterenol treatment in MA51. We confirm here previous reports that isoproterenol dependent relaxations in MA are primarily PKA-independent³⁷ suggesting that this modulation of Kv7.5 may play a role of other aspects of the vascular response to isoproterenol. One further complexity is the relationship of EPAC signalling with $\beta\gamma$ G proteins, recently shown to enhance Kv7.4 channels and necessary for receptor-mediated stimulation in RA smooth muscle cells²².

This study reveals a complex, regulation of Kv7 channels by cAMP dependent signals, which is artery specific. That isoproterenol mediated signalling couples to Kv7 channels via a PKA/AKAP axis in the RA, but an EPAC/Rap axis in the MA, is highly intriguing. Our data shows that EPAC stimulation is capable of producing Kv7 dependent relaxations in the RA, showing that it is not the case that this pathway is redundant here. One possible explanation is that EPAC is known to be under the control of distinct, compartmentalised molecular complexes which display specific cellular distribution. That EPAC dependent signals involve Kv7 channels in the vasculature is another step in unravelling the complexities of vascular

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

None

Sources of Funding

JBS is funded by a British Heart Foundation grant (PG/12/63/29824) awarded to IAG

Abbreviations

AKAP	A-kinase anchoring protein
BK_{Ca}	large conductance Ca ²⁺ activated K ⁺ channel
cAMP	cyclic AMP
EPAC	exchange protein directly activated by cAMP
GEF	guanine nucleotide exchange factor

K_{ATP}	ATP-sensitive K ⁺ channel
MA	mesenteric artery
PKA	protein kinase A
RA	renal artery

References

1. Chadha PS, Zunke F, Zhu HL, Davis AJ, Jepps TA, Olesen SP, Cole WC, Moffatt JD, Greenwood IA. Reduced KCNQ4-encoded voltage-dependent potassium channel activity underlies impaired β -adrenoceptor-mediated relaxation of renal arteries in hypertension. *Hypertension*. 2012; 59:877–884. [PubMed: 22353613]
2. Khanamiri S, Soltysinska E, Jepps TA, Bentzen BH, Chadha PS, Schmitt N, Greenwood IA, Olesen SP. Contribution of Kv7 channels to basal coronary flow and active response to ischemia. *Hypertension*. 2013; 62:1090–1097. [PubMed: 24082059]
3. Morales-Cano D, Moreno L, Barreira B, Pandolfi R, Chamorro V, Jimenez R, Villamor E, Duarte J, Perez-Vizcaino F, Cogolludo A. Kv7 channels critically determine coronary artery reactivity: left-right differences and down-regulation by hyperglycaemia. *Cardiovasc Res*. 2015; 106:98–108. [PubMed: 25616413]
4. Chadha PS, Jepps TA, Carr G, Stott JB, Zhu HL, Cole WC, Greenwood IA. Contribution of Kv7.4/Kv7.5 heteromers to intrinsic and calcitonin gene-related peptide-induced cerebral reactivity. *Arterioscler Thromb Vasc Biol*. 2014; 34:887–893. [PubMed: 24558103]
5. Lee S, Yang Y, Tanner MA, Li M, Hill MA. Heterogeneity in Kv7 channel function in the cerebral and coronary circulation. *Microcirculation*. 2015; 22:109–121. [PubMed: 25476662]
6. Morgado M, Cairrão E, Santos-Silva AJ, Verde I. Cyclic nucleotide-dependent relaxation pathways in vascular smooth muscle. *Cell Mol Life Sci*. 2012; 69:247–266. [PubMed: 21947498]
7. Potet F, Scott JD, Mohammad-Panah R, Escande D, Baró I. AKAP proteins anchor cAMP-dependent protein kinase to KvLQT/IsK channel complex. *Am J Physiol Heart Circ Physiol*. 2001; 282:H2038–2045.
8. Higashida H, Hoshi N, Zhang JS, Yokoyama S, Hashii M, Jin D, Noda M, Robbins J. Protein kinase C bound with A-kinase anchoring protein is involved in muscarinic receptor-activated modulation of M-type KCNQ potassium channels. *Neurosci Res*. 2005:231–234. [PubMed: 15710486]
9. Bal M, Zhang J, Hernandez CC, Zaika O, Shapiro MS. Ca²⁺/Calmodulin disrupts AKAP79/150 interactions with KCNQ (M-type) K⁺ channels. *J Neurosci*. 2010; 30:2311–2323. [PubMed: 20147557]
10. Métrich M, Berthouze M, Morel E, Crozatier B, Gomer AM, Lezoualc'h F. Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. *Pflugers Arch*. 2010; 459:535–546. [PubMed: 19855995]
11. Roberts OL, Dart C. cAMP signalling in the vasculature: the role of Epac (exchange protein activated by cAMP). *Biochem Soc Trans*. 2014; 42:89–97. [PubMed: 24450633]
12. Lezoualc'h F, Fazal L, Laudette M, Conte C. Cyclic AMP sensor EPAC proteins and their role in cardiovascular function and disease. *Circ Res*. 2016; 118:881–897. [PubMed: 26941424]
13. Zieba BJ, Artamonov MV, Jin L, Momotani K, Ho T, Franke AS, Nepl R, Stevenson AS, Khromov AS, Chrzanowska-Wodnicka M, Somlyo AV. The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *J Biol Chem*. 2011; 286:16681–16692. [PubMed: 21454546]
14. Lakshmikanthan S, Zieba BJ, Ge ZD, Momotani K, Zheng X, Lund H, Artamonov MV, Maas JE, Szabo A, Zhang DX, Auchampach JA, et al. Rap1b in smooth muscle and endothelium is required for maintenance of vascular tone and normal blood pressure. *Arterioscler Thromb Vasc Biol*. 2014; 34:1486–1494. [PubMed: 24790136]

15. Chrzanowska-Wodnicka M, White GC 2nd, Quillam LA, Whitehead KJ. Small GTPase Rap1 is essential for mouse development and formation of functional vasculature. *PLoS One*. 2015; 10:e0145689. [PubMed: 26714318]
16. Eid AH, Maiti K, Mitra S, Chotani MA, Flavahan S, Bailey SR, Thompson-Torgerson CS, Flavahan NA. Estrogen increases smooth muscle expression of alpha2C-adrenoceptors and cold-induced constriction of cutaneous arteries. *Am J Physiol Heart Circ Physiol*. 2007; 293:H1955–1961. [PubMed: 17644575]
17. Pöling J, Szibor M, Schimanski S, Ingelmann ME, Rees W, Gajawada P, Kochfar Z, Lörchner H, Salwig I, Shin JY, Wiebe K, et al. Induction of smooth muscle cell migration during arteriogenesis is mediated by Rap2. *Arterioscler Thromb Vasc Biol*. 2011; 31:2297–2305. [PubMed: 21737786]
18. Roberts OL, Kamishima T, Barrett-Jolley R, Quayle JM, Dart C. Exchange protein activated by cAMP (Epac) induces vascular relaxation by activating Ca²⁺-sensitive K⁺ channels in rat mesenteric artery. *J Physiol*. 2013; 591:5107–5123. [PubMed: 23959673]
19. García-Morales V, Cuí as A, Elíes J, Campos-Toimil M. PKA and Epac activation mediated cAMP-induced vasorelaxation by increasing endothelial NO production. *Vascul Pharmacol*. 2014; 60:95–101. [PubMed: 24469067]
20. Hougaard C, Klaerke DA, Hoffmann EK, Olesen SP, Jorgensen NK. Modulation of KCNQ4 channel activity by changes in cell volume. *Biochim Biophys Acta*. 2004; 1660:1–6. [PubMed: 14757214]
21. Chambard JM, Ashmore JF. Regulation of the voltage-gated potassium channel KCNQ4 in the auditory pathway. *Pflugers Arch*. 2005; 450:34–44. [PubMed: 15660259]
22. Stott JB, Povstyan OV, Carr G, Barrese V, Greenwood IA. G-protein $\beta\gamma$ subunits are positive regulators of Kv7.4 and native vascular Kv7 channel activity. *Proc Natl Acad Sci USA*. 2015; 112:6497–6502. [PubMed: 25941381]
23. Jepps TA, Chadha PS, Davis AJ, Harhun MI, Cockerill GW, Olesen SP, Hansen RS, Greenwood IA. Downregulation of Kv7.4 channel activity in primary and secondary hypertension. *Circulation*. 2011; 124:602–611. [PubMed: 21747056]
24. Martelli A, Testai L, Breschi MC, Lawson K, McKay NG, Miceli F, Tagliablatela M, Calderone V. Vasorelaxation by hydrogen sulphide involves activation of Kv7 potassium channels. *Pharmacol Res*. 2013; 70:27–34. [PubMed: 23287425]
25. Sedivy V, Joshi S, Ghaly Y, Mizera R, Zaloudikova M, Brennan S, Novotna J, Herget J, Gurney AM. Role of Kv7 channels in responses of the pulmonary circulation to hypoxia. *Am J Physiol Lung Cell Mol Physiol*. 2015; 308:L48–57. [PubMed: 25361569]
26. Stott JB, Barrese V, Jepps TA, Leighton EV, Greenwood IA. Contribution of Kv7 channels to natriuretic peptide mediated vasodilation in normal and hypertensive rats. *Hypertension*. 2015; 65:676–682. [PubMed: 25547342]
27. Jepps TA, Carr G, Lundegaard PR, Olesen SP, Greenwood IA. Fundamental role for the KCNE4 ancillary subunit in Kv7.4 regulation of arterial tone. *J Physiol*. 2015; 593:5325–5340. [PubMed: 26503181]
28. Salomonsson M, Brasen JC, Braunstein TH, Hagelgyist P, Holstein-Rathlou NH, Sorensen CM. Kv7.4 channels participate in the control of rodent renal vascular resting tone. *Acta Physiol (Oxf)*. 2015:402–414. [PubMed: 25965962]
29. Goodwill AG, Fu L, Noblet JN, Casalini ED, Sassoon D, Berwick ZC, Kassab GS, Tune JD, Dick GM. Kv7 channels contribute to paracrine, but not metabolic or ischemic, regulation of vascular reactivity in swine. *Am J Physiol Heart Circ Physiol*. 2016; 310:H693–704. [PubMed: 26825518]
30. Jepps TA, Olesen SP, Greenwood IA, Dalsgaard T. Molecular and functional characterization of Kv7 channels in penile arteries and corpus cavernosum of healthy and metabolic syndrome rats. *Br J Pharmacol*. 2016; 179:1478–1490.
31. Purves G, Kamishima T, Davies LM, Quayle JM, Dart C. Exchange protein activated by cAMP (Epac) mediated cAMP-dependent but protein kinase A-insensitive modulation of vascular ATP-sensitive potassium channels. *J Physiol*. 2009; 587:3639–3650. [PubMed: 19491242]
32. Randall MD, McCulloch AI. The involvement of ATP-sensitive potassium channels in beta-adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. *Br J Pharmacol*. 1995; 115:607–612. [PubMed: 7582479]

33. Prieto D, Buus C, Mulvany MJ, Nilsson H. Interactions between neuropeptide Y and the adenylate cyclase pathway in rat mesenteric small arteries: role of membrane potential. *J Physiol.* 1997; 508:281–292.
34. Fujii K, Onaka U, Goto K, Abe I, Fujishima M. Impaired isoproterenol-induced hyperpolarization in isolated mesenteric arteries of aged rats. *Hypertension.* 1999; 34:222–228. [PubMed: 10454445]
35. Garland CJ, Yarova PL, Jimenez-Altayo F, Dora KA. Vascular hyperpolarization to β -adrenoceptor agonists evokes spreading dilatation in rat isolated mesenteric arteries. *Br J Pharmacol.* 2011; 164:913–921. [PubMed: 21244369]
36. Omar R, Bottrill FE, Hiley CR, White R. Interaction of cyclic AMP modulating agents with levcromakalim in the relaxation of the rat isolated mesenteric artery. *Eur J Pharmacol.* 2000; 401:85–96. [PubMed: 10915841]
37. White R, Bottrill FE, Siau D, Hiley CR. Protein kinase A-dependent and -independent effects of isoproterenol in rat isolated mesenteric artery: interactions with levcromakalim. *J Pharmacol Exp Ther.* 2001; 298:917–924. [PubMed: 11504785]
38. Huang Y, Kwok KH. Effects of putative K channel blockers on β -adrenoceptor-mediated vasorelaxation of the rat mesenteric artery. *J Cardiovasc Pharmacol.* 1997; 29:515–519. [PubMed: 9156362]
39. Beleznai TZ, Yarova PL, Yuill KH, Dora KA. Smooth muscle Ca^{2+} -activated and voltage-gated K^{+} channels modulate conducted dilation in rat isolated small mesenteric arteries. *Microcirculation.* 2001; 18:487–500.
40. Matsumoto T, Szasz T, Tostes RC, Webb RC. Impaired β -adrenoceptor-induced relaxation in small mesenteric arteries from DOCA-salt hypertensive rats is due to reduced K (Ca) channel activity. *Pharmacol Res.* 2012; 65:537–545. [PubMed: 22388053]
41. Graves J, Poston L. Beta-adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide. *Br J Pharmacol.* 108:631–637.
42. Briones AM, Daly CJ, Jiménez-Altayó F, Martínez-Revelles S, Gonzales JM, McGrath JC, Vila E. Direct demonstration of beta1- and evidence against beta2 and beta3- adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. *Br J Pharmacol.* 2005; 146:679–691. [PubMed: 16113691]
43. Flacco N, Segura V, Perez-Aso M, Estrada S, Seller JF, Jiménez-Altayó F, Noguera MA, D'Ocon P, Vila E, Ivorra MD. Different β -adrenoceptor subtypes coupling to cAMP or NO/cGMP pathways: implications in the relaxant response of rat conductance and resistance vessels. *Br J Pharmacol.* 2013; 169:413–425. [PubMed: 23373597]
44. Chrzanowska-Wodnicka M, Smyth SS, Schoenwaelder SM, Fischer TH, White GC 2nd. Rap1b is required for normal platelet function and hemostasis in mice. *J Clin Invest.* 2005; 115:680–687. [PubMed: 15696195]
45. Chrzanowska-Wodnicka M, Krauz AE, Gale D, White GC 2nd, Vansluys J. Defective angiogenesis, endothelial migration, proliferation and MAPK signalling in Rap1b-deficient mice. *Blood.* 2008;2647–2656. [PubMed: 17993608]
46. Yan J, Li F, Ingram DA, Quillam LA. Rap1a is a key regulator of fibroblast growth factor 2-induced angiogenesis and together with Rap1b controls human endothelial cell functions. *Mol Cell Biol.* 2008; 28:5803–5810. [PubMed: 18625726]
47. Jeyaraj SC, Unger NT, Eid AH, Paul El-Dahdah N, Quillam LA, Flavahan NA, Chotani MA. Cyclic AMP-Rap1a signalling activates RhoA to induce a (2c)-adrenoceptor translocation to the cell surface of microvascular smooth muscle cells. *Am J Physiol Cell Physiol.* 2012; 303:C499–511. [PubMed: 22621783]
48. Motawea HK, Jeyaraj SC, Eid AH, Mitra S, Unger NT, Ahmed AA, Flavahan NA, Chotani MA. Cyclic AMP-Rap1A signalling mediates cell surface translocation of microvascular smooth muscle α 2C-adrenoceptors through the actin-binding protein filamin-2. *Am J Physiol Cell Physiol.* 2013; 305:C829–845. [PubMed: 23864608]
49. Zhang J, Bal M, Bierbower S, Zaika O, Shapiro MS. AKAP79/150 signal complexes in G-protein modulation of neuronal ion channels. *J Neurosci.* 2011; 31:7199–7211. [PubMed: 21562284]

50. Brueggemann LI, Mackie AR, Cribbs LL, Freda J, Tripathi A, Majetschak M, Byron KL. Differential protein kinase C-dependent modulation of Kv7.4 and Kv7.5 subunits of vascular Kv7 channels. *J Biol Chem.* 2014; 289:2099–2111. [PubMed: 24297175]
51. Mani BK, Robakowski C, Brueggemann LI, Cribbs LL, Tripathi A, Majetschak M, Byron KL. Kv7.5 potassium channel subunits are the primary targets for PKA-dependent enhancement of vascular smooth muscle Kv7 currents. *Mol Pharmacol.* 2016; 89:323–334. [PubMed: 26700561]

Highlights

- Kv7 channels contribute to EPAC dependent signals in both rat renal and mesenteric arteries
- EPAC signalling is involved in isoproterenol mediated vasorelaxations of the rat mesenteric artery, but in the renal artery this is a predominantly PKA/ AKAP dependent response
- Isoproterenol stimulation results in increased localisation of Kv7.4 with Rap1a and Rap2 - EPAC effectors – in mesenteric arteries, but not in the renal artery. Here, we see an increased localisation of Kv7.4 and AKAP after stimulation

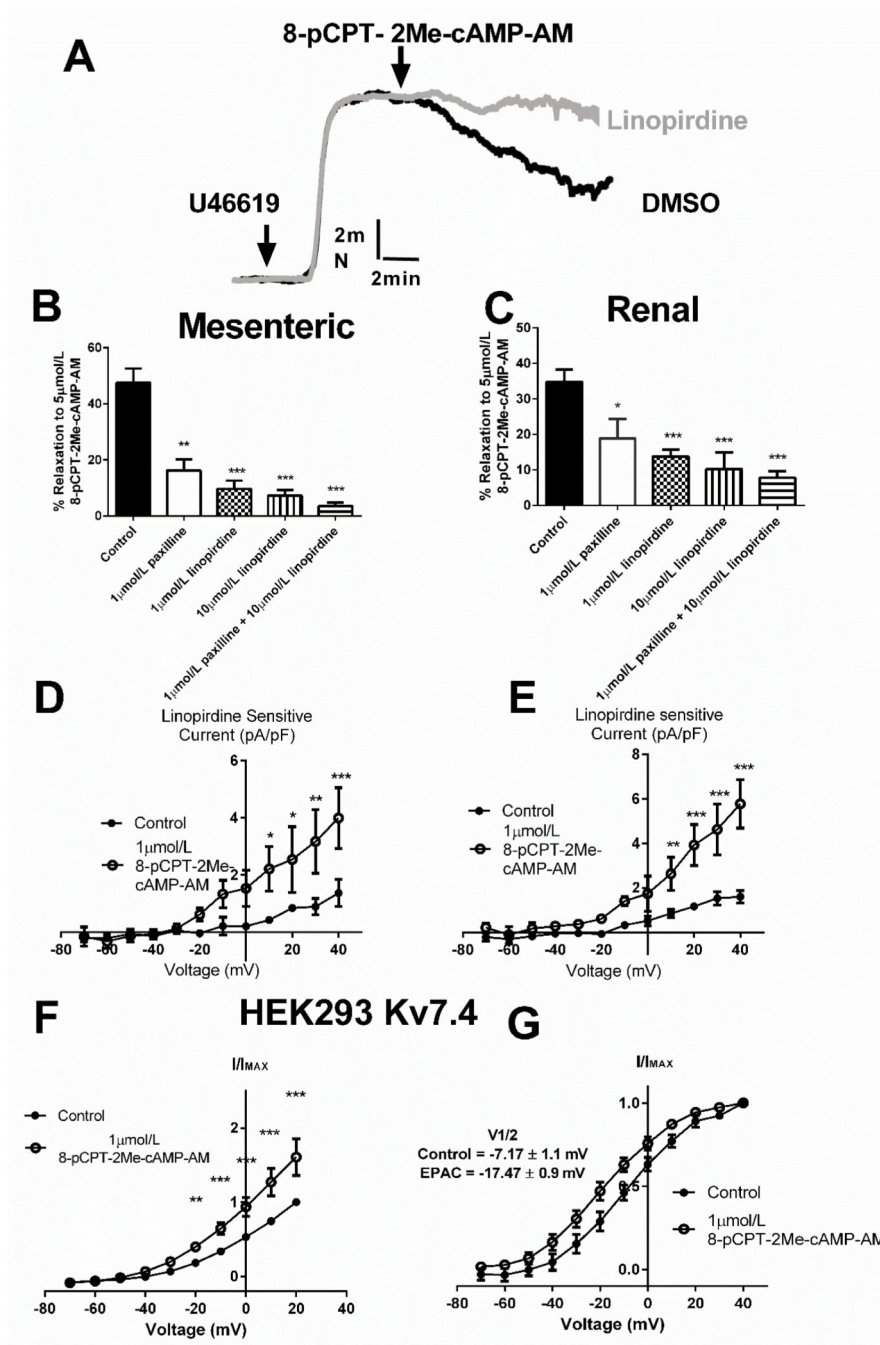


Figure 1. EPAC dependent relaxations of MA and RA involve Kv7 channels

(A) Representative trace of a MA contracted with U46619 and stimulated with 5μmol/L 8-pCPT-2Me-cAMP-AM in DMSO (control, black) and in the presence of 10μmol/L linopirdine (grey). Mean relaxant effect of 5μmol/L 8-pCPT-2Me-cAMP-AM in mesenteric (B) and renal arteries (C) in control or in the presence of 1μmol/L paxilline (BK_{Ca} inhibitor), 1μmol/L and 10μmol/L linopirdine (Kv7 inhibitor), and in combination. Current voltage relationship of the linopirdine sensitive currents (10μmol/L) in control and after stimulation with 1μmol/L 8-pCPT-2Me-cAMP-AM in myocytes from MA (D) and RA (E). (D) Current

voltage relationship of HEK293 Kv7.4 currents in control (closed circles, n=7) (E)
Activation kinetics of Kv7.4 currents in control and after stimulation with 1 μ mol/L 8-pCPT-2Me-cAMP-AM. A one-way ANOVA was performed to analyse isometric tension recording data. For analysis of Kv7.4 currents a Bonferroni post-hoc test was performed following a two-way ANOVA. p<0.05 is denoted (*), p<0.01 is denoted (**) and p<0.001 is denoted (***). Results were deemed non-significant when p>0.05.

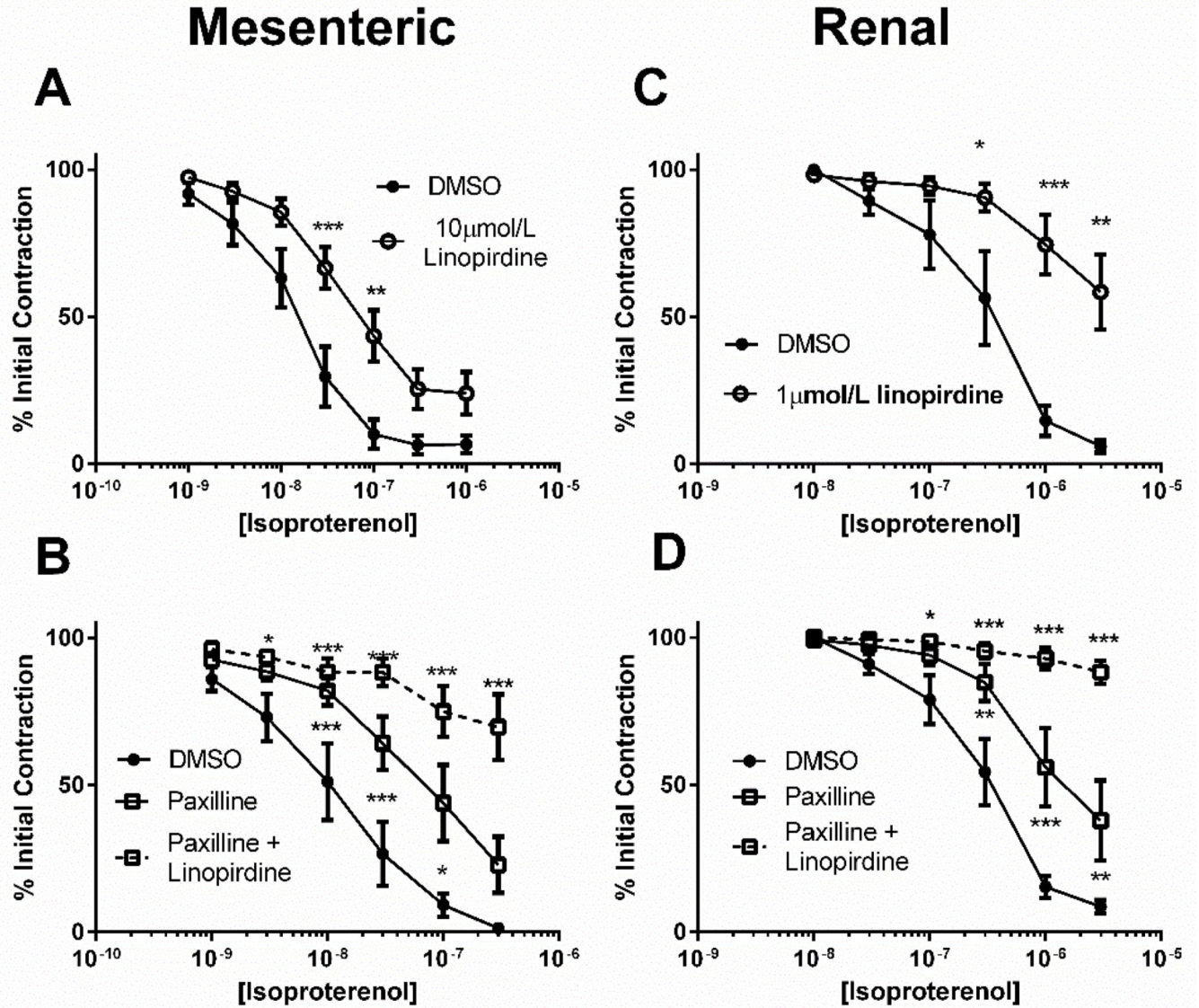


Figure 2. Isoproterenol relaxations of MA and RA involve Kv7 channels

Dose dependent relaxations of MA with isoproterenol (1nmol/L -1μmol/L) in the presence of (A) 10μmol/L linopirdine, (B) 1μmol/L paxilline and both. Dose dependent relaxations of RA with isoproterenol (10nmol/L -3μmol/L) in the presence of (C) 1 μmol/L linopirdine, (D) 1μmol/L paxilline and both. A Bonferroni post-hoc test was performed following a two-way ANOVA. $p < 0.05$ is denoted(*), $p < 0.01$ is denoted(**) and $p < 0.001$ is denoted(***). Results were deemed non-significant when $p > 0.05$.

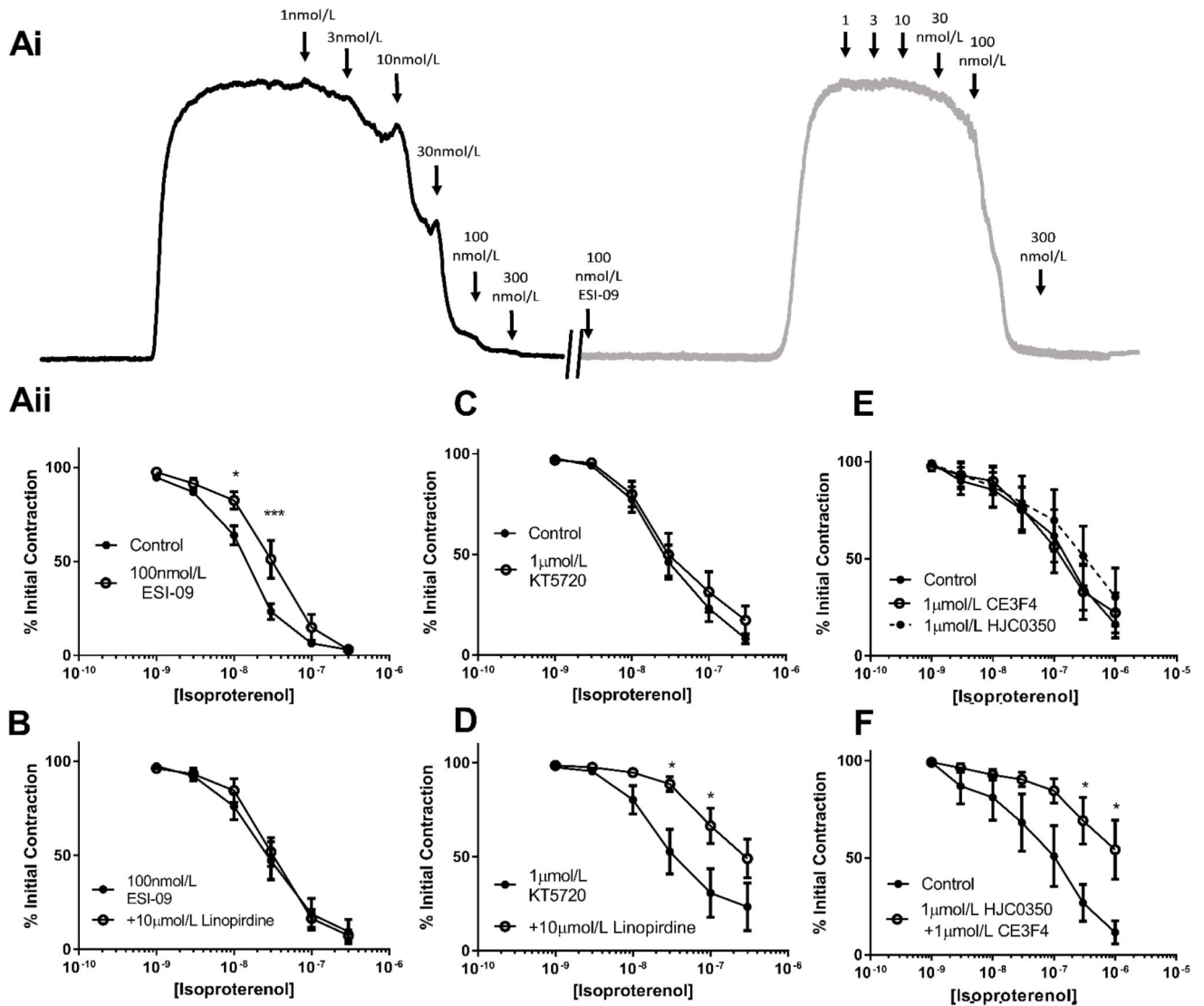


Figure 3. Isoproterenol relaxations in MA are EPAC dependent

Dose dependent relaxations of MA by isoproterenol (1nmol/L-300nmol/L) in the presence of (A) 100nM/L ESI-09 (EPAC inhibitor, n=9), representative trace can be seen in (i) with mean data in (ii), (B) 100nmol/L ESI-09 and 10 μ mol/L linopirdine (n=6), (C) 1 μ mol/L KT5720 (PKA inhibitor, n=10), (D) 1 μ mol/L KT5720 and 10 μ mol/L linopirdine (n=7), (E) 1 μ mol/L CE3F4 (n=6) or 1 μ mol/L HJC0350 (n=6) alone and (F) 1 μ mol/L CE3F4 and 1 μ mol/L HJC0350 in combination (n=5). A Bonferroni post-hoc test was performed following a two-way ANOVA. $p < 0.05$ is denoted (*) and $p < 0.001$ is denoted (***). Results were deemed non-significant when $p > 0.05$.

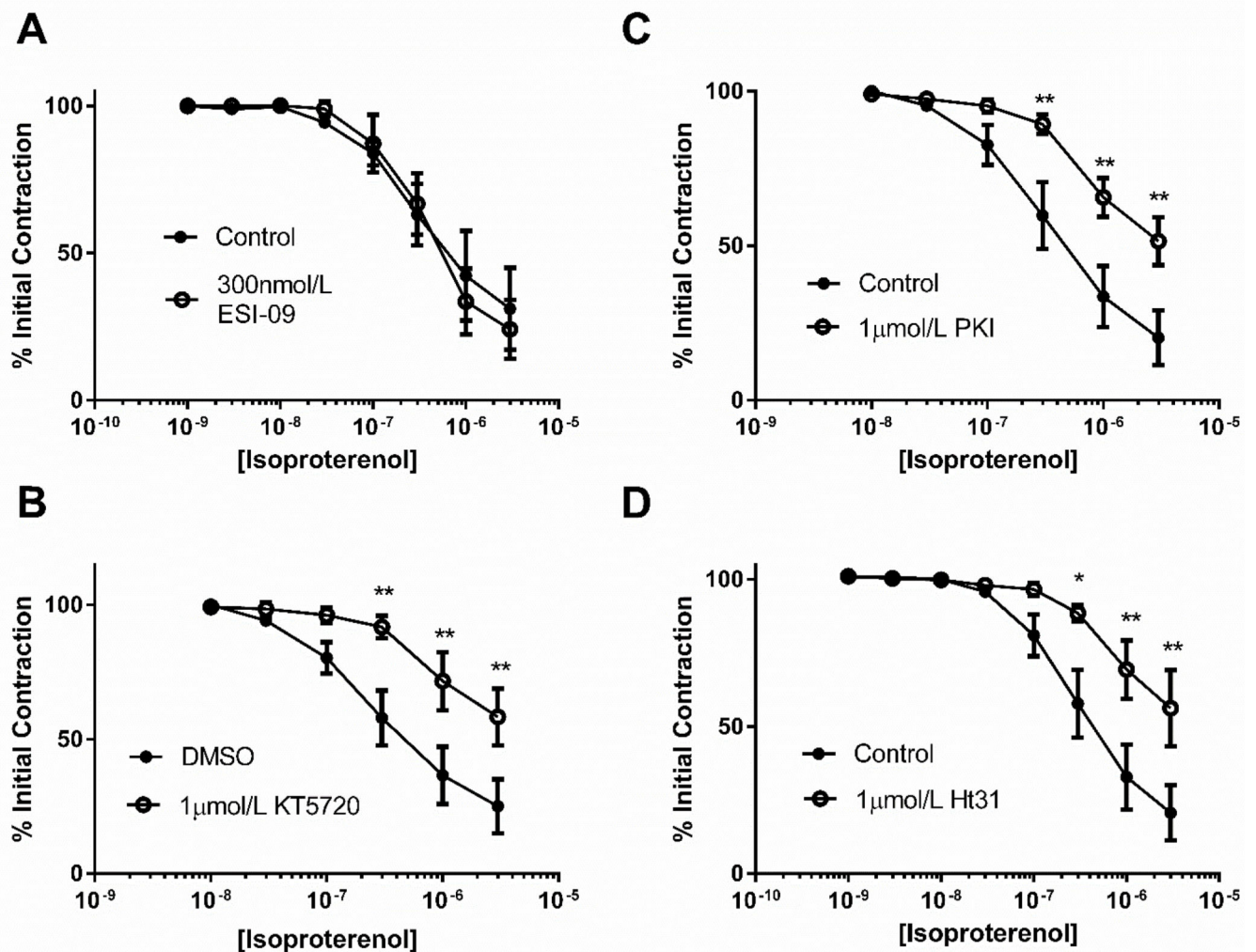


Figure 4. EPAC does not contribute to isoproterenol relaxations in RA

Dose dependent relaxations of RA by isoproterenol (10nmol/L - 3µmol/L) in the presence of 100nmol/L ESI-09 (EPAC inhibitor, n=5), (B) 1µmol/L KT5720 (PKA inhibitor, n=9) (C) 1µmol/L PKI (PKA inhibitor, n=7) and (D) 10µmol/L Ht31 (AKAP inhibitor, n=7). A Bonferroni post-hoc test was performed following a two-way ANOVA $p < 0.05$ is denoted (*), and $p < 0.01$ is denoted (**). Results were deemed non-significant when $p > 0.05$.

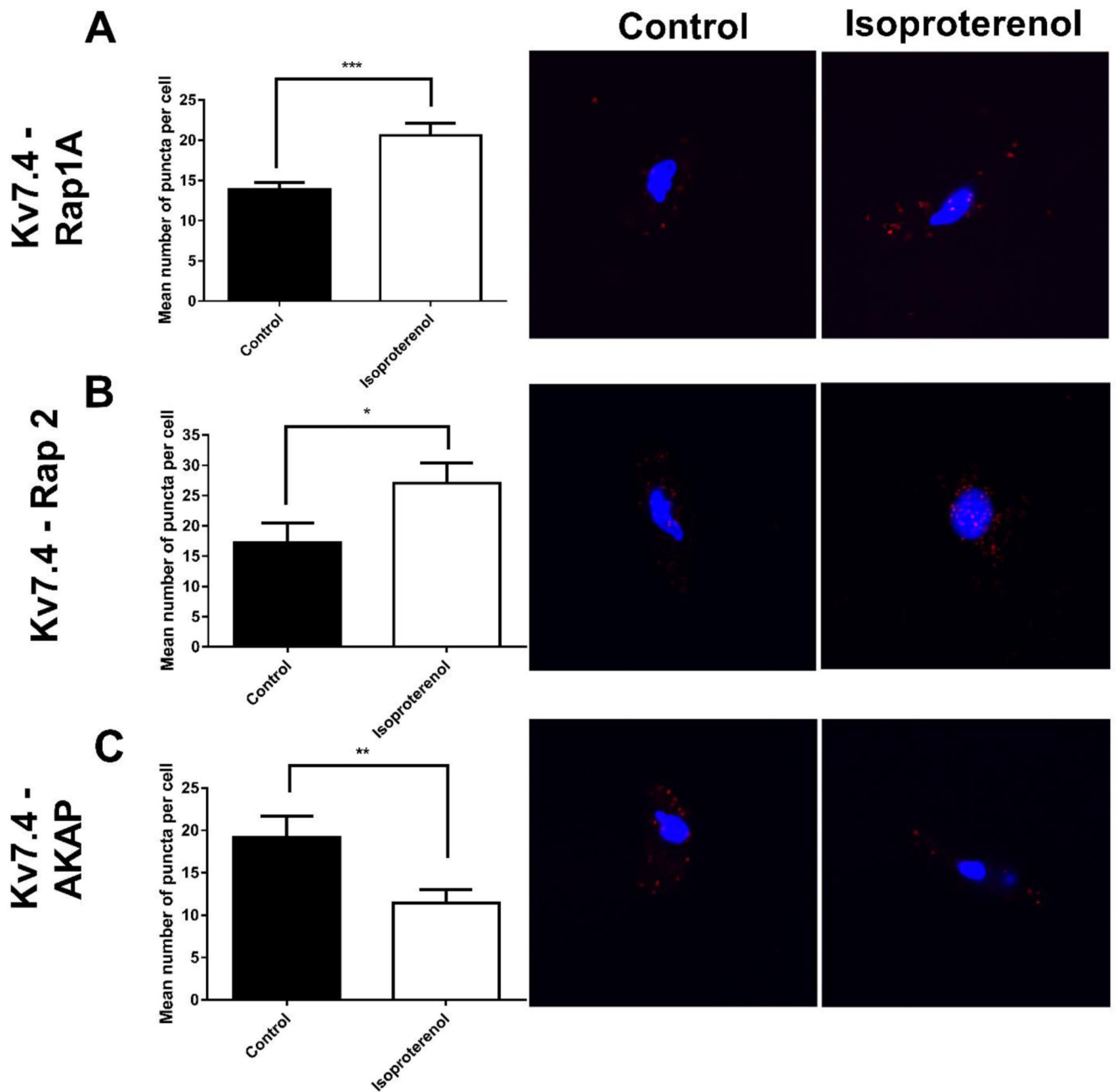


Figure 5. Isoproterenol stimulation in MA alters localisation of Kv7.4 with signalling molecules
 Mean data for the number of PLA puncta and representative images for (A) Kv7.4-Rap1a
 (B) Kv7.4-Rap2 and (C) Kv7.4-AKAP detected in MA myocytes in control and after
 stimulation with 1 μ mol/L isoproterenol. Results were analysed using a one-way ANOVA
 where $p < 0.05$ is denoted (*), $p < 0.01$ is denoted (**) and $p < 0.001$ is denoted (***)

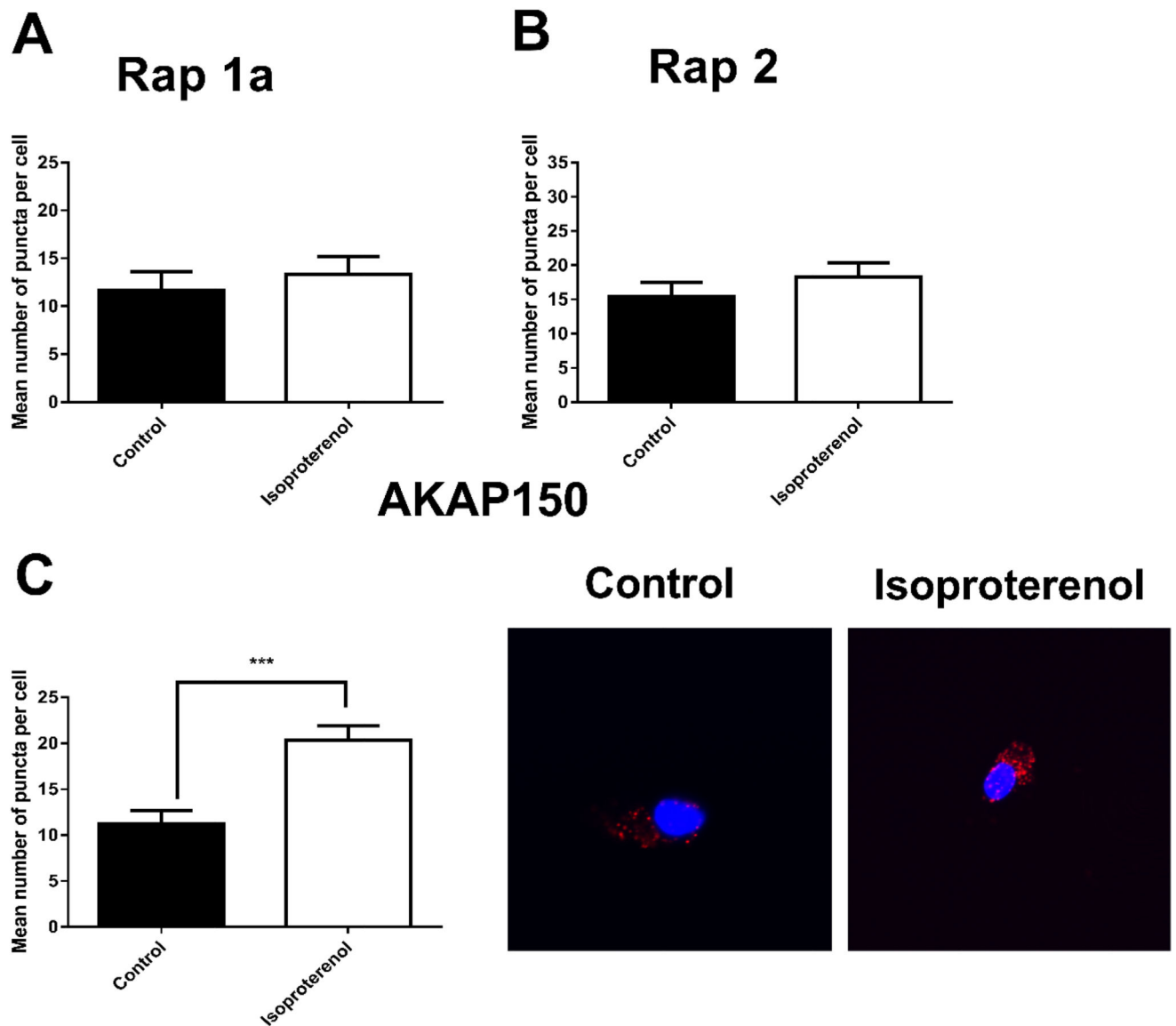


Figure 6. Isoproterenol stimulation in RA myocytes alters localisation of Kv7.4 with signalling molecules

The number of PLA puncta detected in RA myocytes before and after stimulation with (A) Kv7.4-Rap1a, (B) Kv7.4-Rap2 and (C) Kv7.4-AKAP with representative images. Results were analysed using a one-way ANOVA where $p < 0.001$ is denoted (***). Results were deemed non-significant when $p > 0.05$.