Kv7 Channel Activation Underpins EPAC-Dependent Relaxations of Rat Arteries

Jennifer B. Stott, Vincenzo Barrese, Iain A. Greenwood

Objective—To establish the role of Kv7 channels in EPAC (exchange protein directly activated by cAMP)-dependent relaxations of the rat vasculature and to investigate whether this contributes to β-adrenoceptor-mediated vasorelaxations.

Approach and Results—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 signaling modulators. Isolated myocytes were used in proximity ligation assay studies to detect localization of signaling intermediaries with Kv7.4 before and after cell stimulation. 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 localization 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 localization 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. (Arterioscler Thromb Vasc Biol. 2016;36:2404-2411. DOI: 10.1161/ATVBAHA.116.308517.)

Key Words: cyclic nucleotide ■ isoproterenol ■ K channel ■ signaling pathways ■ vascular smooth muscle

The first account of Kv7 channels contributing to physiologically relevant receptor-mediated vasorelaxations showed that pharmacological blockade of Kv7 channels or Kv7.4 knockout resulted in impaired responses to the mixed β-adrenoceptor agonist isoproterenol in the rat renal artery (RA). Subsequently, studies have shown that other vasodilatory agents that also work through increasing intracellular cyclic AMP (cAMP) levels via Gs protein-coupled receptor activation also produce vasorelaxations, which are Kv7-dependent (adenosine and forskolin in coronary artery, CGRP (calcitonin gene-related peptide) and forskolin in cerebral artery). Now that cAMP signaling is well recognized as regulatory to vascular Kv7 channels, the downstream signaling events that are responsible for this regulation need to be established.

CAMP activity stimulates 2 main intracellular signaling molecules—protein kinase A (PKA) and the EPAC (exchange protein directly activated by cAMP). In the vasculature, PKA activity has been extensively researched and is involved in a myriad of regulatory processes, which result in vasorelaxation. One of the prime targets of PKA is the AKAP (A kinase anchoring protein), which is involved in cardiac and neuronal Kv7 channel regulation. By contrast, EPAC is more recently discovered, and its effects are only beginning to be characterized (see Métrich et al, Roberts and Dart, Lezoualc’h et al for recent reviews). EPAC acts as a guanine nucleotide exchange factor and activates several small proteins, most prominently Rap proteins, which have important vascular effects. EPAC stimulation has been shown to contribute to vasorelaxations in rat mesenteric arteries, in part via activation of calcium-activated K channels (BKCa), 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-only Data Supplement.

Results
EPAC Activation Produces Kv7-Dependent Vessel-Specific Relaxation
To examine the possible role of Kv7 channels in EPAC-dependent relaxations in mesenteric artery (MA), we used the EPAC-specific activator 8-pCPT-2Me-cAMP-AM at a concentration selective for EPAC (5 μmol/L). This produced...
relaxations of both the MA and RA (n=13 and n=8, respectively; Figure 1B and 1C). As it has previously been shown that BKCa channels have a role in this process,18 we inhibited this channel with 1 μmol/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 μmol/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 μmol/L (n=6) and 10 μmol/L (n=5), but an additive effect with 1 μmol/L paxilline was not seen (n=4).

Relaxations to 5 μmol/L 8-pCPT-2Me-cAMP-AM were also tested in the presence of the Kv7.1 inhibitor HMR1556 (10 μmol/L) and the EPAC inhibitor ESI-09 (300 nmol/L). HMR1556 had no effect on relaxations in either MA or RA (n=3–6). 300 nmol/L ESI-09 significantly inhibited the relaxation in both beds (n=3–5; Figure 1 in the online-only Data Supplement) 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 μmol/L 8-pCPT-2Me-cAMP-AM in MA endothelium denuded segments, which was assessed by the vasorelaxant response to 10 μmol/L carbachol. Vessels with <20% relaxation to 10 μmol/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; Figure II in the online-only Data Supplement).

To test the effect of EPAC stimulation directly on Kv7 channels, we used myocytes isolated from RA and MA and recorded whole cell K+ currents, which were sensitive to 10 μmol/L linopirdine (in the presence of 1 μmol/L paxilline) before and after application of 1 μmol/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 1D and 1E). We also used HEK293 (human embryonic kidney 293) cells, which stably express Kv7.4—the most abundant Kv7 isoform in the vasculature shown to be enhanced by cAMP20–22 and the isoform that has been most commonly implicated in mediating vasorelaxations.1,3,5,23,30 End point PCR showed that these cells express both the EPAC1 and EPAC2 isoforms (Figure III in the online-only Data Supplement). Kv7.4 channels produce voltage-dependent currents when expressed in HEK293 cells, which increased significantly after addition of 1 μmol/L 8-pCPT-2Me-cAMP-AM (1.6±0.3 times increase maximal current at −20 mV in control; n=7; Figure 1F). This was associated with a leftward shift of the activation curve, with a change in V1/2 from −7.2 mV in control to −17.5 mV after addition of 1 μmol/L 8-CPT-2Me-cAMP (n=7; Figure 1G).

Signalizing Pathways Involved in Isoproterenol Relaxations

We next sought to establish whether EPAC-dependent signaling via Kv7 channels contributes to isoproterenol-mediated vasorelaxations. Isoproterenol produced dose-dependent relaxations of MA, which were significantly attenuated in the presence of 10 μmol/L linopirdine (Figure 2A; n=9) or 1 μmol/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 Kv channels in isoproterenol relaxations has previously been fully characterized.1 In MA, blockade of ATP-sensitive K+ channels (10 μmol/L glibenclamide) had no effect on relaxations while nonspecific K+ blockade (1 mmol/L 4-aminopyridine) enhanced vasorelaxations (n=4–6; Figure IV in the online-only Data Supplement).

In the MA inhibition of EPAC with 100 nmol/L, ESI-09 produced a significant impairment of isoproterenol relaxations (Figure 3A; n=9). In contrast, PKA inhibition by 1 μmol/L KT5720 (Figure 3C; n=10) or 1 μmol/L PKI (protein kinase inhibitor [14-22]-amide; Figure V in the online-only Data Supplement) had no effect on isoproterenol relaxations. Linopirdine (10 μmol/L) inhibited the isoproterenol relaxation in the presence of 1 μmol/L KT5720 (n=7), but not 100 nmol/L ESI-09 (n=6; Figure 3B and 3D). To investigate whether there was any isoform specificity in the EPAC-mediated relaxations, we tested the relaxations to isoproterenol in the presence of 1 μmol/L CE3F4 (EPAC1 inhibitor) and 1 μmol/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 300 nmol/L ESI-09 in the RA had no effect on isoproterenol relaxations (Figure 4A; n=5), while PKA inhibition with 1 μmol/L KT5720 (n=9) or 1 μmol/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 μmol/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 on both MA and RA myocytes stimulated with 1 μmol/L isoproterenol to detect the localization of several signaling 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 (Figure VIA and VIB in the online-only Data Supplement). All antibody combinations were tested in untransfected HEK293 cells and produced low numbers of puncta (<5/cell) in these conditions (Figure VIC in the online-only Data Supplement).

Discussion

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

Since the discovery of EPAC as a downstream mediator of cAMP signaling, 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⁺ desensitization was identified. Subsequently, EPAC-dependent relaxation of rat mesenteric arteries was shown to involve BKCa channel activation. While

Figure 1. EPAC (exchange protein directly activated by cAMP)-dependent relaxations of mesenteric artery (MA) and renal artery (RA) involve Kv7 channels. A, Representative trace of an MA contracted with U46619 and stimulated with 5 μmol/L 8-pCPT-2Me-cAMP-AM in DMSO (control, black) and in the presence of 10 μmol/L linopirdine (gray). Mean relaxant effect of 5 μmol/L 8-pCPT-2Me-cAMP-AM in mesenteric (B) and renal arteries (C) in control or in the presence of 1 μmol/L paxilline (BKCa inhibitor), 1 and 10 μmol/L linopirdine (Kv7 inhibitor), and in combination. Current–voltage relationship of the linopirdine-sensitive currents (10 μmol/L) in control and after stimulation with 1 μmol/L 8-pCPT-2Me-cAMP-AM in myocytes from MA (D) and RA (E). F, Current–voltage relationship of HEK293 (human embryonic kidney 293 cells) Kv7.4 currents in control (closed circles, n=7). G, Activation kinetics of Kv7.4 currents in control and after stimulation with 1 μmol/L 8-pCPT-2Me-cAMP-AM. A one-way analysis of variance (ANOVA) was performed to analyze isometric tension recording data. For analysis of Kv7.4 currents, a Bonferroni post hoc test was performed after a 2-way ANOVA. *P<0.05, **P<0.01, and ***P<0.001. Results were deemed nonsignificant when P>0.05.
EPAC had previously been shown to negatively regulate vascular ATP-sensitive K⁺ channel channels, this enhancement of BKCa 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 signaling in a receptor-mediated vasorelaxant pathway. Isoproterenol is a well-characterized cAMP generator and vasorelaxant. In the MA, the potassium channel(s) underlying this has been debated for some time. Isoproterenol and cAMP-dependent relaxations were initially believed to involve ATP-sensitive K⁺ channel channels; however, it has since been shown that although this results in membrane hyperpolarization, these channels do not contribute directly to vasorelaxation because glibenclamide has no effect on these relaxations (see Omar et al., White et al., Huang and Kwok, and present study). The BKCa channel has also been implicated in the vasoactive properties of isoproterenol (see White et al., Huang and Kwok, Beleznai et al., Matsumoto et al., Beleznai et al., Matsumoto et al., and present study), and we report that like the EPAC-dependent relaxation, this is an effect that is additive to the role of Kv7 channels. Kv7 channels contribute to the EPAC-dependent component, again an interesting parallel with BKCa channels, which were reported to contribute to a PKA-independent component (prior to the discovery of EPAC). Discovering the mechanisms that dually regulate Kv7 and BKCa channels in the MA will be an interesting area of future study. Notably, our study did not show a dependence on the endothelium for the EPAC-dependent relaxation as shown previously. While we saw a wide range of relaxation responses to 5 µmol/L 8-CPT-2Me-cAMP, this was not correlated to the responsiveness to 10 µmol/L carbachol. A similar trend, or lack thereof, was seen with the responsiveness of the MA to 1 µmol/L isoproterenol—this varied considerably between vessels, but no clear correlation was seen between this and the response to carbachol (Figure VII in the online-only Data Supplement). The role of the endothelium in isoproterenol-dependent relaxation

Figure 2. Isoproterenol relaxations of mesenteric artery (MA) and renal artery (RA) involve Kv7 channels. Dose-dependent relaxations of MA with isoproterenol (1 nmol/L to 1 µmol/L) in the presence of (A) 10 µmol/L linopirdine, (B) 1 µmol/L paxilline, and both. Dose-dependent relaxations of RA with isoproterenol (10 nmol/L to 3 µmol/L) in the presence of (C) 1 µmol/L linopirdine, (D) 1 µmol/L paxilline, and both. A Bonferroni post hoc test was performed after a 2-way analysis of variance (ANOVA). *P<0.05, **P<0.01, and ***P<0.001. Results were deemed nonsignificant when P>0.05.

Figure 3. Isoproterenol relaxations in mesenteric artery (MA) are EPAC (exchange protein directly activated by cAMP) dependent. Dose-dependent relaxations of MA by isoproterenol (1–300 nmol/L) in the presence of (A) 100 nM/L ESI-09 (EPAC inhibitor, n=9)—representative trace can be seen in (i), with mean data in (ii); (B) 100 nmol/L ESI-09 and 10 µmol/L linopirdine (n=6); (C) 1 µmol/L KT5720 (PKA inhibitor, n=10); (D) 1 µmol/L KT5720 and 10 µmol/L linopirdine (n=7); (E) 1 µmol/L CE3F4 (n=6) or 1 µmol/L HJC0350 (n=6) alone, and (F) 1 µmol/L CE3F4 and 1 µmol/L HJC0350 in combination (n=5). A Bonferroni post hoc test was performed after a 2-way analysis of variance (ANOVA). *P<0.05 and ***P<0.001. Results were deemed nonsignificant when P>0.05. PKA indicates protein kinase A.
has been debated intensively for many years. 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 proximity ligation assays puncta between Kv7.4 and both Rap1a and Rap2—small G proteins downstream of EPAC. Rap1 proteins have crucial effects within the vasculature, with knockout of a singular isoform resulting in gross cardiovascular defects, such as defective platelet function, angiogenesis, and hypertension, while Rap2 proteins are involved in arteriogenesis. Both Rap1a and Rap 2 are involved in membrane translocation of cellular components in the vasculature, 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 laboratory that Kv7 channels mediate isoproterenol-dependent relaxations in RA. Similar to the MA, we now report that this is in combination with BK Ca channel activity because inhibitors of either channel attenuated the relaxation. However, we did not see an additive effect of BKCa and Kv7 channel inhibition as we had in the MA. The reason for this is unclear, but we speculate that it is because of reduced permeability in RA, which is a much tougher vessel than the MA. We further show that unlike the MA, this relaxation is dependent on PKA, and 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.

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we see an increase in Kv7.4-AKAP localization in RA myocytes after isoproterenol stimulation. AKAP is known to form multifunctional signaling complexes and has been shown to be regulatory to both cardiac (Kv7.1) and neuronal (Kv7.2, 7.3 and 7.5) Kv7 channels. Here, we demonstrate that this could also be an important regulatory mechanism of Kv7 channels in the vasculature, a finding that warrants further study. We investigated the interactions with Kv7.4 because of its crucial role in the regulation of the vasculature, as highlighted by the impact of KCNQ4 knockdown, 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, and Kv7.5 has been shown to be under the control of distinct, compartmentalized molecules, indicating 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, and Kv7.5 has been shown to be under the control of distinct, compartmentalized molecules, indicating that.

EPAC-dependent signals involve Kv7 channels in the vasculature and provide an important regulatory mechanism of Kv7 channels in the vasculature, a finding that warrants further study. We investigated the interactions with Kv7.4 because of its crucial role in the regulation of the vasculature, as highlighted by the impact of KCNQ4 knockdown, 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, and Kv7.5 has been shown to be under the control of distinct, compartmentalized molecules, indicating 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, and Kv7.5 has been shown to be under the control of distinct, compartmentalized molecules, indicating that.

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**Disclosures**

None.

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**Highlights**

- **Kv7 channels contribute to EPAC (exchange protein directly activated by cAMP)-dependent signals in both rat renal and mesenteric arteries.**
- **EPAC signaling is involved in isoproterenol-mediated vasorelaxations of the rat mesenteric artery, but in the renal artery, this is a predominantly protein kinase A/A kinase anchoring protein–dependent response.**
- **Isoproterenol stimulation results in increased localization of Kv7.4 with Rap1a and Rap2—EPAC effectors—in mesenteric arteries, but not in the renal artery. Here, we see an increased localization of Kv7.4 and A kinase anchoring protein after stimulation.**
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Kv7 channel activation underpins EPAC-dependent relaxations of rat arteries

Materials and Methods

Animals Male Wistar rats (175-225g) were culled by cervical dislocation in accordance with the Animals (Scientific Procedures) Act (1986). Renal and mesenteric arteries were dissected of adherent fat and connective tissue and stored on ice in a physiological saline solution (PSS) containing (in mmol/L): 4.5 KCl, 120 NaCl, 1.2 MgSO$_4$

7H$_2$O, 1.2 NaH$_2$PO$_4$ 2H$_2$O, 25 NaHCO$_3$ 5 D-Glucose and 1.25 CaCl$_2$.

Myography Renal and mesenteric arteries were mounted in myographs (Danish Myograph Technologies) for isometric tension recording. Chambers were filled with PSS, aerated with 95% O$_2$/ 5% CO$_2$ at 37°C. After normalisation to 90% of vessel diameter at 100mg Hg, a dose response to the vasoconstrictors methoxamine (renal) and U46619 (mesenteric) was performed to establish an approximate EC$_{80}$ for each individual arterial segment. Vessels were then washed and re-constricted to the approximate EC80 for further experiments to assess the effect of the maximal relaxant effect of the specific cell-permeable EPAC activating analogue 8-pCPT-2Me-cAMP-AM (Tocris, UK) within 5 minutes of application in the absence and presence of ion channel and cell signalling modulators. A separate set of experiments investigated the dose response to isoproterenol in the presence and absence of various ion channel and cell signalling modulators (see Reagents for specific details).

Electrophysiology Dissected renal and mesenteric arteries were used for isolation of individual myocytes. Vessels were bathed for 10 minutes in a nominally Ca$^{2+}$ free solution (in mmol/L: 6 KCl, 120 NaCl, 1.2 MgCl$_2$, 12 D-glucose and 10 HEPES, pH 7.4 with NaOH). Vessels were then incubated at 37°C for 17 (MA) or 23 (RA) mins in Ca$^{2+}$ free solution containing in mg/ml: 1.5 collagenase, 0.75 thermolysin, 1 trypsin inhibitor and 1 bovine serum albumin. Vessels were then washed in Ca$^{2+}$ free solution for 10 mins and then triturated to liberate myocytes. The cell solution was plated on 13mm coverslips and supplemented with an equivalent volume of 2.5mmol/L Ca$^{2+}$ solution to allow the cells to adhere. HEK 293 cells stably transfected with Kv7.4 were maintained in modified Eagle’s medium solution containing 10% foetal bovine serum, 1% penicillin/streptomycin, 1% l-glutamine, 1% non-essential amino acids and 1% sodium pyruvate in an incubator with 5% CO$_2$. Cells were briefly trypsinised on the day of experiments and plated on 13mm coverslips in media at room temperature for 30mins and were then stored in the fridge for use within 8 hours. All current recordings were made using AXOpacht 200B amplifier (Axon Instruments) at room temperature. Whole cell electrical signals were generated and digitized at 1kHz using a Digidata 1322A hosted by a PC running pClamp 9.0 software (Molecular Devices). For recordings cells were placed in an external solution containing (in mmol/L): 140 NaCl, 4 KCl, 2 CaCl, 1 MgCl$_2$ and 10 HEPES. For renal and mesenteric myocyte recording, the external solution was supplemented with 1µmol/L paxilline. Patch pipettes with a resistance of 4-12 MΩ were filled with a pipette solution containing (in mmol/L): 110 K gluconate, 30 KCl, 0.5 MgCl$_2$, 5 HEPES, 0.5 EGTA and 1 Na$_2$ATP. Cells were held at -60mV and currents amplitude was monitored by application of a test pulse to 40mV every 20s. To generate current-voltage relationships a voltage step protocol was used from a holding potential of -60mV testing a range of voltages from -70 to 40mV in 10mv increments at 15s intervals. Drugs were applied in the external solution using a bath perfusion system.

End Point PCR Total RNA was extracted from HEK-Q4 cells and human aortic smooth muscle cells (HASMC) using RNeasy micro-kit (Qiagen, Manchester, UK) according to manufacturer’s instructions, and reverse transcribed as described previously (Stott et al, Hypertension 2015). End-point PCR were carried out in a Multigene thermo-cycler (Appleton Woods, Birmingham, UK) using HotStart Maxima Taq polymerase (Thermo Scientific, Paisley, UK). RT-negative samples and no template controls were run alongside all
reactions to assess contamination. The following cycling conditions were used: initial activation at 94 °C for 5 min, followed by 40 cycles of 95 °C for 1 min, 56 °C for 30 sec and 72 °C for 1 min, and a final extension at 72 °C for 10 min. Primers used were (5'-3'):

EPAC1 For- TCTACTCACCAAGAGGAGC; EPAC1 Rev- CCAGCCCCACCTCATGGTTTC; EPAC2 For- CCTGACAAGGAACACACACCT; EPAC2 Rev- GACTTGCCACATGGCAAACAG; GAPDH For- GAGTCAACCGATTTGGGTCTG; GAPDH Rev- TTTGATTTCAGGGATCTCG.

Proximity Ligation Assay Renal and mesenteric myocytes were isolated as above and the cell solution was plated on 13mm coverslips in a 24 well plate and supplemented with an equivalent volume of 2.5mmol/L Ca\(^{2+}\) solution to allow the cells to adhere. 1ml of solution containing 2.5mmol/L CaCl\(_2\) was then added to each well and cells were placed in an incubator (37 °C, 5% CO\(_2\)) for 30 minutes to equilibrate. Cells were then stimulated with 1µmol/L isoproterenol or H\(_2\)O control for 90s then immediately fixed with 3% PFA on ice for 20 mins and stored in PBS at 4 °C. For the proximity ligation assay, cells were permeabilised with 0.01% Triton X for 5 mins. The Duolink in situ PLA detection kit (Sigma-Aldrich, UK) was used to detect single molecule interactions for Kv7.4 (mouse monoclonal (N43/6, RRID: AB_2131828, UC Davis/NIH NeuroMab Facility) and rabbit polyclonal (ab65797, Abcam)) and the cellular signalling components AKAP 150 (goat polyclonal (sc-6446, Santa Cruz Biotechnology)), Rap1a (mouse monoclonal (NBP2-22527, Novus Biologicals)) Rap1b (rabbit monoclonal (#2326, Cell Signaling Technology) and Rap2 (mouse monoclonal (sc-136138, Santa Cruz Biotechnology)). All antibody combinations were tested in untransfected HEK293 cells to determine the ‘background’ puncta produced by each combination in a cell system (Supplementary Figure 4C). Experiments were performed as per manufacturer’s instructions; primary antibodies were incubated at 1:200 overnight at 4 °C. Red fluorescent oligonucleotides produced as the end product of the procedure were visualised using a Zeiss Confocal LSM 510. Images were analysed using Image J software using the particle detector tool. The number of puncta per cell was calculated as the average of two mid sections in each cell.

Reagents 8-pCPT-2Me-cAMP-AM, Linopirdine, KT5720, PKI, HJC0350 and CE3F4 were all purchased from Tocris, UK. ESI-09 was obtained from BioLog, Germany, Ht31 from Promega and Paxilline from Cambridge Bioscience, UK. All other reagents were from Sigma-Aldrich UK.

Data Analysis

All statistical analyses were performed in GraphPad Prism. A one-way ANOVA multiple comparisons test with a Bonferroni post-hoc analysis was used for 8-pCPT-2Me-cAMP-AM relaxation studies and PLA data. A two-way ANOVA with Bonferroni post-hoc analysis was used for isoproterenol relaxation studies and all electrophysiological studies.
Kv7 channel activation underpins EPAC-dependent relaxations of rat arteries

Supplemental Figure I

Relaxations to 5µmol/L 8-pCPT-2Me-cAMP-AM in the mesenteric (A) and renal (B) arteries in control and in the presence of 300nmol/L ESI-09 or 10µmol/L HMR 1556. Statistical significance was determined by one-way ANOVA. p<0.01 is denoted (**). Results were deemed non-significant when p>0.05
Supplemental Figure II

(A) Original recordings of 2 segments of MA showing initial vessel responses to 300nmol/L U44619 and 10µmol/L carbachol. Vessels were then washed and reconstricted with U46619 before addition of 5µmol/L 8-p-CPT-2Me-cAMP-AM. (B) Mean data of % relaxation to 5µmol/L 8-p-CPT-2ME-cAMP-AM in endothelial intact, and endothelial denuded vessels. Results were deemed non-significant when p>0.05
Supplemental Figure III

Representative end-point PCR showing EPAC1, EPAC2 and GAPDH mRNA expression in human aortic smooth muscle cells (HASMC, positive control) and HEK-Q4 cells; no template control (NTC) are also shown on the left. Expected amplicon size were (in bp): EPAC1=220; EPAC2=270; GAPDH=238.
Supplemental Figure IV

Dose dependent relaxations of MA with isoproterenol (100pmol/L-1µmol/L) in the presence of (A) 10µmol/L glibenclamide or (B) 1mmol/L 4-aminopyridine. Data was analysed by two-way ANOVA with Bonferroni post-hoc analysis. p<0.01 is denoted (**) and p<0.001 is denoted (**). Results were deemed non-significant when p>0.05
Supplemental Figure 5

Dose dependent relaxations of MA with isoproterenol (100pmol/L-1µmol/L) in the presence of 1µmol/L PKI. Data was analysed by two-way ANOVA with Bonferroni post-hoc analysis. Results were deemed non-significant when p>0.05

% Initial Contraction

100
50
0

10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

[Isoproterenol]

Control
1µmol/L PKI
Supplemental Figure 6

The number of PLA puncta detected by Kv7.4 and Rap1b antibodies in mesenteric (A) and renal (B) arterial myocytes in control and after stimulation with 1µmol/L isoproterenol. (C) Antibodies were tested on untransfected HEK23 cells to determine the 'background' number of puncta produced by the antibody pair in a control system. Results were analysed using a one-way ANOVA. Results were deemed non-significant when p<0.05.
Supplemental Figure 7

Relaxations produced in MA by 1μmol/L isoproterenol in control and endothelium denuded vessels. Results were analysed using a one-way ANOVA. Results were deemed non-significant when p<0.05