Kv7 channel activation underpins EPAC-dependent relaxations of rat arteries

Running Title: Kv7 channels contribute to EPAC relaxations

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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.

Abbreviations: A-kinase anchoring protein (AKAP); large conductance Ca^{2+} activated K^+ channel (BK_{Ca}); cyclic AMP (cAMP); exchange protein directly activated by cAMP (EPAC); guanine nucleotide exchange factor (GEF); ATP-sensitive K^+ channel (K_{ATP}); mesenteric artery (MA); protein kinase A (PKA); renal artery (RA)

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 artery[1]. 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 (adenosine[2] and forskolin[3] in coronary artery, CGRP[4] and forskolin[5] 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 vasorelaxation[6]. 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 (5µmol/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 process[18], we inhibited this channel with 1µmol/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 1µmol/L (n=6) and 10µmol/L (n=5), but an additive effect with 1µmol/L paxilline was not seen (n=4).

Relaxations to 5µmol/L 8-pCPT-2Me-cAMP-AM were also tested in the presence of the Kv7.1 inhibitor HMR1556 (10µmol/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 5µmol/L 8-pCPT-2Me-cAMP-AM in MA endothelium denuded segments, which was assessed by the vasorelaxant response to 10µmol/L carbachol. Vessels with <20% relaxation to 10µmol/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 10µmol/L linopirdine (in the presence of 1µmol/L paxilline) before and after application of 1µmol/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 1µmol/L 8-pCPT-2Me-cAMP-AM (1.6 ±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 V1/2

from -7.2mV in control to -17.5mV after addition of 1µmol/L 8-CPT-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 10µmol/L linopirdine (**Figure 2A,** n=9) or 1µmol/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 characterised[1]. In MA, blockade of K_{ATP} channels (10µmol/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 1µmol/L KT 5720 (**Figure 3C,** n=10) or 1µmol/L PKI (Supplementary Figure 5), had no effect on isoproterenol relaxations. Linopirdine (10µmol/L) inhibited the isoproterenol relaxation in the presence of 1µmol/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 1µmol/L CE3F4 (EPAC1 inhibitor) and 1µmol/L HJC0350 (EPAC2 inhibitor). Individually neither had any effect on isoproterenol relaxations (**Figure 3E,** n=6), but in combination they produced a significant impairment (**Figurer 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 1µmol/L KT5720 (n=9) or 1µmol/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, 10µmol/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 1µmol/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 Ca^{2+} desensitisation was identified[13]. Subsequently, EPAC dependent relaxation of rat mesenteric arteries was shown to involve BK_{Ca} channel activation[18]. Whilst EPAC had previously been shown to negatively regulate vascular K_{ATP} channels[31], 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} channels[32], 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 5µmol/L 8-CPT-2Me-cAMP, this was not correlated to the responsiveness to 10µmol/L carbachol. A similar trend, or lack thereof, was seen with the responsiveness of the MA to 1µmol/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 vasculature[15], with knockout of a singular isoform resulting in gross cardiovascular defects such as defective platelet function[44], angiogenesis[45, 46] and hypertension[14], whilst Rap2 proteins are involved in arteriogenesis[17]. 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 artery[1]. 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 MA[51]. We confirm here previous reports that isoproterenol dependent relaxations in MA are primarily PKA-independent[37] 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 cells[22].

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

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Disclosures

None

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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

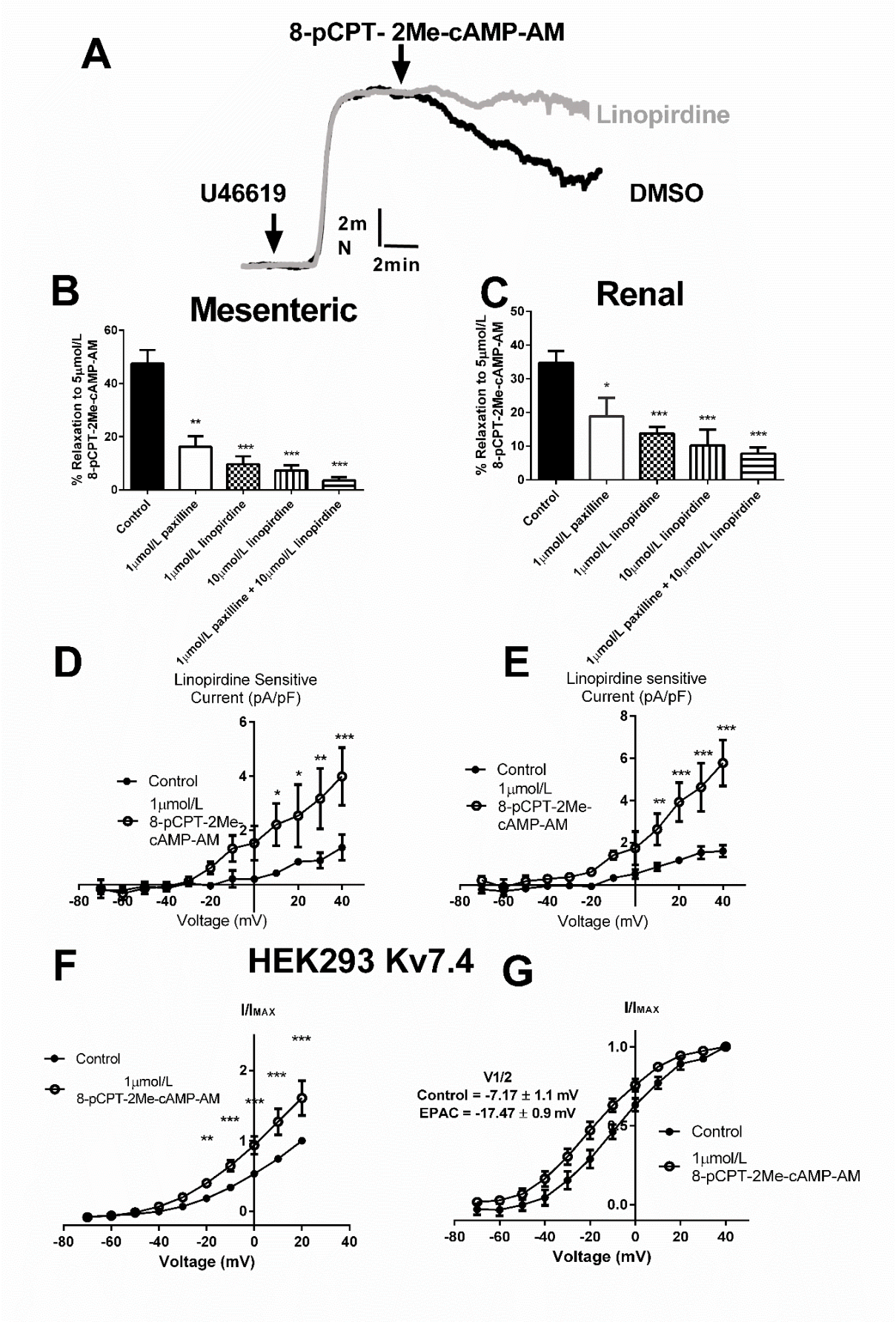

Figure 1 – EPAC dependent relaxations of MA and RA involve Kv7 channels

(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 1µmol/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 $p>0.05$.

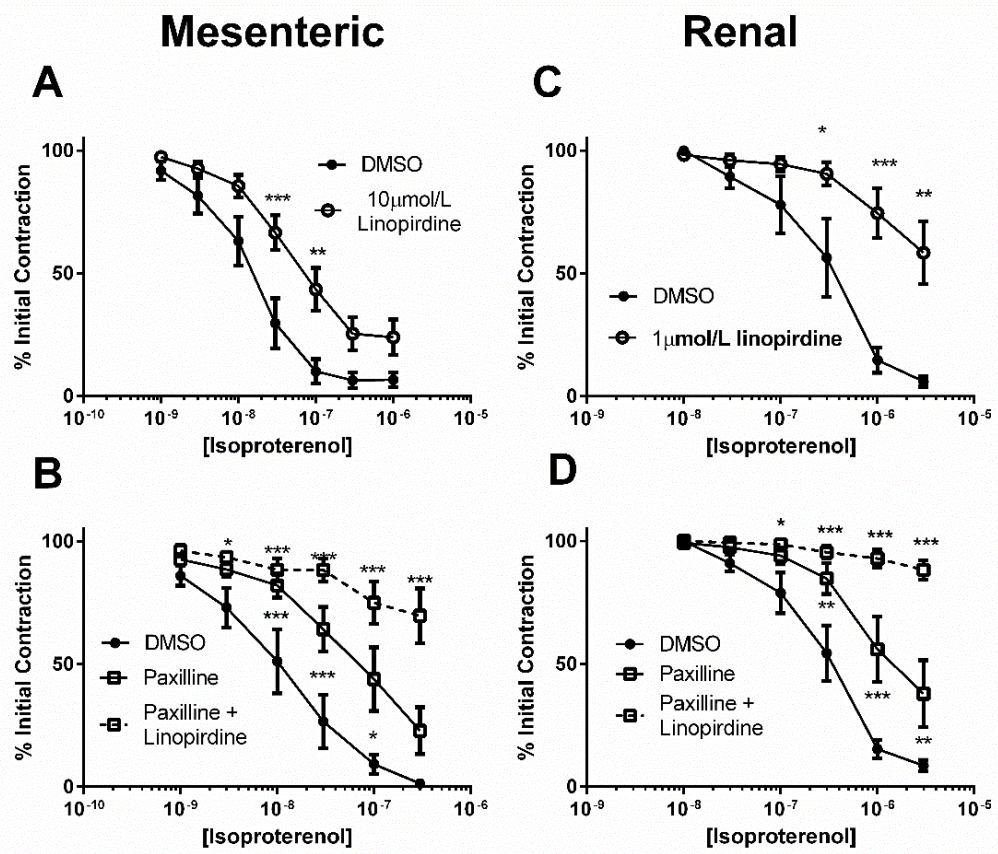

Figure 2 – Isoproterenol relaxations of MA and RA involve Kv7 channels

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 $p>0.05$.

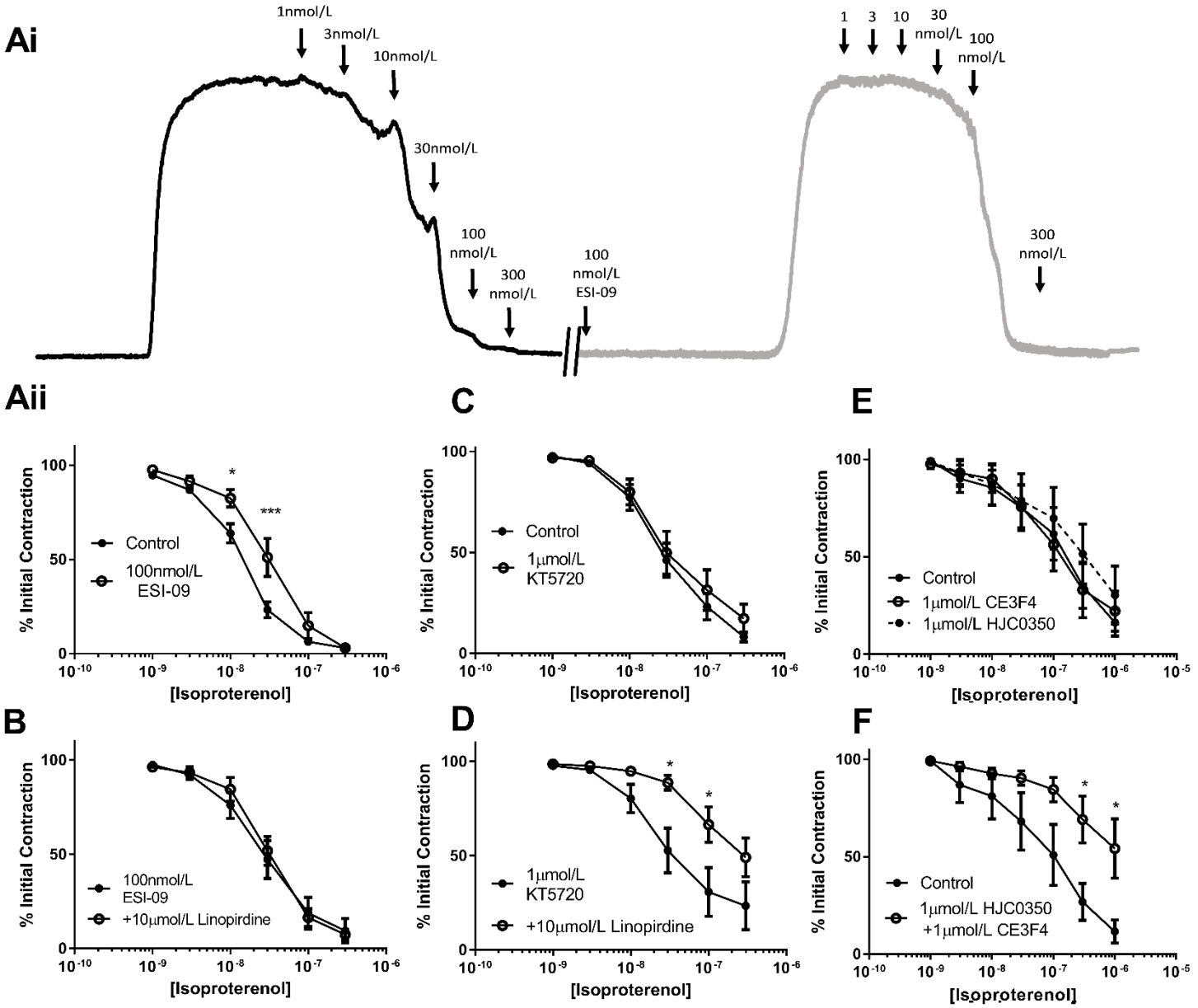
Ai
1nmol/L
3nmol/L
10nmol/L
30nmol/L
100 nmol/L
300 nmol/L
100 nmol/L ESI-09
1
3
10
30 nmol/L
100 nmol/L
300 nmol/L
Aii
B
C
D
E
F
% Initial Contraction
[Isoproterenol]
Control
100nmol/L ESI-09
+10μmol/L Linopirdine
1μmol/L KT5720
1μmol/L CE3F4
1μmol/L HJC0350
1μmol/L HJC0350 +1μmol/L CE3F4

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 10µmol/L linopirdine (n=6), (C) 1µmol/L KT5720 (PKA inhibitor, n=10), (D) 1µmol/L KT5720 and 10µmol/L linopirdine (n=7), (E) 1µmol/L CE3F4 (n=6) or 1µmol/L HJC0350 (n=6) alone and (F) 1µmol/L CE3F4 and 1µmol/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 $p>0.05$.

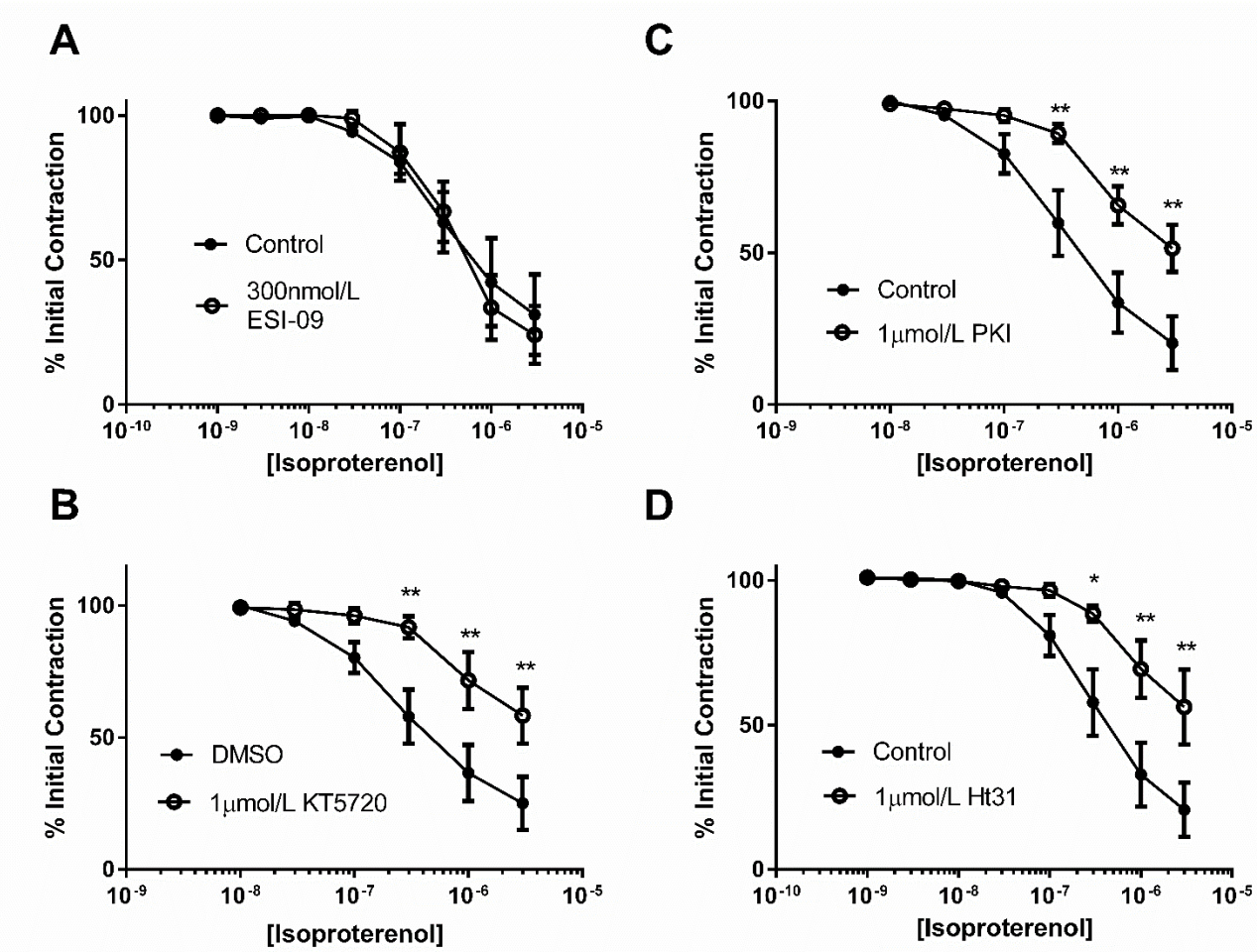

Figure 4 – EPAC does not contribute to isoproterenol relaxations in RA

Dose dependent relaxations of RA by isoproterenol (10nmol/L - 3μmol/L) in the presence of (A) 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 $p>0.05$.

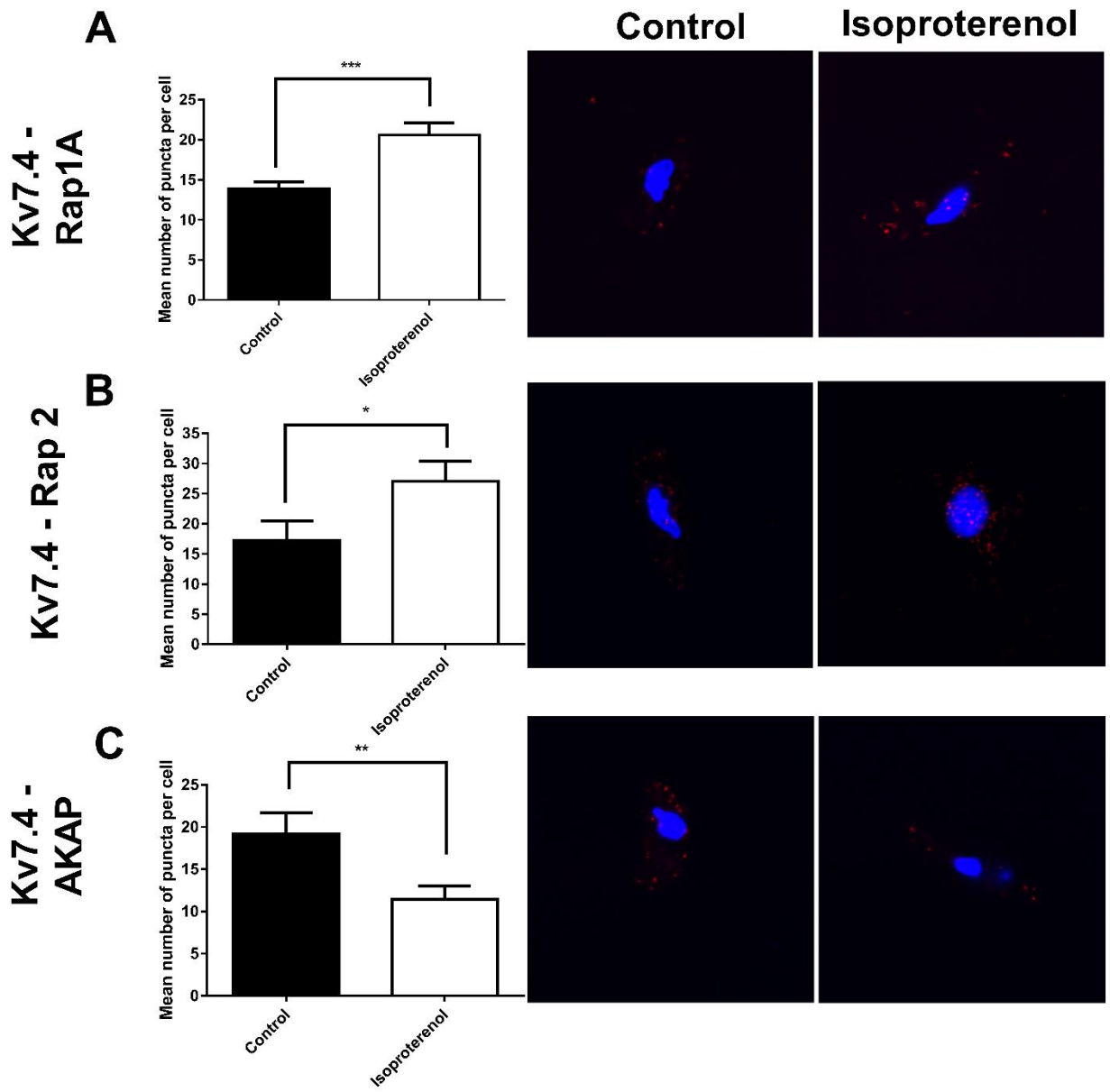

Figure 5 – Isoproterenol stimulation in MA alters localisation of Kv7.4 with signalling molecules

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 1µmol/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 (***)

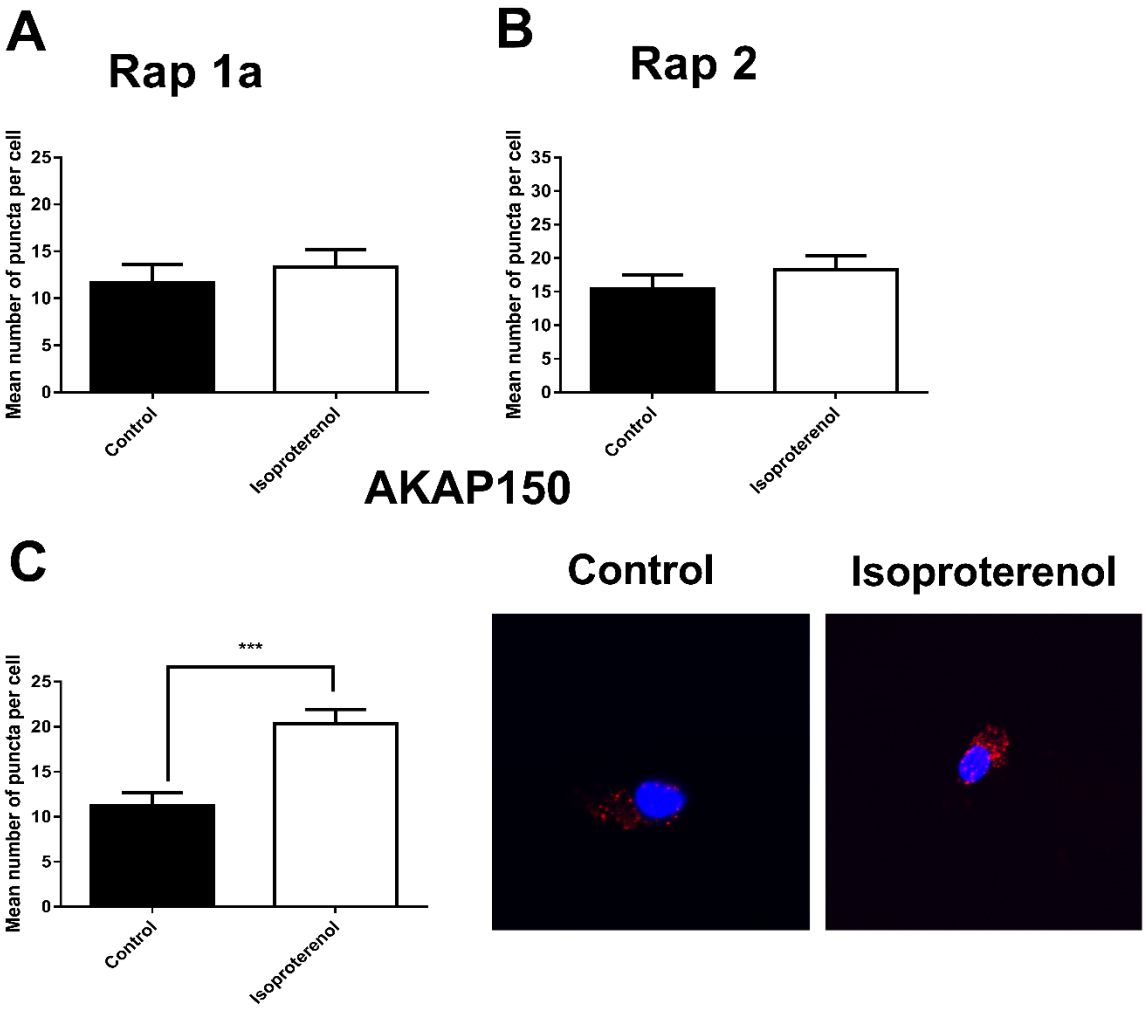

Figure 6 – Isoproterenol stimulation in RA myocytes alters localisation of Kv7.4 with signalling molecules

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 $p>0.05$.