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\textsubscript{4}·7H\textsubscript{2}O, 1.2 NaH\textsubscript{2}PO\textsubscript{4}·2H\textsubscript{2}O, 25 NaHCO\textsubscript{3} 5 D-Glucose and 1.25 CaCl\textsubscript{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\textsubscript{2} 5% CO\textsubscript{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\textsubscript{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\textsuperscript{2+} free solution (in mmol/L: 6 KCl, 120 NaCl, 1.2 MgCl\textsubscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{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 AXOpatch 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\textsubscript{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\textsubscript{2}, 5 HEPES, 0.5 EGTA and 1 Na\textsubscript{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 RNAeasy 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- TCTACTCACCCAAGAGGAGC; EPAC1 Rev- CCAGCCCACCTTCTATGTTTC; EPAC2 For- CCTGACAAGGAGAACACACCT; EPAC2 Rev- GACCTGCCACATGCCAACAG; GAPDH For- GAGTCAACGGATTTGGGTGTGTC; GAPDH Rev- TTGATTGAGGAGGATCTCG.

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.