Functional up-regulation of KCNA gene family expression in murine mesenteric resistance artery smooth muscle

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This study focused on the hypothesis that KCNA genes (which encode K\textsubscript{V}\textalpha\textsubscript{1} voltage-gated K\textsuperscript{+} channels) have enhanced functional expression in smooth muscle cells of a primary determinant of peripheral resistance—the small mesenteric artery. Real-time PCR methodology was developed to measure cell type-specific \textit{in situ} gene expression. Profiles were determined for arterial myocyte expression of RNA species encoding K\textsubscript{V}\textalpha\textsubscript{1} subunits as well as K\textsubscript{V}\textbeta\textsubscript{1}, K\textsubscript{V}\textgamma\textsubscript{9.3}, BK\textsubscript{Ca}\textalpha\textsubscript{1} and BK\textsubscript{Ca}\textbeta\textsubscript{1}. The seven major KCNA genes were expressed and more readily detected in endothelium-denuded mesenteric resistance artery compared with thoracic aorta; quantification revealed dramatic differential expression of one to two orders of magnitude. There was also four times more RNA encoding K\textsubscript{V}\textalpha\textsubscript{2.1} but less or similar amounts encoding K\textsubscript{V}\textbeta\textsubscript{1}, K\textsubscript{V}\textgamma\textsubscript{9.3}, BK\textsubscript{Ca}\textalpha\textsubscript{1} and BK\textsubscript{Ca}\textbeta\textsubscript{1}. Patch-clamp recordings from freshly isolated smooth muscle cells revealed dominant K\textsubscript{V}\textalpha\textsubscript{1} K\textsuperscript{+} current and current density twice as large in mesenteric cells. Therefore, we suggest the increased RNA production of the resistance artery impacts on physiological function, although there is quantitatively less K\textsuperscript{+} current than might be expected. The mechanism conferring up-regulated expression of KCNA genes may be common to all the gene family and play a functional role in the physiological control of blood pressure.

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Voltage-gated K\textsuperscript{+} channels (K\textsubscript{V} channels) are encoded by the large KCNx gene set and have a wide range of properties such that the precise expression profile is instrumental in governing the electrical phenotype of a cell and its response to extrinsic factors. Specific K\textsubscript{V} channel subtypes have established functional roles in shaping action potentials in cardiac muscle, neurones and other cell types (Coetzee et al. 1999; Nerbonne et al. 2001; MacDonald et al. 2001; Catterall et al. 2002; Song, 2002).

The roles of K\textsubscript{V} channels in shaping action potentials may make them surprising functional elements of arterial smooth muscle cells because these cells often exhibit only tonic electrical activity. However, there is substantial evidence to support a major role for this channel type. As early as 1980 it was observed that 4-aminopyridine (a blocker of many K\textsubscript{V} channels) caused tonic depolarization of guinea-pig pulmonary artery (Hara et al. 1980). Although effects of low concentrations of 4-aminopyridine were prevented by the \textalpha-adrenoceptor antagonist phentolamine (suggesting an effect via noradrenaline release from nerve terminals) effects of higher concentrations showed resistance to phentolamine, consistent with the hypothesis that K\textsubscript{V} channels also have a direct inhibitory effect in vascular smooth muscle cells—acting as a tonic physiological break on activation of voltage-gated Ca\textsuperscript{2+} channels (Knot & Nelson, 1995; Cheong et al. 2001a,b, 2002). Numerous patch-clamp studies have shown K\textsubscript{V} channel activity is common in freshly isolated contractile vascular smooth muscle cells in the physiological voltage range of −50 to 0 mV (Okabe et al. 1987; Beech & Bolton, 1989; Gelband & Hume, 1992; Robertson & Nelson, 1994).

As well as K\textsubscript{V} channels, contractile vascular smooth muscle cells contain large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels—the BK\textsubscript{Ca} channels, which have \textalpha-subunits encoded by the Slo1 gene. Expression of BK\textsubscript{Ca} channels is modulated in vascular development, ageing and hypertension (Marijic et al. 2001; Cox & Rusch, 2002) and there is an established role in hyperpolarizing signals in response to elementary intracellular Ca\textsuperscript{2+} release events (Patterson et al. 2002). Suppression of BK\textsubscript{Ca} channel function by disruption of the gene encoding its \textbeta\textsubscript{1}-subunit causes elevation of blood pressure (Patterson et al. 2002). Physiologically, K\textsubscript{V} channels also have an important role.
terminal arterioles under basal conditions $K_v$ channels activate with a more negative threshold than BKCa channels and inhibition of $K_v$, but not BKCa, channels evokes vasoconstriction; induction of pretone with endothelin-1 is necessary to confer sensitivity to blockers of BKCa and $K_v$ channels (Cheong et al. 2002). There are changes in $K_v$ channel gene expression and associated $K^+$ currents in rat models of hypertension, and $K_v$ channels are modulated by many vasoactive substances (Martens & Gelband, 1996; Smirnov & Aaronson, 1996; Berger et al. 1998; Platts et al. 1998; Ren et al. 1999; Cox et al. 2001; Hayabuchi et al. 2001; Liu et al. 2001; Shimoda et al. 2001; Gupte et al. 2002; Heaps & Bowles, 2002; Michelakis et al. 2002; Platoshyn et al. 2002; Irvine et al. 2003).

There are 12 families of KCNx genes encoding $K_v$ channel subunits (Coetzee et al. 1999; Nerbonne et al. 2001; Catterall et al. 2002). Reverse transcriptase PCR studies indicate a large number of these genes are transcribed in blood vessels. Whether these RT-PCR signals originate from smooth muscle cells, endothelial cells, neurones or other cell types is not always completely clear. Nevertheless, the expressed genes include eight KCNA genes encoding $K_v\alpha_{1.1-1.8}$, KCNB genes encoding $K_v\alpha_{2.1-2.2}$, KCNC genes encoding $K_v\alpha_{3.1-3.4}$, KCND genes encoding $K_v\alpha_{4.1-4.3}$, the KCNH2 gene encoding $K_v\alpha_{11.1}$, the KCNQ1 gene encoding $K_v\alpha_{7.1}$, and the KCNS3 gene encoding $K_v\gamma_{9.3}$ (Patel et al. 1997; Archer et al. 1998; Yuan et al. 1998; Xu et al. 1999; Lang et al. 2000; Osipenko et al. 2000; Cheong et al. 2001a,b; Thorneloe et al. 2001; Ohya et al. 2002; Fergus et al. 2003; Ohya et al. 2003). Patch-clamp studies indicate heterogeneity in $K_v$ currents of vascular smooth muscle cells (Archer et al. 1996; Smirnov et al. 2002). Thus KCNx genes may be expressed differently in different blood vessels, and presumably for some purpose. It may not be enough to ask if there is all-or-nothing expression – quantitative differences may be critical. Methodological limitations have hindered progress in this area and so we have developed real-time RT-PCR assays to quantify physiological expression of RNA species encoding $K^+$ channel subunits in arterial smooth muscle cells in situ. These assays were used to determine expression profiles of KCNx genes in a conduit artery and a resistance artery. We focused particularly on $K_v\alpha_{1}$-encoding genes because of data indicating these genes have an important function in arterioles and other resistance arteries (Cheong et al. 2001a,b; Lu et al. 2002; Pozeg et al. 2003; Albarwani et al. 2003). Thus we sought to test the hypothesis that discrete KCNx genes, especially KCNA genes, are differentially expressed in resistance arteries – not in absolute terms, but quantitatively – and that this translates to functional signals. The mesenteric artery used in this study contributes a resistance at least a thousand times higher than the conduit of the thoracic aorta.

**Methods**

**Dissection of tissues**

Eight-week-old male C57/BL6 mice were killed by CO$_2$ asphyxiation and cervical dissociation in accordance with the Code of Practice, UK Animals (Scientific Procedures) Act 1986. The thoracic aorta and mesenteric artery (approximately 0.75 and 0.2 mm external diameter, respectively) were removed and placed in ice-cold Hanks’ solution. Fat was removed completely by dissection and blood cells were flushed from the lumen with Hanks’ solution. In some cases endothelium was removed by brief luminal perfusion with 0.1% (v/v) Triton X-100 in water, and the adventitia were removed (‘medial layer only’) by fine dissection. For the isolation of brain RNA, small cubes of cerebral cortex were cut in ice-cold Hanks’ solution and snap-frozen.

**RNA isolation and RT-PCR**

Individual aortae or six mesenteric arteries were snap frozen on liquid N$_2$ immediately after dissection. RNA was extracted using Tri reagent (Sigma), 250 µg glycogen added and cells disrupted using a homogenizer. RNA precipitates were re-suspended in water and digested with 6 units DNase I (Ambion) for 60 min at 37°C. RNA was quantified using Ribogreen (Molecular Probes). In all experiments 0.6 µg total RNA was reverse transcribed at 65°C for 30 min in 20 µl reactions using 6 units C. therm polymerase (Roche) and gene-specific primers (1 µM). From the same sample, 0.6 µg RNA was used as a genomic DNA control in a reaction without reverse transcriptase. cDNA templates were purified on QIAquick columns (Qiagen) and quantitative real-time PCR (Bustin, 2000; Peirson et al. 2003) performed using SYBR Green I on a Roche Lightcycler. DNA amplification was for 35–40 cycles with an initial 10 min at 95°C followed by 10 s at 95°C, 6 s at 55°C, and 14 s at 72°C. All PCR primers are given in Table 1. Fluorescence was acquired at 72°C. After PCR cycling, melt-curves were generated by temperature ramps from 65 to 95°C. PCR cycle crossing-points ($C_p$) were determined by fit-points methodology (Lightcycler software 3.5). PCR efficiency ($E$) was calculated as shown in Fig. 1, where $E = 10^{(-1/\text{slope})}$. Relative abundance of target RNA was calculated from ($E_{\beta\text{-actin} \ C_p}$)/($E_{\text{target} \ C_p}$). β-Actin RNA abundances were nevertheless not different.
between samples (Cp values were: aorta intact, 22.2 ± 0.5; endothelium-denuded aorta, 22.9 ± 0.3; media layer of aorta, 21.9 ± 0.3; endothelium-denuded mesenteric artery, 22.2 ± 0.5; n = 4, P > 0.05, ANOVA, e.g. Fig. 1E). All quantified amplicons had identity confirmed by direct sequencing (Lark, UK).

**Western blotting and immunostaining**

Protocols were standard and largely as previously described (Cheong et al. 2001b). Lysates for Western blots were prepared from intact aortae. Antibodies were rabbit polyclonal anti-rat Kvα1.1 (458–475) or anti-rat Kvα1.3 (456–474) (1: 500; Cheong et al. 2001b) or mouse monoclonal Cy3-conjugated smooth muscle α-actin antibody (1: 200; Sigma). Control reactions were run in parallel in the absence of primary antibody. Sections were mounted in Vectashield (Vector Laboratories) with or without 4’,6’-diamino-2-phenylindole hydrochloride (DAPI). Staining was viewed using a Plan Apochromat × 63 objective (NA 1.4) on a Zeiss Axiovert microscope equipped with a 12-bit charge-coupled device camera (Orca-ER, Hamamatsu, Japan). Images were sampled at five focal planes separated by 0.5 µm, background subtracted, and haze removed by a deconvolution algorithm (Openlab software, Improvision; Coventry, UK).

**Wire myography**

Vessels were mounted on two 40-µm diameter wires for isometric tension recording in a 410A dual wire myograph.

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**Figure 1. Expression profiling by real-time RT-PCR**

A–C, the method using aortic β-actin as an example. A, end-point PCR ‘melt-curve’ showing change of SYBR Green fluorescence with temperature and (inset) the PCR products on an agarose gel (predicted size, Table 1); + and – indicates plus and minus reverse transcriptase reaction; M, DNA markers (bp). B, SYBR Green fluorescence against PCR cycle for the reaction in A and dilutions of cDNA as indicated. C, for triplicate reactions on a cDNA sample, mean ± S.E.M. Cp (i.e. intersection with the horizontal dotted line shown in B). The fitted line has a correlation coefficient of 0.99. D, tension in parallel aortic rings – one with endothelium removed. PE, phenylephrine (10 µM); and ACh, acetylcholine (1 µM). E, real-time PCR for aortic RNA encoding eNOS relative to β-actin (**P < 0.01). F, sections of intact aorta and medial-layer stained with smooth muscle α-actin antibody (red) and DAPI (blue, showing all cell nuclei). Autofluorescence of elastic laminae is in green. Scale bar, 20 µm.
system (Danish Myo Technology, Denmark). The bath solution was at 36°C and gassed continuously with air–5% CO2. It contained (mm): NaCl, 125; KCl, 3.8; NaHCO3, 25; MgSO4, 1.5; KH2PO4, 1.2; d-glucose, 8; CaCl2, 1.2; EDTA, 0.02.

**Patch-clamp recording**

Aortae were incubated in Hanks’ solution containing 0.15 mg ml−1 collagenase, 0.1 mg ml−1 protease, 0.13 mg ml−1 hyaluronidase and 475 Units elastase for 1 h at 4°C followed by 15 min at 37°C. Mesenteric arteries were incubated for 30 min at 37°C in 1 mg ml−1 collagenase, 0.5 mg ml−1 protease and 1 mg ml−1 hyaluronidase. Enzymes were removed and tissue agitated with a Pasteur pipette. Myocytes were used within 8 h. Recordings were made at room temperature using conventional whole-cell recording. Signals were amplified and sampled using an Axopatch 200B amplifier and pCLAMP 8 software (Axon Instruments). Signals were filtered at 1 kHz and sampled at 2 kHz. Patch pipettes had resistance of 3–5 MΩ. Hanks’ solution contained (mm): NaCl, 137; KCl, 5.4; CaCl2, 0.01; Na2HPO4, 0.34; K2HPO4, 0.44; d-glucose, 8; Hepes, 5. The bath solution for Kv current contained (mm): NaCl, 135; KCl, 5; d-glucose, 8; Hepes, 10; MgCl2, 4 (for BKCa current, 1.5 mM CaCl2 and 1.2 mM MgCl2 replaced 4 mM MgCl2). The patch pipette solution contained (mm): NaCl, 5; KCl, 130; Hepes, 10; Na2ATP, 3; MgCl2, 2; EGTA, 5 (for BKCa current, 0.05 EGTA replaced 5 mM EGTA). The pH of all solutions was titrated to pH 7.4 using NaOH.

**Heterologous expression of Kv channels**

*Xenopus laevis* were killed by an overdose of tricaine anaesthetic followed by destruction of the brain and spinal cord. cRNA was prepared from linearized cDNA templates encoding human Kvα1.6 (accession number NP002226), rat Kvα2.1 (accession number P15387) and rat Kγ9.3 (accession number O88759). Oocytes were prepared and injected with cRNA as previously described (Cheong et al. 2001a). For Kγ9.3 and Kvα2.1 coexpression studies cRNAs were injected in a ratio of 3 : 1. Recordings were made 2 days after injection using a GeneClamp 500 amplifier (Axon Instruments) for two-electrode voltage clamp (TEVC). The extracellular bathing solution during recordings was Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 10 mM Hepes, pH 7.4).

**Chemicals**

Correolide Compound C (′Correolide C, Merck) and penitrem A (Sigma) were prepared as 10 mM stocks in 100% DMSO. Arachidonic acid (Sigma) was prepared as a 100 mM stock in 100% ethanol. The concentration of DMSO/ethanol in experiments did not exceed 0.01% (v/v). All other chemicals were from Sigma (Poole, UK).

**Statistics**

All data are given as means ± s.e.m. and significance was determined by Student’s unpaired t test and ANOVA where P < 0.05 was accepted as significant. The n values indicate the number of independent experiments from separate animals except for the patch-clamp experiments where n is the number of cells from which recordings were made.

**Results**

Irrespective of the amplicon detection method, quantitative PCR depends on a highly specific reaction. Co-amplification of other products adversely affects the specific reaction, altering the PCR cycle cross-point (CP) that is the basis of the quantification. Specificity of all reactions was confirmed by melt-curve and gel electrophoresis (Figs 1A and 2). PCR efficiency also has major impact on quantification. Efficiency was determined for each experiment by dilution of the cDNA sample (Fig. 1B). Only straight-line fits of data with correlation coefficients ≥ 0.99 were accepted, showing the efficiency values were constant over the ranges of cDNA concentrations studied (e.g. Fig. 1C). To determine if signals originated from endothelial cells (Cheong et al. 2001a) we adopted a method for endothelium denudation (see Methods), which completely abolished relaxant responses to 1–100 μM acetylcholine without damaging contractile function (Fig. 1D, n > 10 intact and denuded aortae). This was combined with real-time PCR on the assumption that a shift in the CP value will occur if RNA originated from the endothelial cells. Analysis of RNA from vessels tested by myography showed that endothelium-denudation reduced the mean abundance of RNA encoding endothelial nitric oxide synthase (eNOS) by 92% (n = 3) (Fig. 1E). Isolation of the aortic media (i.e. removal of the adventitia as well as endothelium) reduced eNOS abundance by a further 7% (n = 3) (Fig. 1E). Staining of tissue sections confirmed endothelium and adventitia had been deleted (Fig. 1F).

Specific quantitative PCR reactions were developed for a range of RNA species (Fig. 2). RNAs encoding Kvα1.4, Kvα1.6 and Kvα1.7 were detected but abundances were low in aorta and quantitative reactions could not be developed (see below). Expression of protein was
confirmed for $K_v\alpha1.1$–6, $K_v\alpha2.1$ and $BK_{Ca}\alpha1$, $BK_{Ca}\beta1$ (Fig. 3, or data not shown). $K_V1.4$ and $K_V1.6$ proteins signals were weak, consistent with the RT-PCR analysis. RNA abundances for species encoding $K_v\alpha1.1$, $K_v\alpha1.2$, $K_v\alpha1.5$, $K_v\beta1$ and $K_v\alpha2.1$ were not significantly different between intact aorta and medial layer of aorta (Fig. 3A). In other words, removal of the endothelium and adventitia did not cause a shift in the $C_P$ values, suggesting RNA for these genes was not present at significant levels in endothelial or adventitial cells. Therefore, the measurements give the in situ RNA amounts in physiological aortic smooth muscle cells. By contrast, the abundance of RNA encoding $K_v\alpha1.3$ was lower in the medial layer-only samples, as well as in samples with only the endothelium removed (Fig. 3B). Therefore, $K_v\alpha1.3$ RNA occurs in endothelial cells and presumably at a higher concentration compared with the smooth muscle cells. It follows that $K_v\alpha1.3$ protein should be in endothelial and smooth muscle cells. This was confirmed by antibody labelling experiments (Fig. 3C). The RT-PCR data also indicate that $K_v\alpha1.1$ is in the smooth muscle. Consistent with this, $K_v\alpha1.1$ protein was only detected in the smooth muscle cells (Fig. 3C).

Comparisons were made between the abundances of RNA species encoding $K_v\alpha1.1$–3 and $K_v\alpha1.5$ in aorta and mesenteric artery (Fig. 4A and B). There was 20–140 times more RNA encoding $K_v\alpha1.1$–3 and $K_v\alpha1.5$ in the mesenteric artery. RNA encoding $K_v\alpha1.4$, 6 and 7 was more readily detected in mesenteric artery, but not quantified (Fig. 4C). Therefore, seven $KCNA$ gene family members are more expressed at the RNA level in the resistance artery. $K_v\alpha1$ subunits heteromultimerize and so the sum of these RNA species is relevant. Assuming abundances of RNAs encoding $K_v\alpha1.4$, 6 and 7 were half those of RNA encoding $K_v\alpha1.5$ the sum of the $K_v\alpha1$-encoding RNAs in mesenteric artery was 55 times that

Figure 2. Specificity of detection in aorta samples using $F_1/R_1$ PCR primer sets (Table 1)  
Melt curve analyses are shown with inset agarose gels.
of the aorta (the difference is 43 times if Kᵥα1.4, -6 and -7 are excluded). There were also differences for other K⁺ channel genes but not as marked as for Kᵥα1 (Fig. 5A and B). RNA encoding Kᵥα2.1 was four times more abundant in mesenteric artery but RNA encoding its associated γ-subunit (Kᵥγ9.3) was not proportionately increased. Abundances of RNA species encoding Kᵥβ1, BKᵥα1 and BKᵥβ1 were significantly lower in mesenteric artery (Fig. 5A and B).

Our technical approach for quantifying RNA expression in different blood vessels was validated using additional PCR primers sets for Kᵥα1.2, Kᵥα1.3 and BKᵥα1 (F2 and R2, Table 1). Using these primer sets, Kᵥα1.2 expression relative to β-actin was 0.0053 ± 0.0008 in aorta compared with 0.0674 ± 0.0109 in mesenteric artery, 13 times more in mesenteric artery (P < 0.01, n = 4). Kᵥα1.3 expression was 0.001 ± 0.0001 in aorta compared with 0.064 ± 0.0133 in mesenteric artery, 64 times more in mesenteric artery (P < 0.01, n = 4), and BKᵥα1 expression was 0.827 ± 0.1428 in aorta compared with 0.315 ± 0.0302 in mesenteric artery, 3 times more in aorta (P < 0.01, n = 4). These data sets are not significantly different from those shown in Figs 4 and 5. Therefore, variables such as PCR efficiency (Table 1) do not impact substantially on the outcome.

The above data show RNA levels of Kᵥα1 genes are up-regulated in mesenteric artery smooth muscle cells. It follows that there might be more functional Kᵥα1 channel protein in these cells. To test this idea the amplitudes of functional Kᵥα1 signals in aortic and mesenteric smooth muscle cells must be quantified under the same conditions. Although studies of contractile function can reveal roles of Kᵥα1 channels (e.g. Cheong et al. 2001a,b) the two vessels cannot be compared under conditions giving the same membrane potential and intracellular Ca²⁺ concentration, factors that determine the amplitude of Kᵥα1 signals. Therefore, we chose to measure ionic currents through Kᵥα1 channels in freshly isolated smooth muscle cells under voltage-clamp. To separate Kᵥα1 current from other signals we required a method for specific block of all Kᵥα1 channels. We previously used correolide but found its effect occurred slowly, hampering the generation of quantitative data (Cheong et al. 2001a). Therefore, we

![Figure 3. Smooth muscle origin of signals and protein localization](image-url)

F₁/R₁, PCR primer sets were used (Table 1). A, mean ± s.e.m. relative RNA abundances for species encoding Kᵥα1.1, Kᵥα1.2, Kᵥα1.5, Kᵥβ1 and Kᵥα2.1 comparing intact aorta (black bars) and medial layer only (white bars). ***P < 0.01, n = 4. B, mean ± s.e.m. relative RNA abundances for Kᵥα1.3, comparing medial layer-only (white bar), endothelium-denuded adventitia intact (hatched bar) and intact aorta (black bar). ****P < 0.01, n = 4. C, cross-sections of aorta labelled with anti-Kᵥα1.1 antibody (left, green) or anti-Kᵥα1.3 antibody (right, green) with autofluorescence of elastic laminae in blue. Endothelial cells were positive for Kᵥα1.3 (arrows) but not Kᵥα1.1. The scale bar applies to both images. Lower panels show Western blots for aorta lysates stained for Kᵥα1.1 (predicted mass, 57 kDa) or Kᵥα1.3 (predicted mass, 58 kDa).
explored the effects of Compound C, a derivative of correolide (Koo et al. 1999), favouring this over the use of toxin inhibitors of Kvα1 because Kvα1.5 is ‘toxin-resistant’, compromising toxin-block of other Kvα1 sub-units (Cheong et al. 2001a,b). We refer to Compound C as ‘correolide-C’. Correolide-C blocks Kvα1.6 channels expressed in Xenopus oocytes (Fig. 6A and B). To explore the specificity of correolide-C we tested it against Kvα2.1 and Kvα2.1 + Kvγ9.3, which are expressed in the mouse blood vessels (see above). There was no effect of correolide-C (Fig. 6C and D). The data suggest correolide-C is a fast-acting and specific inhibitor of Kvα1 channels.

Correolide-C had a marked inhibitory effect on voltage-dependent K⁺ current in isolated smooth muscle cells, causing steady-state block of 88.0 ± 6.23% and 68.8 ± 9.17% at 0 mV and +40 mV in mesenteric artery myocytes, and 79.0 ± 6.8% and 66.9 ± 8.82% at 0 mV and +40 mV in aortic myocytes (n = 8 for each, 5–6 animals) (Fig. 7A–D). Correolide-C-sensitive current density was approximately twice as large in myocytes from mesenteric artery compared with aorta (4.25 ± 0.71 pA pF⁻¹ cf. 2.05 ± 0.6 pA pF⁻¹ at 0 mV and 10.0 ± 1.93 pA pF⁻¹ cf. 4.58 ± 1.02 pA pF⁻¹ at +40 mV, n = 8 cells for each; P < 0.05). Therefore, higher Kvα1 RNA levels in mesenteric artery are associated with greater, but more modest, functional Kvα1 protein expression.

Although, in the above experiments, we used a Ca²⁺-free bath solution and a relatively high Ca²⁺ buffering capacity in the patch pipette, current through BKCa channels may contribute when recording from smooth muscle cells (e.g. Cheong et al. 2002). Therefore, we checked whether correolide-C has an effect on the smooth muscle BKCa current. The basal BKCa current was small but could be induced strongly by the addition of arachidonic acid.

Figure 4. Conduit versus resistance artery for RNA encoding Kvα1

Data are for endothelium-denuded vessels. Adventitia were intact. F₁/R₁ PCR primer sets were used (Table 1). A, SYBR Green fluorescence plotted against PCR cycle for typical reactions. B, mean ± S.E.M. abundances for aorta (white bars) and mesenteric artery (black bars) (**P < 0.01 mesenteric versus aorta, n = 4). C, end-point PCR products on agarose gels for RNA species indicated. DNA standards were run on the left side of each gel. Templates were from aorta (‘A’), mesenteric artery (‘M’) and mouse brain samples. Presence (+) or absence (–) of reverse transcriptase reaction.

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(Fig. 8). The signal was sensitive to the BK<sub>Ca</sub> inhibitor penitrem A (Cheong et al. 2002) but completely resistant to correolide-C (Fig. 8).

**Discussion**

Real-time quantitative RT-PCR methodology has been developed and validated for specific RNAs expressed physiologically in smooth muscle cells of murine arteries. Expression of all of the KCNA gene family members, with the exception of one distantly related member (Lang et al. 2000), was shown to be markedly up-regulated in mesenteric resistance artery compared with the conduit of the thoracic aorta. In contrast, expression of other K<sup>+</sup> channel genes was down-regulated such that, for example, there was more than a 100-fold increase in the ratio of total K<sub>V</sub>α1 to BK<sub>Ca</sub>α1. Use of a novel specific inhibitor of K<sub>V</sub>α1 channels enabled measurement of the total K<sub>V</sub>α1 signal in freshly isolated smooth muscle cells and this showed enhanced RNA expression translates partially to physiological function.

A critical issue in RT-PCR studies is the cell type from which the signal originates – in this case the arterial smooth muscle cell in its physiological contractile state. To achieve this focus we adopted a novel deletion approach. This involves validated removal of the endothelium and adventitia followed by quantitative measurement of gene expression. If RNA from the endothelium or adventitia contributes to the RT-PCR signal a shift of the C<sub>P</sub> value is expected to occur if either layer is removed. For some RNA species we observed such a shift and for others we did not. A criticism of this method might be that it is uncertain whether medial layer samples are devoid of contamination from non-smooth muscle cell types. For example, although isolation of aortic media reduced the eNOS signal by 99%, the RNA species was still detected. This might be due to expression of eNOS in smooth muscle cells (Teng et al. 1998). However, the key factor in the

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**Figure 5. Expression of RNAs encoding non-Kvα1 subunits**

Protocols and labelling are as for Fig. 4A and B. F<sub>i</sub>/R<sub>i</sub>; PCR primer sets were used (Table 1). B, **P < 0.05, ***P < 0.01 mesenteric versus aorta, n = 4.
deletion method is quantification. If the concentration of a specific RNA species were greater in endothelial cells than would be a rightward shift in the PCR crossing-point (Cp) after removal of the endothelium. This was strikingly the case for Kα1.3-encoding RNA, but not other RNA species encoding K+ channels. For genes encoding channels such as Kα1.2 our deletion approach enables highly quantitative in situ RT-PCR with RNA isolated from freshly dissected snap-frozen tissue in which the smooth muscle cells are in situ within their elastic laminae. There is no significant contamination from endothelial or adventitial cells and there is the significant advantage that multiple genes can be analysed and compared in the same sample.

Kα1.2 and 1.5 communoprecipitate, and heterologous coexpression confers properties comparable to those of a component of the native K+ current in portal vein myocytes (Kerr et al. 2001). Expression data have indicated these two subunits dominate in vascular smooth muscle (Cox et al. 2001; Cox & Rusch, 2002; Albarwani et al. 2003). However, there is also expression of other Kα1 subunits in vascular smooth muscle (reviewed in Cheong et al. 2001b) and our new quantitative data reveal in situ expression of RNA species encoding seven of the Kα1 subunits in murine aorta and mesenteric artery. Although Kα1.2- and 1.5-encoding RNAs are expressed, they do not stand out, and RNA encoding Kα1.5 is difficult to detect in aorta. We have also detected Kα1.1–1.6 in the medial layer of human saphenous vein and thus the expression of several Kα1 subunits is not peculiar to mouse (S. J. Fountain, N. Quinton, C. Munsch & D. J. Beech, unpublished observations). Consistent with the expression of Kα1.1 we observe that 5 mM tetraethylammonium (TEA+) inhibits the correolide-C-sensitive current (A. Cheong, P. Jackson...
Kvα1.1 is the most TEA$^+$ sensitive of the Kvα1 subunits (Coetzee et al. 1999). Dendrotoxin-K inhibits Kvα1.1 but has only a weak effect on the correolide-C-sensitive current (A. Cheong & D. J. Beech, unpublished observations). We presume this is because the coexpression of many other Kvα1 subunits – especially Kvα1.5 – inhibits toxin binding (Hatton et al. 2001).

In a quantitative analysis of the impact of Kvα1, all subunits should be considered because they heteromultimerize into channel complexes. It follows that disruption of only one KCNA gene may have little effect on the vasculature because all the Kvα1 subunits have similar electrophysiological properties. This may be why mice with a single disruption of a KCNA gene are not obviously hypertensive (Nerbonne et al. 2001). Although at this general level KCNA genes may have equivalent functions in vascular smooth muscle it is also apparent that there is differential expression between different blood vessels. The functional relevance of such differences is, however, unknown. We can only speculate that there may be regulatory implications in terms of effects of phosphorylation, subunit assembly, implications for fine-tuning of the membrane potential, or impact on cell-cycle progression as well as contractile function (Chittajallu et al. 2002; Zhu et al. 2003).

We show that there is not only up-regulation of RNA levels in resistance artery but that this translates to an increase in functional Kvα1 protein at the plasma membrane (i.e. K$^+$ current). Therefore, we assume

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**Figure 6. Specificity of correolide-C (Cor. C, 1 μM) as a blocker of Kvα1**

A, current–voltage (I–V) relationships for Kvα1.6-expressing Xenopus oocytes before (○) and after (●) application of 1 μM Cor. C. Cells were held at −80 mV and I–V relationships generated by applying 0.5 s incremental 10 mV depolarizing pulses at 0.1 Hz. Mean ± s.e.m. normalized currents (n = 4 for each data set). Currents for control water-injected oocytes are also shown (□), n = 4 for each. The smooth curves are fitted modified Boltzmann functions (Cheong et al. 2001a). B, time series for Kvα1.6 current at 0 mV. Typical current traces are inset with the voltage step above. C–D, lack of effect of Cor. C on Kvα2.1- or Kvα2.1 + Kvγ9.3-mediated currents in Xenopus oocytes. C, comparison of Kvα2.1 alone (○) with coinjected Kvα2.1 + Kvγ9.3 (●) conductance–voltage relationships verifying expression of Kvγ9.3 (n = 4 for each). The fitted Boltzmann functions indicated the half-activation voltage shifted significantly (P < 0.05) from +3.1 ± 1.0 mV to −10.5 ± 0.5 mV (n = 4), consistent with previous studies (e.g. Kerchensteiner & Stocker, 1999). D, upper panels show representative currents elicited by steps to 0 mV for Kvα2.1 and Kvα2.1 + Kvγ9.3-injected oocytes with and without Cor. C. Lower panels show mean ± s.e.m. data (n = 4, ***P < 0.01) for currents in the presence of Cor. C or 5 mM tetraethylammonium (TEA) normalized to the amplitude of current before application of the blocker.

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the up-regulated RNA production is physiologically important in resistance artery function. A doubling in the amplitude of K⁺ current will have major inhibitory impact on voltage-dependent Ca²⁺ entry, especially in the context of a high resistance plasma membrane such as that of the vascular smooth muscle cell (Nelson & Quayle, 1995; Quinn et al. 2000). It is also striking, however, that there is the quantitative discrepancy between RNA and K⁺ current; the sum of the Kᵥα_l-encoding RNAs in resistance artery was about 50 times that of the conduit, whereas the correolide C-sensitive (Kᵥα_l) current amplitude was about two-times bigger. There should not be surprise at this quantitative discrepancy. Studies of yeast and bacteria have previously revealed similar differences between RNA levels, protein levels and protein function (Gygi et al. 1999; Glanemann et al. 2003). A static correlation between levels of mRNA, protein and active protein is highly unlikely given the existence of such an array of complex post-translation mechanisms. It is only now, as we start to measure RNA levels accurately and relate them to proteins and their function, that we are can see such relationships. Ours is amongst the first of such studies, although a recent real-time PCR study of BKCa channels in myometrial smooth muscle has also revealed a perhaps surprising relationship between RNA and protein (Eghbali et al. 2003). At this stage we can only speculate on why there should be an apparent inefficiency in translation or suppressive post-translational effect in the mesenteric artery. One explanation could relate to our observation that there is lower expression of Kᵥβ1 RNA because Kᵥβ1 protein is a chaperone for Kᵥα_l-subunits (Manganas & Trimmer, 2000; Campomanes et al. 2002). But this may only be part of a large system in which there is regulation also at the levels of transcription, translation, protein trafficking to the membrane and protein degradation. Our data indicate there may be a stop-tap at the higher levels, restricting the impact of higher transcriptional activity. However, it is also premature to rule out the impact of

Figure 7. Amplitude of functional Kᵥα_l signals in smooth muscle cells from aorta and mesenteric artery
Correolide-C was applied at 1 µM. A–D, whole-cell patch-clamp recordings from smooth muscle cells using the ‘Kᵥ’ recording solutions and a holding potential of −60 mV. A, typical current traces from an aortic cell. B, time-series plot for experiment in A and showing current densities measured at the ends of the voltage steps to 0 and +40 mV. C and D, as in A and B, except for mesenteric artery.

Figure 8. Lack of effect of correolide-C on Ca²⁺-activated K⁺ channels
Whole-cell recording from an aortic myocyte using the ‘BKCa’ recording solutions. The cell was held at 0 mV and depolarized to +40 mV for 0.5 s and to +100 mV during a 0.5 s ramp. Arachidonic acid (AA, 10 µM) was applied to enhance BKCa current. Correolide-C (1 µM) had no effect. Presence of BKCa current was confirmed by application of penitrem A (100 nM).
acute regulation on channel activity because β-subunits could be in the membrane but non-functional. This type of effect was striking for the BKCa channel (Fig. 8).

Thyrotropin-releasing hormone, insulin-like growth factor-1, Bcl-2 oncprotein, c-Jun immediate early gene, chronic hypoxia, cyclic-adenosine-monophosphate and glucocorticoids all affect expression of genes encoding Kᵥα1 channels of vascular myocytes or other cell types (Mori et al. 1993; Allen et al. 1998; Levitan & Takimoto, 1998; Zhang et al. 2000; Ekhterae et al. 2001; Platoshyn et al. 2001; Yu et al. 2001; Gamper et al. 2002). Whether any of these underlie the up-regulated expression in mesenteric artery is unknown. Chronic hypoxia, however, seems unlikely from a physiological perspective and because it affected KCNA gene expression in pulmonary but not mesenteric myocytes (Platoshyn et al. 2001). Our study firmly establishes the principle that in situ expression of genes encoding Kᵥα1 channels in smooth muscle is physiologically up-regulated upon progression to resistance mesenteric artery. The effect is dramatic and consistent with a ‘competitive’ RT-PCR study of two Kᵥα1-encoding genes in endothelium-intact rat arteries (Cox et al. 2001). Importantly we now show this is a smooth muscle phenomenon, that it extends to essentially the whole family of Kᵥα1-encoding genes, and that it translates to a physiological signal. The fact that up-regulation is common across the family of Kᵥα1-encoding genes should facilitate efforts to elucidate the underlying mechanism. Intriguingly, KCNA genes are clustered primarily in loci of two chromosomes in mouse and human: KCNA6, -1 and -5 in series on human chromosome 12, and KCNA8, -2 and -3 in series on human chromosome 1. A consequence might be that elements of their transcriptional control are common. The findings of this study support this supposition and indicate that such a mechanism would have a role in the physiological control of blood pressure and local tissue perfusion.

References


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