Heteromultimeric Kv1 Channels Contribute to Myogenic Control of Arterial Diameter

Frances Plane, Rosalyn Johnson, Paul Kerr, William Wiehler, Kevin Thorneloe, Kuniaki Ishii, Tim Chen, William Cole

Abstract—Inhibition of vascular smooth muscle (VSM) delayed rectifier K+ channels (K_{DR}) by 4-aminopyridine (4-AP; 200 μmol/L) or correolide (1 μmol/L), a selective inhibitor of Kv1 channels, enhanced myogenic contraction of rat mesenteric arteries (RMAs) in response to increases in intraluminal pressure. The molecular identity of K_{DR} of RMA myocytes was characterized using RT-PCR, real-time PCR, and immunocytochemistry. Transcripts encoding the pore-forming Kvα subunits, Kv1.2, Kv1.4, Kv1.5, and Kv1.6, were identified and confirmed at the protein level with subunit-specific antibodies. Kvβ transcript (β1.1, β1.2, β1.3, and β2.1) expression was also identified. Kv1.5 message was ≈2-fold more abundant than that for Kv1.2 and Kv1.6. Transcripts encoding these three Kv1α subunits were ≈2-fold more abundant in 1st/2nd order conduit compared with 4th order resistance RMAs, and Kvβ1 was 8-fold higher than Kvβ2 message. RMA K_{DR} activated positive to −50 mV, exhibited incomplete inactivation, and were inhibited by 4-AP and correolide. However, neither α-dendrotoxin or e-dendrotoxin affected RMA K_{DR}, implicating the presence of Kv1.5 in all channels and the absence of Kv1.1, respectively. Currents mediated by channels because of coexpression of Kv1.2, Kv1.5, Kv1.6, and Kvβ1.2 in human embryonic kidney 293 cells had biophysical and pharmacological properties similar to those of RMA K_{DR}. It is concluded that K_{DR} channels composed of heteromultimers of Kv1 subunits play a critical role in myogenic control of arterial diameter. (Circ Res. 2005;96:216-224.)

Key Words: delayed rectifier potassium channel ■ KCNA ■ vascular smooth muscle ■ myogenic contraction ■ arterial diameter

The ability of small resistance arteries to develop myogenic tone in response to elevations in intraluminal (or transmural) pressure is an essential autoregulatory mechanism and an important determinant of peripheral vascular resistance, regional blood flow, and blood pressure. Pressure-induced depolarization of vascular smooth muscle (VSM) leading to increased intracellular Ca2+ ([Ca2+]i) via voltage-dependent activation of Ca2+ channels is required for myogenic tone development.1,2 However, the depolarization does not evoke regenerative action potentials, rather incremental changes in diameter are achieved by graded, steady-state depolarizations.3 Our understanding of the ionic basis of this precise control of myogenic depolarization is incomplete.

Increased intraluminal pressure is thought to activate non-selective cation or Cl− channels of VSM cells,2 with the level of depolarization attributable to these channels precisely controlled by an activation of VSM K+ channels.3 Compelling evidence for a contribution of large Ca2+ -activated K+ channels (BKCa) to this feedback control comes from studies using specific inhibitors (eg, iberiotoxin) and transgenic mice lacking the BKCa modulatory β1 subunit.4 However, whether other channels are also involved in controlling myogenic depolarization in VSM is not clear.

Voltage-gated delayed rectifier channels (K_{DR}) are expressed by VSM cells of several vascular beds3 and could also play a role in control of myogenic contraction. This view is supported by studies of cerebral arteries; myogenic reactivity was enhanced by 4-aminopyridine (4-AP)-induced inhibition of K_{DR}.5 Moreover, changes in the activity and/or expression of K_{DR} channel subunits are reported to occur in diseases (eg, hypertension,6,7 pulmonary hypertension,8–10 and diabetes11) characterized by increased myogenic reactivity.12–14

Our understanding of arterial VSM K_{DR} is incomplete and complicated by variations in the reported properties of the channels, as well as the channel subunits expressed.5–10,15–21 Whether these differences are attributable to regional variations in channel type and/or level of expression, or alternatively, to varied recording conditions, cell quality and/or selectivity of tools used is unknown. K_{DR} are composed of pore-forming Kvα and modulatory Kvβ subunits arranged in an octameric complex. We provided the first evidence that Kv1.2, Kv1.5, and Kvβ1.2 coassemble to form heteromultimeric K_{DR} in rabbit portal vein (RPV) VSM.16,17 Expression of transcripts encoding these subunits was reported for...
resistance arteries, as well as additional Kv1 subunits, including Kv1.1 and Kv1.6.6,7,15,18–23 However, parallel evidence of subunit protein expression and comparison of the properties of native and recombinant channels was not always performed to justify the conclusion that each subunit contributed to the functional channels and/or control of arterial function.6,7,15,18–22

The aims of this study were 3-fold: (1) assess the role of Kv1-containing KDR in the myogenic response of rat mesenteric arteries (RMAs) using 4-AP (200 μmol/L) and correolide23–25 (1 μmol/L) to inhibit the channels, (2) identify the expression profile of Kv1 and Kvβ subunits in RMAs, and (3) compare the properties of RMA KDR and channels resulting from heterologous coexpression of the subunits present in RMA myocytes.

Materials and Methods
Male Sprague-Dawley rats (Charles River, Montreal, Canada) were housed and killed according to the standards of the Canadian Council on Animal care and a protocol reviewed by the Animal Care Committee of the Faculty of Medicine, University of Calgary. Details of the methods and materials used in this study are similar to previous publications16,17,26 and/or are in the online data supplement available at http://circres.ahajournals.org.

Results
Control of Myogenic Response by Kv1-Containing KDR
Second order conduit RMAs dilated passively in response to increased transmural pressure (20 to 120 mm Hg) and did not develop active myogenic tone; ie, the pressure-response curves for control and nifedipine-treated (10 μmol/L) tissues were identical (Figure 1A). KDR inhibition with 4-AP (200 μmol/L) led to active myogenic tone development and arterial diameter was unchanged at pressures ≤40 mm Hg (no significant difference in the values >40 mm Hg). In contrast, 4th order resistance RMAs developed active tone at pressures >60 mm Hg, and the control pressure-response curve was different from the passive dilation in nifedipine (Figure 1B). 4-AP enhanced the magnitude of change in diameter with increasing pressure in these resistance RMAs, and in 6 of 7 vessels, spontaneous oscillations in diameter were observed (Figure 1B).

Similar results were obtained for RMAs in the presence of correolide (1 μmol/L; gift of Merck & Co. Inc, Rahway, NJ) (Figure 2). Enhanced responses to increased intraluminal pressure were observed between 60 to 120 mm Hg in 2nd and 4th order RMAs after correolide treatment compared with
control conditions. Oscillations in the diameter of 4th order vessels were observed in correolide, but less often than with 4-AP (ie, 2 of 6 tissues).

Expression of Kv1 and Kvβ Subunits by RMA VSM

Kv1 subunit expression by intact 4th order RMAs was examined by RT-PCR using subunit-specific primers. Products of appropriate size were obtained for Kv1.1, Kv1.2, Kv1.4, Kv1.5, and Kv1.6 after 35 cycles (Figure 3A). Consistent results were obtained except for Kv1.1, which was detected in only 3 of 6 arteries, but was always apparent in brain RNA. Kv1.1 and/or Kv1.6 expression was reported for cerebral and mesenteric arteries,6,7,15,19,24,25 but not for RPV.17 RNA samples were obtained from 200 to 300 individually selected RMA myocytes to confirm VSM cell-specific expression and control for contamination by message of non-VSM origin (eg, endothelium and neurons). Kv1.6 mRNA was consistently detected, but Kv1.1 expression was not evident (Figure 3B). Primers for the endothelial cell and neuronal markers, endothelin-1 and Erg3, respectively, confirmed the absence of contaminating message (Figure 3B). The expression of Kvβ1.1, β1.2, β1.3, and Kvβ2.1 in intact RMAs was also identified by RT-PCR (Figure 3C).

The abundance of Kv1 message normalized to β-actin mRNA in 1st/2nd (combined) and 4th order RMAs was determined by real-time PCR (see online data supplement for method and primer verification). The relative abundance of Kv1.5 message was ~2-fold greater than that for Kv1.2 and Kv1.6 in 1st/2nd and 4th order RMAs, and the abundance of all three transcripts was significantly higher in 1st/2nd order RMAs (Figure 3D). The similarity in nucleotide sequence of Kvβ1.1-β1.3 and of Kvβ2.1-β2.2 precluded generation of subunit-specific primers, so pan-Kvβ1 and -Kvβ2 primers were used. Kvβ1 message abundance was higher than that of Kvβ2 in 1st/2nd and 4th order RMAs (Figure 3D).

Kv1 protein expression in 4th order RMA myocytes was identified using subunit-specific antibodies. Anti-Kv1.1 and anti-Kv1.6 were first checked for specificity using human embryonic kidney (HEK293) cells cotransfected with cDNAs encoding the subunits (Kv1.1 and Kv1.6 cDNAs; gifts of Drs H. Duff [University of Calgary, Canada] and O. Pongs...
[Universität Hamburg, Germany], respectively) and green fluorescent protein (GFP) (Figure 3E; the specificity of the anti-Kv1.2, -Kv1.4, and -Kv1.5 was previously confirmed). Polyclonal anti-Kv1.1 and anti-Kv1.6 from Calbiochem and Alomone, respectively, appropriately recognized Kv1.1 and Kv1.6 subunit expression, and the immunofluorescence was blocked by preabsorption with antigenic peptide. Two additional anti-Kv1.1s from Upstate Biotechnology (monoclonal) and Chemicon (polyclonal; Figure 3E) were also tested, but these only demonstrated nonspecific reactivity.

Kv1.2, Kv1.4, Kv1.5, and Kv1.6 immunofluorescence was detected in RMA myocytes and blocked by antigenic peptide or absent in 2nd antibody alone (Figure 3F). However, no immunoreactivity was apparent for RMAs when Calbiochem anti-Kv1.1 was used (Figure 3F). Similarly, a lack of Kv1.1 protein expression by rat cerebral arterial myocytes was also evident using this antibody (Figure 3F), contrary to previous reports.

**Comparison of RMA K<sub>DR</sub> and Kv1 Currents**

RMA myocytes (4th order) were isolated using methods (see online data supplement) that yielded healthy, relaxed cells or overdigested, contracted cells (Figure 4A and 4B). Mean cell capacitance of relaxed myocytes was 27.5±2.0 pF (n=31) and significantly higher than the 18.8±1.3 pF (n=35; P<0.01) identified for overdigested, contracted cells. Sustained K<sub>DR</sub> currents, similar to those of other VSM cells.
were apparent in both cell types, and a small transient outward current component (inactivation in less than 50 ms) was occasionally apparent (see inset in Figure 7). However, the density of KDR current (at +30 mV) was significantly higher at 27.4±1.5 (n=19) pA/pF for relaxed cells and it activated at a more negative voltage of ~−40 mV compared with overdigested myocytes at 4.7±0.6 pA/pF (n=13; P<0.01) (Figure 4C) and ~−30 mV (Figure 4C and 4E). This indicates that cell isolation can influence VSM KDR current, and for this reason, only relaxed myocytes with >0 pA/pF KDR current were used.

RMA KDR currents showed voltage-dependent activation and inactivation (Figure 4A and 4D). A single exponential Boltzmann function was used to fit normalized tail current amplitude and the voltage required for half-maximal (V0.5) activation was ~−10 mV (Figures 4D and 5A and Table). Steady-state availability was assessed using a standard double-pulse protocol and inactivation was found to be incomplete, with a residual component of noninactivating net KDR remaining after 10 seconds at >+10 mV (Figure 4D and 4E). The V0.5 for inactivation determined by fits of normalized end-pulse amplitude with a Boltzmann function was −39.4±1.8 mV (k=11.1; n=7). The time constants of activation, inactivation, and deactivation were determined by curve fitting with single or double exponential functions. Values for activation and inactivation are given in the Table.

The properties of currents attributable to cotransfection of combinations of cDNAs encoding Kv1.2, Kv1.5, Kv1.6, and Kvβ1.2 were determined (Table). Figure 5 shows data for coexpression of all four subunits; the V0.5 for activation was more negative, but RMA KDR and Kv1.2-Kv1.5-Kv1.6-Kvβ1.2 currents activated, inactivated, and deactivated with similar time constants (Table), and the tail currents were overlapping when scaled to an identical amplitude (Figure 5). In the absence of Kvβ1.2, the V0.5 and deactivation time constant values were both different from RMA KDR (Table 1). Currents attributable to coexpression of Kv1.2 and Kv1.5 or Kv1.5 and Kv1.6, expression of these pairs of subunits as tandem constructs (joined by a polyglycine linker of 7 or 5 residues, respectively),16 or coexpression of Kv1.2 and Kv1.6 did not deactivate at a rate consistent with RMA KDR, and the Kv1.5-Kv1.6 channels exhibited slower activation (Table).

Inhibition of VSM KDR by 4-AP is associated with an apparent positive shift in the voltage of activation (ie, the V0.5 shifts by ~+10 mV).16 4-AP (1 mmol/L) reduced RMA KDR current by ~34% (Table) and caused a similar shift in V0.5 of +10.7±1.8 mV (n=7) (Figure 5). Kv1.2-Kv1.5-Kv1.6-Kvβ1.2 currents were also inhibited by ~34% (Table) and showed a similar shift in activation of +12.1±0.7 mV (n=3) in the presence of 4-AP (Figure 5D). Paired combinations of coexpressed subunits exhibited a shift in V0.5, but the extent of current suppression by 4-AP was >50% (Table).

Figure 6 compares the inhibition by correolide (1 μmol/L) of KDR currents of 2nd and 4th order RMA myocytes. The inhibition occurred at a voltage consistent with the level of membrane potential reported for the vessels, ie, −30 to −50 mV (Figure 6A and 6B). Although KDR current density (at +30 mV) for myocytes of 2nd (28.5±1.5 pA/pF, n=11) and 4th order arteries (32.9±2.5 pA/pF, n=10) was identical (P>0.05), the level of current suppression by correolide was significantly greater at 57.3±2.8% (n=3) compared with 37.4±3.6% (n=3; P<0.01), respectively (Figure 6C).

α-Dendrotoxin (α-DTX; 50 nmol/L) was used as it inhibits homo- and heteromultimeric channels composed of Kv1.2 and Kv1.6 (as well as Kv1.1), but not channels containing Kv1.5.30 α-DTX suppressed Kv1.2-Kv1.6 current at 50 nmol/L (Figure 7A), but it had no effect on RMA KDR or currents attributable to coexpression of Kv1.2, Kv1.5, Kv1.6, and Kvβ1.2 (Figure 7B and 7C). The contribution of Kv1.1 to RMA KDR was assessed using κ-dendrotoxin (κ-DTX), a specific inhibitor of homo- and heteromultimeric Kv channels containing Kv1.1.27,28 κ-DTX (50 nmol/L) suppressed cur-
rents attributable to Kv1.1 expression in HEK293 cells, but it did not affect RMA KDR (Figure 7D and 7E).

Discussion

This study provides novel evidence for the contribution of heteromultimeric KDR composed of Kv1.2, Kv1.5, Kv1.6, and Kvβ subunits to the control of myogenic contraction of arterial resistance vessels. This view is based on an analysis of arterial myogenic responses in the presence of Kv1 channel inhibition, identification of Kv1 subunit message and protein expression, quantification of transcript abundance, and a comparative assessment of biophysical and pharmacological properties of RMA KDR and recombinant channels composed of the subunits expressed by RMA myocytes.

Comparison of Properties of RMA KDR to Those of Recombinant Channels Attributable to Coexpression of Varied Combinations of Kv1.2, Kv1.5, Kv1.6, and Kvβ1.2

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<th>Act V_{E/K}, mV</th>
<th>Act Time Constants</th>
<th>Deact Time Constants</th>
<th>Slow Inact Time Constant, ms</th>
<th>α-DTX</th>
<th>4-AP, %</th>
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<td></td>
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<td>T1, ms</td>
<td>T2, ms</td>
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<tr>
<td>RMA KDR</td>
<td>−10.4±0.4 (15)#</td>
<td>1.7±0.1 (6)</td>
<td>6.2±2.3 (6)</td>
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<td>Kv1.2−1.5−1.6−β1.2</td>
<td>−21.7±0.7* (15)</td>
<td>1.1±0.1 (7)</td>
<td>5.2±0.2 (7)</td>
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<tr>
<td>Kv1.2−1.5−1.6</td>
<td>−15.5±0.5* (3)</td>
<td>1.3±0.3 (3)</td>
<td>4.1±0.5 (3)</td>
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<tr>
<td>Kv1.2−1.5 coexp</td>
<td>−15.2±0.6* (5)†</td>
<td>1.3±0.1 (6)</td>
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<td>Kv1.5−1.2 tandem</td>
<td>−25.5±1.4* (7)†</td>
<td>1.1±0.1 (5)</td>
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<tr>
<td>Kv1.2−1.6 coexp</td>
<td>−15.7±0.5* (5)†</td>
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<tr>
<td>Kv1.5−1.6 tandem</td>
<td>−19.3±1.1* (8)</td>
<td>1.4±0.3 (7)</td>
<td>42.0±3.1* (7)</td>
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#Parentheses indicate no. of myocytes/cells used. *Significantly different (P<0.05) from RMA KDR by ANOVA followed by Dunnett test. †Value from Kerr et al.†

†Percent 4-AP block at +20 mV with 1 mmol/L. §5 of 8 cells had an additional fast T of 1023±101 ms when fit with two exponentials.
Kv currents of RMA myocytes are due, at least in part, to heteromultimeric channel complexes composed of Kv1.2, Kv1.5, Kv1.6, and Kv\(\beta\)1 and/or Kv\(\beta\)2, but not Kv1.1. Expression of Kv1.1 to 1.6 in arteries was previously shown by RT-PCR using RNA obtained from intact segments in several studies, but whether all of the identified subunits contributed to functional KvDR was not determined. Our analysis of intact RMAs showed expression of Kv1.2, Kv1.4, Kv1.5, and Kv1.6 message and protein, with Kv1.5 message being \(\approx 2\) fold more abundant than that encoding Kv1.2 and Kv1.6. Expression of Kv\(\beta\)1 (\(\beta\)1.1, \(\beta\)1.2, and \(\beta\)1.3) and Kv\(\beta\)2 (\(\beta\)2.1) was also identified. Evidence for a contribution of Kv1.1 to RMA KvDR was not obtained, however, contrary to studies of small arteries and arteries. Message encoding Kv1.1 was inconsistently identified in RMAs and could not be identified in RNA of isolated RMA myocytes. Additionally, no evidence of Kv1.1 protein or functional contribution to RMA KvDR was obtained with anti-Kv1.1 or \(\kappa\)-DTX, a toxin that inhibits Kv1.1-containing channels regardless of subunit composition or stoichiometry. Kv1.1 mRNA (unpublished observation, 2004) and/or protein expression as well could not be identified in rat cerebral arteries and isolated myocytes.

The properties of RMA KvDR were characterized for comparison with currents attributable to coexpression of Kv1 and Kv\(\beta\) subunits. The voltage-dependence and kinetics of RMA KvDR were similar to those previously identified for arterial KvDR, but the reported values exhibit considerable variability, eg, \(V_{0.5}\) values for activation range from \(-33.7\) mV to \(+3\) mV. In this study, the amplitude of KvDR current at \(+30\) mV was significantly higher at \(>500\) pA compared with previous studies at \(<500\) pA. Activation threshold was more negative at \(\approx -45\) mV, and the kinetics of deactivation were slower than previously reported. We also did not observe the complete inactivation of KvDR current described in some studies rather \(\approx 20\%\) of the current persisted during prolonged steps to positive voltages. These differences could reflect a species- and/or vessel-dependent expression of KvDR with varied subunit composition and properties. Alternatively, the variability may derive from differences in the quality of the isolated myocytes used. The importance of cell quality is indicated by our data showing that cell capacitance and KvDR current density were lower and the voltage threshold for KvDR activation more positive for contracted, overdigested cells compared with relaxed, healthy RMA myocytes. This suggests that careful attention must be paid to cell quality to achieve an accurate analysis of the properties of arterial KvDR. In the absence of appropriate care, erroneous conclusions may be made regarding the operating voltage range over which the channels are active and contribute to control of membrane potential. Also, meaningful comparisons of the properties of native and recombinant channels may be precluded if the former are affected by the cell isolation procedure.

Detailed comparisons of currents attributable to native and recombinant channels can be complicated by variations in the biophysical properties of the recombinant channels in different heterologous expression systems, especially voltage-dependence of activation and inactivation. With the exception of the \(V_{0.5}\) of activation, the properties of channels attributable to coexpression of Kv1.2, Kv1.5, and Kv1.6 with Kv\(\beta\)1.2 in HEK293 cells mimicked those of RMA KvDR and provide evidence of the subunit composition of the native channels. (1) The kinetics of activation, deactivation, and inactivation of RMA KvDR were similar to currents resulting from coexpression of Kv1.2, Kv1.5, Kv1.6, and Kv\(\beta\)1.2. On the other hand, paired heteromultimeric combinations of these Kv\(\alpha\) subunits, or all three subunits in the absence of Kv\(\beta\)1.2, deactivate more quickly and/or activate more slowly than RMA KvDR, implying that the native channels may contain all four subunits. (2) RMA KvDR and channels attributable to coexpression of Kv1.2, Kv1.5, Kv1.6, and Kv\(\beta\)1.2 displayed a lack of sensitivity to \(\alpha\)-DTX, a toxin that inhibits homo- and heteromultimeric Kv1.1, Kv1.2, and Kv1.6 channels, but not Kv1.5-containing channels. This indicates that homo- and heteromultimeric Kv1.2 and Kv1.6 channels are likely not present; ie, all RMA KvDR and Kv1.2-Kv1.5-Kv1.6-Kv\(\beta\)1.2 channels contained Kv1.5 and were insensitive to \(\alpha\)-DTX. That Kv1.5 contributes to RMA KvDR is consistent with the demonstrated presence of this subunit in KvDR of other vessels. (3) The extent of inhibition by 4-AP of RMA KvDR and Kv1.2-Kv1.5-Kv1.6-Kv\(\beta\)1.2 currents was identical at...
approximately 34%, but 4-AP had a greater effect on channels attributable to other heteromultimeric combinations of these subunits at >50%. Significantly, RMA K_{DR} were less sensitive to 4-AP compared with RPV K_{DR} current ( > 50% block with 1 mmol/L), but consistent with the findings of other studies of RMAs (5 to 10 mmol/L)\textsuperscript{1,3,4,5} and cerebral (5 mmol/L)\textsuperscript{17} arterial K_{DR}. (4-AP almost completely inhibited human mesenteric K_{DR} current, but the control current amplitude was only \( \approx 100 \text{ pA} \)).\textsuperscript{25} The differences in 4-AP sensitivity of VSM K_{DR} currents may be attributable to varied levels of expression of 4-AP–sensitive Kv1-containing and 4-AP–resistant non-Kv1 channels, eg, Kv2 channels.\textsuperscript{33} Alternatively, differences in Kv1 subunit composition could be involved, eg, the presence of Kv1.6 which has a higher IC\textsubscript{50} for 4-AP block.\textsuperscript{38} In agreement with this view, Kv1.2-Kv1.5-Kv1.6 channels expressed by RPV exhibit >50% block by 1 mmol/L 4-AP\textsuperscript{16,17} compared with the 34% inhibition shown here for Kv1.2-Kv1.5-Kv1.6- Kv1.2 channels. Interestingly, the sensitivity of Kv1.5-Kv1.6 and Kv1.2-Kv1.5 channels was identical and greater than RMA K_{DR} and Kv1.2-Kv1.5-Kv1.6-Kv1.2 channels, suggesting the combination of Kv1.6 with Kv1.2 and Kv1.5 may affect 4-AP sensitivity rather than the presence of Kv1.6 alone. Further experiments are required to identify the mechanism involved. (4) 4-AP inhibition of RPV K_{DR} was associated with a positive shift in the voltage-dependence of activation,\textsuperscript{46} eg, the same was shown here for RMA K_{DR}. Homomultimeric Kv1.5 channels do not exhibit a shift in activation, but homomultimeric Kv1.2\textsuperscript{1,16} and Kv1.6 (unpublished observation, 2004), as well as Kv1.2- \textit{K}v1.5,\textsuperscript{36} Kv1.5- \textit{K}v1.6 (unpublished observation, 2004), and Kv1.2- \textit{K}v1.5- \textit{K}v1.6- \textit{K}v\textbeta1 (present study) channels all mimic VSM K_{DR}. This indicates that RMA K_{DR} must contain Kv1.5 in association with Kv1.2 and/or Kv1.6. (5) RMA K_{DR} do not display fast inactivation as observed for Kv1 subunits when expressed with Kv1.4 or Kv\textbeta1 subunits.\textsuperscript{39,40} This lack of fast inactivation is consistent with the presence of Kv1.6 in RMA K_{DR}; the N-terminus of Kv1.6 has a motif, the NIP domain, that inhibits pore block by binding the positively charged inactivation ball of Kv1.4 and Kv\textbeta1 subunits.\textsuperscript{39,40} Although Kv1.4 is expressed in RMA myocytes, we do not believe that this subunit contributes to RMA K_{DR} as Kv1.4-containing channels display significantly faster activation.\textsuperscript{39,40}

This study indicates for the first time the importance of K_{DR} channels composed of Kv1 subunits to the normal function of arterial resistance vessels via their role in controlling myogenic reactivity. Previous studies indicating a role for K_{DR} channels in control of myogenic contraction used 4-AP at 0.3 to 1 mmol/L.\textsuperscript{5} Kv1 channels are sensitive to this concentration of 4-AP, but so too are Kv channels of other subfamilies with overlapping and, in some cases, coincident sensitivities to 4-AP; eg, Kv3.\textsuperscript{3,15} For this reason, we also used the putative Kv1 channel blocker, correolide, to assess the role of Kv1-containing K_{DR} in control of the myogenic response. Inhibition of K_{DR} by correolide was previously shown to elicit depolarization and constriction of cerebral arteries and/or arterioles,\textsuperscript{5,15,24} but its effect on myogenic reactivity was not assessed. We found that correolide mimicked the effect of 4-AP to enhance myogenic reactivity of RMAs and to inhibit RMA K_{DR} over a voltage range consistent with that associated with myogenic contraction of RMAs.\textsuperscript{1,5} Moreover, RMA K_{DR} currents activated positive to \(-50 \text{ mV}\) and showed incomplete inactivation; this is consistent with the view that the channels are capable of steady-state activity and contribute to control of membrane potential over the range reported for myogenic depolarization.\textsuperscript{3} Taken together, these data provide evidence for the participation of Kv1 subunit–containing K_{DR} in controlling the response of resistance arterial VSM to changes in transmural pressure. Whether additional subunits from other Kv subfamilies (eg, Kv2, which is expressed in RMAs; unpublished observation, 2004) also contribute to control of myogenic reactivity must be addressed in the future.

The ability of resistance arteries to develop myogenic tone varies between vascular beds, the response being most prominent in areas with the greatest degree of autoregulation. For example, cerebral and skeletal muscle arteries show a pronounced decrease in diameter with increasing pressure,\textsuperscript{3,41} whereas RMAs of similar diameter do not.\textsuperscript{3,42} However, smaller resistance RMAs do show a pressure-induced increase in [Ca\textsuperscript{2+}], similar to that of cerebral vessels\textsuperscript{41} and their pressure-diameter curve is flattened at higher pressures (ie, \( \approx 60 \text{ mm Hg} \)). This indicates that although they do not display a decrease in diameter, they do show active tone development and maintenance of diameter rather than passive dilation.\textsuperscript{3,42} Consistent with previous reports,\textsuperscript{43} 2nd order conduit RMAs did not exhibit active constriction and only dilated passively in response to elevated pressure. However, in the presence of 4-AP or correolide, these vessels maintained a stable diameter with increasing transmural pressure. This suggests that K_{DR} activation inhibits pressure-induced alterations in diameter in conduit arteries; ie, the presence of K_{DR} composed of Kv1 subunits is sufficient to prevent myogenic tone development. In contrast, 4th order resistance RMAs maintained a stable diameter when subjected to increased transmural pressure, and in the presence of K_{DR} inhibition, myogenic tone development was enhanced and accompanied by rhythmic oscillations in diameter. This indicates that a negative feedback regulation of VSM depolarization via voltage-dependent activation of Kv1-containing K_{DR} contributes to the control of myogenic contraction in resistance RMAs and may be necessary for stable, graded changes in arterial diameter. Interestingly, correolide produced a greater inhibition of K_{DR} current of 2nd versus 4th order RMA myocytes. This implies that the component of K_{DR} current attributable to Kv1-containing channels may be smaller in resistance compared with conduit RMAs, a view that is consistent with the lower abundance of Kv1 message in 4th compared with 1st/2nd order vessels as detected by real time PCR. It is possible that the amplitude of the negative feedback regulation provided by Kv1 subunit-containing K_{DR} may be an important determinant of the level of myogenic reactivity exhibited by arteries of different size and/or vascular origin. A reduced contribution of Kv1-containing K_{DR} would permit greater depolarization by inward current activated in response to increased transmural pressure, and thereby, greater active tone development. Whether differences in other ionic currents are also present and contribute to variability in myogenic reactivity requires further study.
In contrast to the specialized nature of the cerebral vascular system, the mesenteric arterial bed is relatively nonspecialized and representative of the peripheral vasculature in general. Our demonstration of a role for \( K_{\text{Ca}} \) in permitting stable myogenic alterations in the diameter of resistance RMs suggests that this may be a mechanism of generalized importance for appropriate function of the peripheral arterial vasculature and, therefore, for normal blood pressure regulation.

Acknowledgments

This work was supported by the Canadian Institutes of Health Research (MT-13505), The Wellcome Trust (UK), a Natural Sciences and Engineering Research Council PGS A award to R.J. and AHFMR and HFSC studentship awards to T.C. We thank Claude Velletelle and Susan Li for technical assistance. This work is dedicated to the memory of Prof Burton Horowitz, University of Nevada (Reno).

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Heteromultimeric Kv1 Channels Contribute to Myogenic Control of Arterial Diameter

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In an article published by Plane et al (Circ Res. 2005;96:216–224.), the reference section contained errors in some citations. Corrections to text (italicized) and cited literature (numbers are those of Plane et al) are given below:

Page 216–217: “Expression of these transcripts... including Kv1.1 and Kv1.6.6,15,18,19,20,23.”

Page 218: “Kv1.1 and/or Kv1.6 expression was reported for cerebral and mesenteric arteries.6,10,15,20,21,22,31”

Page 219: “Similarly a lack of protein expression by mesenteric arterial myocytes was also evident using this antibody, contrary to previous reports.10,23”

Page 222: “Evidence for a contribution of Kv1.1... contrary to some other studies of small arteries.6,8,10,20,23”

Page 223: “Inhibition of KDR by correolide was previously shown to elicit depolarization and/or constriction of cerebral arteries and/or arterioles.15,22”
Online Supplement

**Intact arterial vessel pressure myography:** Male Sprague-Dawley rats (250-350 g; Charles River, Montréal, Quebec, Canada) were maintained and killed by halothane inhalation and exsanguination according to the standards of the Canadian Council on Animal care and a protocol reviewed by the Animal Care Committee of the Faculty of Medicine, University of Calgary. The mesenteric arterial bed was carefully removed and placed in Krebs buffer containing (in mmol/L): NaCl 120, NaHCO₃ 25, KCl 4.8, NaH₂PO₄ 1.2, MgSO₄ 1.2, Glucose 11, CaCl₂ 2.5. Second and 4th order arteries were removed, cleaned of adherent fat, cannulated and mounted in a pressure myograph (Living Systems, Burlington, USA) for measurement of diameter. All drugs were added to the superfusate and all arteries were endothelium denuded, as indicated by the absence of a response to acetylcholine. Arteries were allowed to equilibrate for 60 mins and then subjected to increasing pressure steps from 20 to 120 mmHg. After the last step, intraluminal pressure was reduced to 10 mmHg, and 4-AP (200 µmol/L) or correolide (1 µmol/L) were added to the perfusate for 10 or 50 mins, respectively, before the pressure ramp was repeated. Following superfusion with control Krebs solution, nifedipine (10 µmol/L) was added to the perfusate and the pressure ramp was repeated to determine the passive diameter of the artery at each pressure step.

**Myocytes isolation from rat mesenteric and cerebral arteries:** Rats were killed as described above and the mesenteric arterial bed and brain were carefully removed and placed in an ice-cold dissection solution (SMDS) containing (in mmol/L): NaCl 120, NaHCO₃ 25, KCl 4.2, KH₂PO₄ 0.6, MgCl₂ 1.2 Glucose 11, CaCl₂ 0.01. Short segments of 2nd and 4th order RMA as well as rat cerebral arteries (RCA; including basilar, middle, and posterior cerebral arteries) were dissected free of connective tissue and single myocytes dispersed from the tissue samples using an isolation method modified from Porter et al., 1998. briefly,
arterial segments were placed in calcium-free SMDS containing papain (0.1-2.0 mg/ml; Worthington Biochemical Corporation, New Jersey), bovine serum albumin (BSA; 1-2 mg/ml; Sigma) and dithiothrietol (1 mg/ml Sigma) for 10-15 minutes at 37°C. The tissues were then washed in SMDS and placed in SMDS containing collagenase (1 mg/ml of a mixture of types F and H in a ratio of 70:30; Sigma) and BSA (1 mg/ml) for 10-15 minutes at 37°C. The tissues were then washed in ice-cold SMDS, triturated using a wide-bore glass pipette and kept on ice until used. This method consistently produced large numbers of long spindle-shaped, relaxed myocytes (see figure 4 in manuscript) which reversibly responded to agonist (phenylephrine) stimulation.

In some experiments, RMA myocytes were prepared by methods employed in other studies. However, the cells obtained were not relaxed, rather they consistently had a contracted, over-digested appearance (as is apparent in figure 4 in the manuscript).

**Patch clamp electrophysiology:** Whole-cell currents due to K<sub>DR</sub> channels of RMA myocytes and heterologous expression of Kv subunits in HEK293 cells were recorded by standard whole-cell patch clamp technique and analyzed as previously described. The pipette solution contained (in mmol/L): potassium gluconate 110, KCl 30, Na<sub>2</sub>ATP 5, MgCl<sub>2</sub> 0.5, BAPTA 10, N-[hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) 5 and GTP 1 (pH 7.2) and recordings were made using Krebs or HEPES-buffered (in mmol/L; NaCl 120, NaHCO<sub>3</sub> 3, KCl 4.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgCl<sub>2</sub> 0.5, Glucose 10, HEPES 10 and CaCl<sub>2</sub> 1.8) external saline solution. Cell capacitance was determined for each myocyte and employed to normalize all current amplitude data for cell size. Data were compared by paired Student’s t test or repeated measures ANOVA followed by Dunnett’s post hoc test. A level of P<0.05 was considered to be statistically significant.

**Molecular biology:** Standard methods for RT-PCR were used as previously described.
Specific primers for Kv1α (Kv1.1 to 1.6) and Kvβ (Kvβ1.1, β1.2, β1.3 and Kvβ2.1) subunits were used for RT-PCR of mRNA isolated from intact 4th order RMA and cerebral arteries or, where stated, from 200-300 enzymatically dissociated and individually selected VSM cells of 4th order RMA (see table 1 for primers). Rat or mouse brain mRNA was used as a positive control to confirm the utility of all primers and integrity of each RT-PCR reaction performed. All PCR products were sequenced to confirm their identity. The presence of contaminating RNA from neurons and endothelial cells was assessed using primers for the neuronal K+ channel, Erg37 and endothelial constrictor peptide, endothelin-1 (ET-1), respectively.

Real time PCR analysis of transcript abundance relative to β-actin message was performed with a Bio-Rad iCycler. Intact 1st/2nd order conduit (combined), as well as 4th order resistance vessels were collected from each rat (n = 4), cleaned of fat and placed in RNase- and DNase-free collection tubes. Total RNA was extracted using an RNeasy Mini kit with DNase treatment (Qiagen, Valencia, CA, USA) and first strand cDNA synthesized using the Sensiscript RT kit (Qiagen) with oligo d(T) primer. To optimize the specificity of all reactions, real-time PCR was initially performed on each primer set using rat brain cDNA, SYBR-Green (Qiagen) and a range of annealing temperatures between 52 and 62°C. On the basis of melt curve analysis, one ml of each reaction product was placed on a DNA 500 lab chip and examined using an Agilent Technologies 2100 Bioanalyzer (online Figure 1). A single peak of appropriate size was obtained for each reaction product with no evidence of additional amplicons. A second aliquot of product was then electrophoresed on a 1.5% (w/v) agarose gel, extracted using a gel extraction kit (Qiagen) and sequenced by the University of Calgary Core DNA facility to confirm the identity of the amplicon. Using the identified ideal annealing temperature for each reaction to be performed, real-time PCR reaction efficiency was then determined for all primer sets using serial dilutions of brain cDNA as a template. Only those
primer sets with efficiencies of >90% and not differing by more than 5% were employed in this study. The determined efficiencies at an annealing temperature of 58.2°C for the primer sets employed were: β-actin, 96.6%; Kv1.2, 99.6%; Kv1.5, 95.7%; Kv1.6, 95.9%; Kvβ1, 94.9%; Kvβ2, 95.3%. The optimal real-time PCR reaction consisted of a hot start at 95 °C for 15 min, followed by 40 cycles of 94°C for 15 s, 58.2°C for 30 s and 72°C for 30 s. A melt curve analysis was performed for each reaction and found to yield a single peak and a lack of evidence of dimer formation (Online Figure 2). Threshold cycle was then determined using the Bio-Rad iCycler and software provided with the instrument (Online Figure 3).

Quantification of transcript abundance in 1st/2nd and 4th order RMA was accomplished using the \(2^{\Delta\Delta Ct}\) method\(^9\) and β-actin expression as a reference for cDNA level normalization. Forward and reverse primers used for quantitative PCR in this study are indicated in table 2.

**Immunocytochemistry:** Identification of Kv1 subunit protein expression was performed as previously described\(^{17}\) using commercial antibodies against Kv1.1 (Calbiochem; Upstate Biotechnology; Chemicon), Kv1.2 and Kv1.4 (Upstate Biotechnology), as well as Kv1.5 and Kv1.6 (Alomone laboratories).
Online Supplement References


Online Figure Legends

**Online Figure 1.** Amplicon characterization of β-actin, Kv1 and Kvβ primer sets for real-time PCR by Agilent Technologies 2100 Bioanalyzer. Single amplicons for each of the primer sets used in the study with indicated size in bp were generated. The small peaks on the left and right in each panel indicate the low and high bp standards.

**Online Figure 2.** Melt curve analysis of β-actin, Kv1 and Kvβ primer sets used for real-time PCR. Red tracings are control curves with no template added. Note lack of evidence of dimer formation for all the primer sets.

**Online Figure 3.** Representative threshold cycle determinations for Kv1 (left panels) and Kvβ (right panels) subunits of 1<sup>st</sup> (top) and 4<sup>th</sup> (bottom) order RMA. Threshold cycle was also determined for β-actin in each experiment for normalization of transcript abundance via the $2^{\Delta\Delta Ct}$ method. Dashed line represents threshold.
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**Table 1:** Forward and reverse primers used for RT-PCR of intact RMA and/or isolated RMA myocyte mRNAs

<table>
<thead>
<tr>
<th></th>
<th><strong>Forward (5'-3')</strong></th>
<th><strong>REVERSE (5'-3')</strong></th>
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<tr>
<td>Kv1.1 (720 bp)</td>
<td>GCATCGACAAACACCACGGTC</td>
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<td>AAGGGGCTGACAGATCCTG</td>
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<td>Kv1.6 (1612 bp)</td>
<td>TAACGGGAATTCATGGATCGGAGAAATCCCT</td>
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<tr>
<td>Erg3 (429 bp)</td>
<td>CCCAAGGTTAAAGAGAGGGACACA</td>
<td>AGCGGCACCATATTATGAGTATC</td>
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Table 2: Forward and reverse primers used for quantitative real-time PCR of intact RMA mRNAs.

<table>
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<tr>
<th></th>
<th>Forward (5'-3')</th>
<th>REVERSE (5'-3')</th>
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<td>Kv1.5: (96 bp)</td>
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<td>Kv1.6: (92 bp)</td>
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<td>Kvβ1: (110 bp)</td>
<td>TCAACCAAGGCATGGCAATG</td>
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<td>Kvβ2: (123 bp)</td>
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<td>β-Actin: (93 bp)</td>
<td>TATGAGGTCACGCCTCCC</td>
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