Molecular Composition of 4-Aminopyridine–Sensitive Voltage-Gated K⁺ Channels of Vascular Smooth Muscle

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Abstract—Voltage-gated K⁺ channels (Kv) play a critical role in regulating arterial tone by modulating the membrane potential of vascular smooth muscle cells. Our previous work demonstrated that the dominant 4-aminopyridine (4-AP)–sensitive, delayed rectifier Kv current of rabbit portal vein (RPV) myocytes demonstrates similar 4-AP sensitivity and biophysical properties to Kv1α-containing channels. To identify the molecular constituents underlying the 4-AP-sensitive Kv current of vascular myocytes, we characterized the expression pattern of Kv1α subunits and their modulatory Kvβ subunits in RPV. The mRNAs encoding pore-forming subunits Kv1.2, Kv1.4, and Kv1.5 were detected by reverse transcriptase–polymerase chain reaction (RT-PCR), whereas Kv1.1, Kv1.3, and Kv1.6 transcripts were undetectable. Kvβ1.1, β1.2, β1.3, β2.1, and β2.2 messages were expressed, whereas Kvβ3.1 and β4 mRNAs were undetected by RT-PCR. Kv1.2, Kv1.4, Kv1.5, Kvβ1.2, β1.3, and β2.1 proteins were detected in RPV by Western blotting and/or immunocytochemistry of freshly isolated myocytes. We provide the first evidence, from coimmunoprecipitation studies, for the formation of heteromultimeric Kv channel complexes composed of Kv1.2, Kv1.5, and Kvβ1.2 subunits in vascular smooth muscle. (Circ Res. 2001;89:1030-1037.)

Key Words: vascular smooth muscle  ■  Kv1.5  ■  Kv1.2  ■  Kvβ subunits  ■  voltage-gated K⁺ channel

Control of arterial diameter and peripheral vascular resistance is dependent on the development of tone by vascular smooth muscle cells. Membrane potential of vascular myocytes is a major determinant of tone development, as it controls the magnitude of steady-state Ca²⁺ influx across the membrane through voltage-gated Ca²⁺ channels. The level of membrane potential is the result of a dynamic balance between opposing inward (depolarizing) and outward (hyperpolarizing) ionic currents. Hyperpolarizing K⁺ current in vascular myocytes is primarily due to the activity of four classes of K⁺ channels: inward rectifier, ATP-sensitive, long-conductance Ca²⁺-activated, and voltage-gated K⁺ (Kv) channels. Alterations in K⁺ channel activity modulate the level of membrane potential and, thereby, cytosolic free Ca²⁺ concentration and contractile filament activation.

Voltage-dependent outward current of vascular myocytes is due to at least three components: a slowly inactivating 4-aminopyridine (4-AP)–sensitive delayed rectifier (Kᵥₒᵣ) current; a 4-AP–insensitive outward current; and a rapidly inactivating, 4-AP–sensitive transient A-type current (Kᵥₐᵣ). 4-AP–sensitive Kᵥₒᵣ channels are thought to contribute to the control of membrane potential in vascular myocytes. This view is supported by (1) electrophysiological evidence that Kᵥₒᵣ channels are active at voltages consistent with the membrane potential of vascular myocytes and (2) pharmacological evidence indicating that submillimolar 4-AP inhibits Kᵥₒᵣ currents of vascular myocytes and elicits depolarization and contraction of intact blood vessels. Moreover, the activity of vascular 4-AP–sensitive Kᵥₒᵣ channels is modulated by agonists that signal through protein kinases A and C and may contribute to the physiological regulation of arterial diameter by locally released or circulating vasoactive factors.

The molecular identity of vascular 4-AP–sensitive Kᵥₒᵣ channels is not established, but recent reports describe the expression of several transcripts encoding Kv channel subunits in vascular tissues. Kv channels are composed of pore-forming Kvα and modulatory Kvβ subunits. Kvα proteins with six transmembrane segments are encoded by nine related families, Kv1 to Kv9, within the superfamily of Kv channel genes. Kv1 to Kv4 proteins assemble to form homotetrameric channels, or coassemble with members of the same family to produce heterotetrameric channel complexes. Subunit proteins of the Kv5 to Kv9 families do not form functional channels, but they coassemble with Kv2 or Kv3 subunits into functional complexes with unique properties. Additional functional diversity and appropriate trafficking to the membrane are obtained by association of the
Kv1α subunits with Kvβ subunits.\textsuperscript{20–23} Four genes encoding Kvβ subunits, Kvβ1 to 4, as well as splice variants of the Kvβ1 and β2 genes, have been identified.\textsuperscript{20–23} The assembly pattern of Kvα and β subunits provides a mechanism for obtaining diverse populations of channels with unique properties for appropriate cellular function.

Approximately 70% of the \( K_{\infty} \) current, as well as the \( K_{\text{TO}} \) current, of rabbit portal vein (RPV) myocytes is inhibited by 4-AP with an IC\(_{50}\) in the submillimolar range.\textsuperscript{3,5,8,9,11,24} This level of 4-AP sensitivity and the biophysical properties of the currents are consistent with those previously identified for Kv1-containing,\textsuperscript{11,17,22} current patterns of Kv1 and Kv2–4-containing,\textsuperscript{26–31} Kv channels. The properties of whole-cell and unitary currents due to expression of Kv1.5 cloned from RPV closely mimicked those of native RPV K\( \text{DR} \) channels.\textsuperscript{11} However, homomultimeric Kv1.5 channels do not share functional identity with vascular \( K_{\infty} \) because (1) differences in rate and voltage dependence of inactivation are apparent;\textsuperscript{15} (2) RPV Kv1.5 channels in inside-out membrane patches of mouse L cells or human embryonic kidney 293 (HEK) cells do not exhibit modulation by purified protein kinase A in the presence of ATP;\textsuperscript{32} and (3) 4-AP treatment is associated with a positive shift in the voltage dependence of activation of native \( K_{\infty} \), but not Kv1.5 channels, as described in another study published in this issue of Circulation Research by Kerr et al.\textsuperscript{24} This lack of functional identity indicates that Kv1.5 cannot be the only Kv1 subunit contributing to K\( \text{DR} \) channels of vascular myocytes.

In this study, reverse transcriptase–polymerase chain reaction (RT-PCR), Western blotting, and immunocytochemistry were performed to identify the expression profile of Kv1 and Kvβ subunits in RPV. Furthermore, the pattern of subunit coassembly was assessed by coimmunoprecipitation. We demonstrate expression in RPV of Kv1.2, Kv1.4, and Kv1.5 pore-forming subunits, as well as Kvβ1 and β2 modulatory subunits. Additionally, we present the first direct evidence that Kv1.2 and Kv1.5 coassemble to form heteromultimeric Kv channels in vascular myocytes.

**Materials and Methods**

Standard methods for RT-PCR, antibody production, immunocytochemistry, Western blotting, and immunoprecipitation were used. Full-length clones were prepared for Kv1.2, Kvβ1.1, β1.2, β1.3, β2.1, and β2.2 of RPV via RT-PCR for expression in HEK cells. GenBank accession numbers are the following: for Kv1.2, AF284420; for Kvβ1.1, AF131934; for β1.2, AF131935; for β1.3, AF131936; for β2.1, AF247701; and for β2.2, AF247702. Western blotting, immunocytochemistry, and immunoprecipitation were performed using commercial antibodies against Kv1.2, Kv1.4, and Kv1.6 (Upstate Biotechnology), as well as Kv1.5 (Alomone Laboratories). Novel peptide-directed rabbit polyclonal antibodies against RPV β1.1, β1.2, β1.3, β2.1, and β2.2 raised in conjunction with Macromolecular Resources (Colorado State University, Fort Collins, Colo) were also used after affinity purification. The rabbit Kv1.2- and Kvβ-subunit amino acid sequences and details of all methods used are included in the online data supplement available at http://www.circres.saha.org.

**Results**

**RT-PCR for Kv1α and Kvβ Subunits of RPV**

Specific primers for Kv1α subunits (Kv1.1 to 1.6) were used for RT-PCR of mRNA isolated from RPV. Rat or mouse brain mRNA was utilized as a positive control to confirm the function of all primers and integrity of each RT-PCR reaction performed. All PCR products were sequenced to confirm their identity. RT-PCR products of appropriate size for Kv1.2 (1500, 1418, 449, and 367 bp), Kv1.4 (188 bp), and Kv1.5 (923 and 591 bp) were obtained from RPV mRNA after 35 cycles of PCR (Figure 1). The RT-PCR product representing the full-length coding sequence of RPV Kv1.2 was cloned into pcDNA3 for use in subsequent heterologous expression experiments. The amino acid sequence of rabbit Kv1.2 exhibits a very high level of identity with Kv1.2 expressed in other species (online Figure 1, available at http://www.circres.saha.org). Products for Kv1.1 (710 bp), Kv1.3 (653 bp), and Kv1.6 (1590 bp) were detected utilizing brain mRNA after 35 cycles, but not in RPV, mRNA. D, 188-bp product of Kv1.4 was detected in rat brain and RPV mRNA. E, 923- and 591-bp products of Kv1.5 were detected in RPV mRNA. F, 1580-bp product of Kv1.6 was detected in rat brain, but not in RPV, mRNA.

![Figure 1. Kv1α transcript expression by RPV.](http://www.circre-saha.org) A, 710-bp product of Kv1.1 was detected by RT-PCR in rat brain (RB), but not in RPV, mRNA. B, 1500-, 1418-, 449-, and 367-bp products of Kv1.2 were detected in rat brain and RPV mRNA. C, 653-bp product of Kv1.3 was detected in mouse brain (MB), but not in RPV, mRNA. D, 188-bp product of Kv1.4 was detected in rat brain and RPV mRNA. E, 923- and 591-bp products of Kv1.5 were detected in RPV mRNA. F, 1580-bp product of Kv1.6 was detected in rat brain, but not in RPV, mRNA.
N-termini (online Figure 2), as previously described for Kvβ subunits cloned from other species. A novel 2.2 splice variant that lacked 14 amino acids within the N-terminus of 2.1 was identified in RPV, and was subsequently reported to be expressed in glioma and astrocytes. We consistently noted the additional presence of a 435-bp product when using the 2 primers (Figure 2A, arrow). This product was sequenced and found to correspond to the 3 end of the Kv1 coding sequence. RT-PCR for 3.1 and 4 subunits yielded 1213-bp and 750-bp products, respectively, from brain mRNA, but not from RPV (Figure 2), even after 70 cycles of PCR.

Figure 2. Kvβ transcript expression by RPV. A, 1206-, 1277-, and 1260-bp products for Kvβ1.1, β1.2, and β1.3, as well as 1102- and 1062-bp products of Kvβ2, were detected by RT-PCR in RPV mRNA. Arrow indicates 435-bp product of Kvβ1.2 detected using Kvβ2 primers. B, 1213-bp product of Kvβ3.1 was detected in rat brain (RB), but not in RPV, mRNA. C, 750-bp product of Kvβ4 was detected in mouse brain (MB), but not in RPV, mRNA.

Western Blotting for Kvα and Kvβ Subunits
Commercial antibodies were used to confirm the expression in RPV of Kv1.2, Kv1.4, and Kv1.5 subunit proteins. Western blotting experiments were conducted using HEK cells transfected with Kv1.2, Kv1.4, or Kv1.5 cDNA to confirm the specificity of the antibodies (online Figure 3, available at http://www.circresaha.org). Immunoreactive bands of appropriate molecular weight were apparent in transfected, but not untransfected, HEK cells. Multiple bands were observed for Kv1.4 and Kv1.5 and may represent products with different posttranslational modification. Blotting of RPV protein with anti-Kv1.5 revealed an immunoreactive band in RPV with a molecular weight similar to that of the Kv1.5 products expressed in HEK cells, and the immunoreactivity was eliminated by competition with antigenic peptide (Figure 3). Blotting of RPV protein for Kv1.2 and Kv1.4 failed to detect specific immunoreactivity to these two subunits (data not shown). However, the presence of Kv1.2 subunit in RPV protein was subsequently detected by immunoprecipitation followed by Western blotting (see below).

Three peptide-directed rabbit polyclonal antibodies were raised to specifically recognize the splice variants of the rabbit Kvβ1 gene based on the unique N-terminal domains of β1.1, β1.2, and β1.3 (online Figure 2). Two additional antibodies were raised, one specific for Kvβ2.1 based on the 14-amino acid sequence present in β2.1, but not β2.2, and a Kvβ2 antibody raised against a peptide based on the common region of the N-termini of Kvβ2.1 and β2.2, but not present.

Figure 3. Western blots for RPV Kv1.5, Kvβ1.2, and β2.1. A, Anti-Kv1.5 detected Kv1.5 (arrow) in RPV. Immunoreactive bands were absent after antigenic peptide preabsorption (+peptide). B, Kvβ1.2 was detected in RPV. Immunoreactive bands were absent after preabsorption with respective antigenic peptide (+peptide). C, Kvβ2.1 and β2.2 were detected in RPV. Immunoreactive bands were absent after preabsorption with antigenic peptide (+peptide).

Figure 4. Western blotting for Kvβ subunits of HEK cells. Anti-Kvβ1.1 (A), anti-β1.2 (B), anti-β1.3 (C), anti-β2 (D), and anti-β2.1 (E) immunoreactive bands (arrows) were detected in protein of transfected, but not untransfected, HEK cells, with the exception of the band detected in HEK cells with anti-Kvβ2.1 (E). E, Right panel, Lack of anti-β2.1 immunoreactive bands in HEK cells transfected with Kvβ2.1 or β2.2 and untransfected HEK cells after preabsorption with respective antigenic peptide.
in the Kvβ1 subunits (online Figure 2). The antibodies were first utilized for Western blotting of HEK cells expressing the RPV Kvβ subunit clones (Figure 4). No cross-reactivity of the Kvβ antibodies was apparent (data not shown). Kvβ1.1, β1.2, and β1.3 antibodies recognized proteins of ≈36 kDa in transfected, but not in untransfected, HEK cells, and the immunoreactivity was eliminated by competition with antigenic peptide (Figures 3 and 4). Small differences in molecular weight were apparent, as expected on the basis of the distinct amino acid sequences of these subunits (online Figure 2). Anti-Kvβ2 recognized a band in lysates of both Kvβ2.1- and β2.2-transfected, but not in untransfected, HEK cells (Figure 4). These bands were at a lower molecular weight than those identified with anti-Kvβ1, consistent with the different amino acid sequences of the Kvβ2 splice variants and Kvβ1 subunits (online Figure 2). Western blotting with anti-Kvβ2.1 detected a band of ≈33 kDa in HEK cells expressing Kvβ2.1, consistent with the molecular weight observed with anti-Kvβ2 (Figure 4). However, an identical band was also observed with anti-Kvβ2.1 in HEK cells transfected with Kvβ2.2, and in untransfected HEK cells (Figure 4) that were reported to lack endogenous Kvβ expression. Preabsorbing anti-Kvβ2.1 with antigenic peptide eliminated the immunoreactive band in extracts of cells transfected with Kvβ2.1, as well as the band in Kvβ2.2-transfected and untransfected HEK cell extract (Figures 3 and 4). RT-PCR and PCR product sequencing confirmed the endogenous expression of both Kvβ2 splice variants in untransfected HEK cells (data not shown). This Kvβ2 subunit expression by HEK cells may reflect differences in passage number or culture conditions compared with those used previously.

Kvβ1.2 and β2.1 proteins were detected by Western blotting of RPV lysate with antibodies to Kvβ1.2 and β2.1, respectively. Immunoreactive bands corresponding to the proteins identified in transfected HEK cells were observed and eliminated by preabsorption with the respective antigenic peptides (Figure 3). Western blotting with antibodies to Kvβ1.1, β1.3, and β2 did not demonstrate specific immunoreactivity in RPV protein extracts. This may be due to the relatively lower titer of these three antibodies observed in transfected HEK cells (data not shown).

**Immunocytochemistry of RPV Kv1α and Kvβ Subunits**

We previously detected expression of Kv1.5 in isolated RPV myocytes by immunocytochemistry. In this study, we demonstrate immunolabeling of myocytes with antibodies to Kv1.2, Kv1.4, and the Kvβ1.2 and β1.3 subunits. The specificity of the monoclonal Kv1.2 and Kv1.4 antibodies was first determined: HEK cells were cotransfected with cDNAs encoding the subunits and green fluorescent protein (GFP), the latter a marker of successfully transfected cells. The Kv1.2 and Kv1.4 antibodies recognized the expression of the subunits, as indicated by the consistent correlation of Cy3 secondary antibody immunofluorescence with GFP fluorescence in the HEK cells and lack of cross-reactivity of anti-Kv1.2 and anti-Kv1.4 with Kv1.4- and Kv1.2-transfected cells, respectively (online Figure 4, available at http://www.circresaha.org). On the basis of these control experiments, the antibodies were then used to identify the expression of Kv1.2 and Kv1.4 protein in RPV myocytes (Figure 5). Immunofluorescence was not detected in cells exposed to secondary antibody alone (Figure 5).

The function of our Kvβ antibodies for immunocytochemical detection of subunit expression was assessed. The Kvβ1.2, β1.3, and β2 antibodies recognized Kvβ1.2, β1.3, and β2.1/β2.2 expression, respectively, in HEK cells; GFP fluorescence and immunofluorescence were correlated and the immunolabeling was eliminated by preabsorption with antigenic peptide (Figure 6). The Kvβ1.1 and β2.1 antibodies were unable to identify β1.1 or β2.1 expression, respectively, and were not used in subsequent experiments on RPV myocytes. Exposure of RPV myocytes to Kvβ1.2 and β1.3 antibodies resulted in intense immunofluorescence that was blocked by peptide preabsorption (Figure 5). However, the anti-Kvβ2 immunostaining was not blocked by peptide preabsorption. This may be due to low antibody titer for identification of the subunit in HEK cells coupled with high background fluorescence and/or a low level of Kvβ2 protein expression in the myocytes compared with the overexpression in HEK cells.
Coimmunoprecipitation of Kv Subunits From RPV

The results of the preceding experiments indicated the expression of Kv1.2, Kv1.4, Kv1.5, Kvβ1.2, β1.3, and β2.1 transcripts and proteins in RPV. These subunits were reported to coassemble in other cell types, but whether similar heteromultimeric channels are present in smooth muscle has not been determined, although data consistent with this possibility exist. The ability of the Kv1.2, Kv1.5, and Kvβ1.2 antibodies to immunoprecipitate protein from lysates of HEK cells was confirmed by Western blotting with these antibodies (Figure 7). Specific subunit bands were not apparent in untransfected HEK cell lysates immunoprecipitated with the antibodies. Additionally, no cross-reactivity of the Kv1.2 and Kv1.5 antibodies was apparent; anti-Kv1.5 did not immunoprecipitate Kv1.2 subunits from HEK cells expressing Kv1.2, and anti-Kv1.2 did not immunoprecipitate Kv1.5 subunits from HEK cells expressing Kv1.5 (Figures 7B and 7D). Anti-Kv1.2, -β1.3, -β2, and -β2.1 failed to immunoprecipitate subunit protein from transfected HEK cells and were not used for analysis of RPV protein. However, bands of appropriate molecular weight for Kv1.2, Kv1.5, and Kvβ1.2 were identified in Western blots of immunoprecipitates of RPV protein obtained using Kv1.2, Kv1.5, and Kvβ1.2 antibodies, respectively (Figure 8). The bands for Kv1.2 and Kv1.5 were not observed when RPV protein was immunoprecipitated with anti-Kv1.6 (Figure 8), which is not ex-
pressed in RPV (Figure 1). Significantly, immunoprecipitation of RPV protein with anti-Kv1.2 or anti-Kvβ1.2, followed by blotting with anti-Kv1.5, detected the presence of Kv1.5 in both immunoprecipitates, and blotting with anti-Kvβ1.2 revealed the presence of this subunit in the immunoprecipitate obtained using anti-Kv1.5 (Figure 8).

Discussion

This study provides the first comprehensive examination of the expression of Kv1α- and Kvβ-subunit mRNAs and proteins, the first evidence for the expression of Kvβ-subunit proteins, and the novel finding of the heteromultimeric association of Kv1α and Kvβ proteins in vascular smooth muscle cells. This subunit association may be predicted to produce channels that possess functional properties that are distinct from homomultimeric channels, an issue that is addressed in the study by Kerr et al.24 The molecular biological, biochemical, and immunocytochemical data presented in this study indicate that Kv1.2 and Kv1.5 are the only delayed rectifier-type Kv1α pore-forming subunits expressed by RPV myocytes and that these subunits associate to form heteromultimeric Kv channels. In the study by Kerr et al.24 we present complementary evidence that whole-cell currents due to heteromultimeric, but not homomultimeric, channels composed of Kv1.2 and Kv1.5 possess functional identity with native 4-AP-sensitive K_{DR} channels of RPV.24 The combination of these observations provides compelling evidence that the dominant 4-AP-sensitive K_{DR} channel complex of RPV vascular myocytes is a heteromultimer consisting of Kv1.2 and Kv1.5 subunits.

This study provides novel insights into vascular Kv channel subunit composition. Kv gene expression has been studied previously using vascular and nonvascular smooth muscle tissues, and distinct patterns of expression of Kv subunit transcripts were reported for vessels of different vascular beds and for different regions within the gastrointestinal tract.10–16,36–39 We have shown that vascular myocytes of RPV express mRNAs encoding Kv1.2, Kv1.4, and Kv1.5 pore-forming subunits, as well as the modulatory Kvβ subunits β1.1, β1.2, β1.3, β2.1, and β2.2. The expression of Kv1.2, Kv1.4, and Kv1.5 in RPV is consistent with previous reports indicating the presence of transcripts encoding these subunits in several smooth muscle tissues.10–16,36–39 Moreover, transcripts encoding Kvβ subunits were also shown to be expressed in smooth muscle of pulmonary artery, mesenteric artery, and gastrointestinal tract.10,12,13,38 However, Kv1.1, Kv1.3, and Kv1.6 were reported to be expressed in pulmonary10 and/or mesenteric arteries,12,13 but were not detected in RPV. Additionally, splice variants of Kvβ1 (ie, β1.1 and β1.2) were found in smooth muscle cells of the gastrointestinal tract, but β2.1 expression was not detected.38 It seems likely that these differences reflect the presence of vessel- or tissue-specific patterns of Kv channel subunit expression. Differences in the voltage dependence of activation and inactivation, in the rates of inactivation, and in the pharmacology (eg, IC_{50} of 4-AP inhibition) of K_{DR} current of myocytes derived from different smooth muscle tissues have been reported.1,2,3,5,12 These differences may reflect vessel-specific expression of Kv1α and Kvβ subunits, the differential assembly of Kv1α family and Kvβ subunits into distinct populations of heteromultimeric channels containing varied mixtures of subunits, and/or the additional expression of Kvβ subunits of the Kv2 to 9 families.

However, substantive conclusions concerning Kv subunit expression in cells can only be achieved through the use of complementary techniques to identify the expression of mRNAs encoding the subunits and the subunit proteins themselves. In this study, RT-PCR was used to identify the expression in RPV of Kv1α and Kvβ subunits at the mRNA level. This identification was followed by Western blotting and/or immunoprecipitation experiments to identify the expression of subunit proteins and to demonstrate the ability of the antibodies to identify proteins of appropriate molecular weight. Finally, immunocytochemistry was used to identify the specific expression of Kv subunit proteins by freshly isolated myocytes. Parallel control experiments were also conducted using a heterologous cell type expressing the subunits to confirm the specificity of the antibodies used. This complementary approach minimizes the possibility of artifactual identification of subunit expression due to (1) contaminating mRNA derived from cell types, such as neurons (eg, Kv1.1) and lymphocytes derived from the blood (eg, Kv1.3); (2) nonspecific immunolabeling by Kv subunit antibodies; and (3) the use of phenotypically distinct, cell-cultured myocytes. In addition, in the Kerr et al study, we exploited a feature of 4-AP inhibition and lack of charybdotoxin sensitivity of native K_{DR} current to provide the first functional evidence for the presence of heteromultimeric channels composed of Kv1.2 and Kv1.5 subunits in vascular myocytes.

Vascular myocytes exhibit the following two components of Kv current that are suppressed by submillimolar 4-AP: rapidly inactivating K_{TO} and slowly inactivating K_{DR} currents.1–6,11,24 We attribute the K_{DR} component to the expression of Kv1.4, but it is unlikely that this subunit contributes substantially to the K_{DR} current. Whole-cell currents, as a result of the expression of Kv1.4 in heterologous cell types,25,40 exhibit several characteristics consistent with those of vascular K_{TO} currents.1,6 For example, the inactivation kinetics are identical and the activation threshold of the K_{TO} current at −65 mV is consistent with that reported for Kv1.4 current,25,40 but inconsistent with the properties of fast-inactivating Kv3 and Kv4 channels that activate at more positive voltages.27–30 The view that Kv1.4 subunits have a minimal contribution to K_{DR} channel complex(es) of RPV myocytes is suggested by the ability of even a single Kv1.4 subunit to induce rapid inactivation of channels involving the association of Kv1.4 with Kv1.2 or Kv1.5 subunits.25

This study provides evidence that Kv1.2 and Kv1.5 are the primary Kv1α subunits contributing to 4-AP-sensitive K_{DR} channels of RPV myocytes. Kv1.2 and Kv1.5 are the only Kv1α subunits expressed in RPV that display delayed activation and slow inactivation.11,17,22–25,36 The dominant component of Kv current in RPV myocytes exhibits half-maximal activation at ≈−15 mV and inactivates over a slow time course that occurs with time constants of ≈0.5 and 3 seconds.2,4,5,8,9,11 These properties are similar to those of homo- and heterotetrameric Kv1.2 and Kv1.5 chan-
nels.11,17,22–26,36 The coimmunoprecipitation of Kv1.2 and Kv1.5 from RPV provides the first evidence for the association of these subunits in vascular myocytes. The data presented in this study indicate, therefore, the potential contribution of homomultimers of Kv1.2 and Kv1.5, as well as Kv1.2 and Kv1.5 heteromultimeric channels. However, the data cannot determine whether both homo- and heteromultimeric channels are present in the myocytes. In the Kerr et al24 study, we determined whether both homo- and heteromultimeric channels or that the inactivation ball is immobilized. For example, the inactivation rate of native whole-cell KDR currents of RPV myocytes is immobilized by some other-as-yet unidentified protein(s).10

In conclusion, we have identified the expression of Kv1.2, Kv1.4, Kv1.5, β1.2, β1.3, and β2.1 mRNA and protein in RPV smooth muscle cells. We also provide the first evidence for the formation of heteromultimeric Kv channels in vascular myocytes. The data presented here combined with the pharmacological evidence in the Kerr et al24 study, indicate that the 4-AP–sensitive KDR current of RPV is predominantly due to heteromultimeric channels composed of Kv1.2 and Kv1.5. In light of the uniform expression of these subunits by myocytes of several vascular and nonvascular smooth muscle tissues, as well as cardiac muscle,10–14,16,17,37–39 it seems likely that there is a widespread distribution of KDR channels with identical Kv1.2 and Kv1.5 subunit composition and functional properties in these cell types.

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References


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ONLINE DATA SUPPLEMENT

**RT-PCR and Cloning:** New Zealand white rabbits were injected in the ear artery with a lethal dose of halothane, prior to removal of the portal vein. Portal veins were dissected free of connective tissue and the endothelium was removed prior to flash freezing in liquid nitrogen. A Poly-A-Pure kit (Ambion) was utilized to extract mRNA from RPVs and freshly isolated rat or mouse brains, followed by RT-PCR with a Retroscript kit (Ambion) using SuperTaq Plus (Ambion) and subunit specific primers. Primers were designed for conserved regions (differing by 4 or less nucleotides) based upon the DNA sequences of multiple species aligned utilizing Genetic Computer Group software (Ver. 9). They were as follows (5'-3'):

- **Kv1.2**
  
  GCTAGAAGCTTATGACAGTGGACTACCAGGAA
  ATACGAGATCTTCAGACATCAAGTTACACATTTGG,  **Kv1.5**
  TGGCGCTTTGAGACGCAGCT
  GCCCAGCTCCCCCATGGA, AAGGGGCTGCAGATCTCTG, TCACAACTCGGGTTCCGGCT
  **Kv1.6**
  AACTGGGATATCTCAAACCTCGGTAGCATTCT, TAACGGAAATTCATAGATCGGAGAAAATCCCT,  **Kvβ1.1**
  CCGTGGAAATTCATGCAAGTCTCCCATAGCCTG,  **Kvβ1.2**
  GGCTGCAATTCCATGATCTGAATCTGCTATAAACCTGCC,  **Kvβ1.3**
  GATTAGAAATTCATGCTGCGACGCCGGAC,  **Kvβ2**
  GCACGAATTCATGATCCGGAATCAACCAC,  **Kvβ1/β2**
  GTCCGAGATATCTCTATGCTATAGTCCCTTICTGC,  **Kvβ3.1**
  CTGGAGAAATTCTCGAGGTGTCTATCGGC,  **Kvβ4**
  GCCGAGAATTCATGTCAGAGGGATAGGCTTG, and  **Kvβ3/4**
  GCCAAGATATCCTCTTTTTTGGTGGATCCATT.

Additional primers for Kv1.1, Kv1.2, Kv1.3 and Kv1.4 were as previously described.¹

All primers were designed based on pile-ups of the known sequences of Kv1α and Kvβ subunits from different species. The regions of the sequences chosen for the primers differed by less than 5 nucleotides within the interior of the primer sequence. For this reason failure to identify product following 70 cycles of PCR was likely not due to species-specific variation in the rabbit subunit sequences.
Full-length clones were prepared for Kv1.2, Kvβ1.1, β1.2, β1.3, β2.1 and β2.2 via RT-PCR using primers to the ends of the 5' and 3' coding regions. 5' and 3' imposed primer sequences including stop and start codons were then verified by 5' and 3' RACE. GenBank accession numbers are as follows: Kv1.2: AF284420; Kvβ1.1: AF131934; Kvβ1.2: AF131935; Kvβ1.3: AF131936; Kvβ2.1: AF247701; and Kvβ2.2: AF247702.

**Antibody Production:** Peptide-directed rabbit polyclonal antibodies were raised against RPV Kvβ1.1, β1.2, β1.3, β2.1 and β2.2 subunits. Peptides with the following sequences were synthesized by the Peptide Synthesis Facility (University of Calgary): Kvβ1.1 (CIACTEHNKLKSRNGEDRLLSSKQS), β1.2 (CADIPSPKLGLPKSSESALK), β1.3 (CSGGSKDRSPKKEVENKDS), β2.1 (CYSTRYGSPKRQLQF) and β2.2 (CMYPESTTGSRARLSLRQTGSPGMI), conjugated to KLH and injected into rabbits by Macromolecular Resources (University of Colorado). Antisera was purified by HiTrap Protein A (Pharmacia) followed by affinity chromatography against their respective antigenic peptides coupled to Sulfolink resin (Pierce).

**Immunocytochemistry:** RPV smooth muscle myocytes were isolated using methods previously described.² HEK 293 cells were co-transfected with cDNAs encoding the Kv1α and Kvβ subunits in pcDNA3 and a cDNA encoding green fluorescent protein utilizing Fugene 6 (Boehringer Mannheim). Immunocytochemistry was performed on freshly isolated RPV myocytes and 24 hour post-transfected HEK cells. Cells were washed with phosphate-buffered saline (PBS), fixed for 20 min with 3.7% formaldehyde, and permeabilized for 5 min with 0.1% Triton X-100. Cells were then washed with PBS, blocked for 1 h in 1% bovine serum albumin (BSA)/PBS, and labeled overnight at 4 °C with primary antibodies
in 0.5% BSA/PBS. Cells were washed with 0.5% BSA/PBS and labeled for 1 hr with anti-rabbit or anti-mouse IgG-conjugated Cy3 secondary antibodies (Jackson Labs). Unbound secondary was then washed prior to visualizing immunolabeled cells.

**Western blotting and immunoprecipitation:** HEK 293 cells were harvested and RPV tissues were homogenized in extraction buffer containing: 20 millimole/L Tris pH 7.5, 138 millimole/L NaCl, 3 millimole/L KCl, 1 millimole/L ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 2 millimole/L [ethylene dinitrilo]tetraacetic acid, 1 millimole/L benzamidine, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin A, 1 millimole/L phenylmethyl-sulfonylfuoride, 1 millimole/L dithiothreitol and 1% Triton X-100, followed by centrifugation at 16 000 x g. The protein content of supernatants was determined (DC kit, BioRad) prior to SDS-PAGE on 7.5-15 or 7.5-20 % polyacrylamide gels. Proteins were transferred to 0.2 μm nitrocellulose membranes, which were blocked with 5% non-fat dried milk (NFDM) in 0.05% Tween-20 Tris buffered saline (TBST). Membranes were labeled with primary antibodies in 1% NFDM TBST and were washed with 0.05% TBST prior to labeling with 1:10000 or 1:5000 anti-rabbit IgG-HRP or anti-mouse IgG-HRP, respectively (Chemicon). Unbound secondary antibody was washed from membranes prior to exposure to Kodak XB-1 blue film. For immunoprecipitation experiments all incubations were performed while tumbling at 4° C. RPV and HEK cell protein extracts were pre-cleared for 1 h with 25 μL of Protein A Sepharose (Pharmacia) and incubated overnight with 5 μg of antibody. Antibody-bound Kv channel complexes were then captured by the addition of 25 μL of Protein A Sepharose and incubated for 2 h to facilitate binding. Protein A Sepharose was spun down at 2000 x g, washed twice with extraction buffer, and once with Triton X-100 free extraction buffer. Immunoprecipitated channel complexes were then
eluted from the beads using SDS-PAGE sample buffer prior to electrophoresis and Western blotting.
ONLINE SUPPLEMENT REFERENCES


ONLINE FIGURE LEGENDS

Figure 1: Comparison of RPV Kv1.2 amino acid sequence with Kv1.2 of other mammalian species. Sequence differences are shown in yellow.

Figure 2: Amino acid sequences of RPV Kvβ1 and β2 subunits. Full length coding sequences of rabbit Kvβ1.1, β1.2, β1.3, β2.1 and β2.2 are indicated. Yellow indicates differences in amino acid sequence and blue highlights the sequences of the peptides utilized to generate the subunit-specific rabbit polyclonal antibodies employed in this study.

Figure 3: Western blotting for Kv1.2, Kv1.4 and Kv1.5 subunits in HEK cells. (A) Anti-Kv1.2 blotting of lysate of Kv1.2 transfected and untransfected HEK cells. Arrow indicates Kv1.2 band. (B) Anti-Kv1.4 blotting of lysate of Kv1.4 transfected and untransfected HEK cells. Arrow indicates multiple Kv1.4 bands. (C) Anti-Kv1.5 blotting of lysate of Kv1.5 transfected and untransfected HEK cells. Arrow indicates multiple bands for Kv1.5.

Figure 4: Immunocytochemistry of Kv1α and GFP co-transfected HEK cells. (A & B) GFP and anti-Kv1.2 immunofluorescence of cells successfully co-transfected with GFP and Kv1.2. (C & D) GFP fluorescence but lack of anti-Kv1.2 immunostaining of HEK cells co-transfected with GFP and Kv1.4. (E & F) GFP and anti-Kv1.4 immunofluorescence of cells successfully co-transfected with GFP and Kv1.4. (G & H) GFP fluorescence but lack of anti-Kv1.4 immunostaining of HEK
cells co-transfected with GFP and Kv1.2.
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