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Abstract—Small arteries play an essential role in the regulation of blood pressure and organ-specific blood flow by contracting in response to increased intraluminal pressure, ie, the myogenic response. The molecular basis of the myogenic response remains to be defined. To achieve incremental changes in arterial diameter, as well as blood pressure or organ-specific blood flow, the depolarizing influence of intravascular pressure on vascular smooth muscle membrane potential that elicits myogenic contraction must be precisely controlled by an opposing hyperpolarizing influence. Here we use a dominant-negative molecular strategy and pressure myography to determine the role of voltage-dependent Kv1 potassium channels in vasoregulation, specifically, whether they act as a negative-feedback control mechanism of the myogenic response. Functional Kv1 channel expression was altered by transfection of endothelium-denuded rat middle cerebral arteries with cDNAs encoding c-myc epitope-tagged, dominant-negative mutant or wild-type rabbit Kv1.5 subunits. Expression of mutant Kv1.5 dramatically enhanced, whereas wild-type subunit expression markedly suppressed, the myogenic response over a wide range of intraluminal pressures. These effects on arterial diameter were associated with enhanced and reduced myogenic depolarization by mutant and wild-type Kv1.5 subunit expression, respectively. Expression of myc-tagged mutant and wild-type Kv1.5 subunit message and protein in transfected but not control arteries was confirmed, and isolated myocytes of transfected but not control arteries exhibited anti-c-myc immunofluorescence. No changes in message encoding other known, non-Kv1 elements of the myogenic response were apparent. These findings provide the first molecular evidence that Kv1-containing delayed rectifier K^+ (K_{DR}) channels are of fundamental importance for control of arterial diameter and, thereby, peripheral vascular resistance, blood pressure, and organ-specific blood flow. **(*Circ Res*. 2006;99:53-60.)**

Key Words: myogenic response ■ delayed rectifier potassium channels ■ vascular smooth muscle ■ Kv1 channels

The intrinsic ability of resistance arteries to contract in response to elevations in intraluminal (or transmural) pressure, the myogenic response, was first described over 100 years ago by Bayliss.[1–4] This phenomenon is now well recognized to be an essential autoregulatory mechanism.[2–4] Myogenic tone development depends on L-type Ca^{2+} channel (Cav1.2) activity within vascular myocytes.[5] The resulting rise in intracellular free Ca^{2+} concentration via these Ca^{2+} channels[6] activates cross-bridge cycling and contractile force development that may be enhanced and/or maintained by a Ca^{2+} sensitization of the contractile machinery.[3,4]

A current working hypothesis suggests that the activation of L-type Ca^{2+} channels during the myogenic response is the result of low amplitude, steady-state depolarization of the vascular smooth muscle (VSM) cells attributable to increased intraluminal pressure.[2,3,6] However, very precise control of the extent of myogenic depolarization by an opposing hyperpolarizing influence is required to prevent regenerative Ca^{2+} influx and action potential initiation and to permit incremental, steady-state changes in arterial diameter that are essential for appropriate regulation of blood pressure and organ-specific blood flow.[2,3,6] A negative-feedback mechanism involving the activation of smooth muscle K^+ channels is thought to be required for control of myogenic depolarization.[2,3,7,8] However, present understanding of the molecular basis of this fundamental example of physiological regulation in the field of vascular biology is limited. We have addressed this issue in the present study because an abnormal myogenic response contributes to several clinically relevant conditions, such as hypertension, diabetes, end-stage renal failure, coronary vasospasm, and delayed cerebral vasospasm following hemorrhagic stroke. Significant new insight into the molecular basis of the myogenic response will not only define the processes that contribute to abnormal control of arterial diameter in disease but also provide a rational basis for novel therapies aimed at restoration of normal blood pressure and organ-specific blood flow.

Previous studies provide evidence for an important role of large conductance, Ca^{2+}-activated K^+ channels (BK_{Ca}) in the negative-feedback regulation of myogenic depolarization.[7,8] However, smooth muscle cells also express other types of K^+ channels, for example, voltage-dependent, delayed rectifier potassium (K_{DR}) channels, that exhibit steady-state activation within the range of membrane potential (E_m) of the myogenic

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response (ie, approximately −55 to −30 mV).[2] Specifically, expression of Kv1 and Kv2 pore-forming and modulatory Kvβ subunits that form K_{DR} channels has been demonstrated for VSM cells of several vessels.[9–13] Pharmacological evidence that Kv1- and Kv2-based K_{DR} can contribute to control of E_m, resting tone, and/or myogenic response of resistance arteries was previously obtained in experiments using 4-aminopyridine (4-AP)[10–14] and correolide for Kv1 inhibition[10–12] and stromatoxin for Kv2 inhibition.[15] However, 4-AP at >1 mmol/L affects Kv2, ATP-sensitive K^+ channels, and BK_{Ca},[2] as well as intracellular pH and Ca^{2+} release from internal stores[16,17]; correolide binds to Kv2.1 channels, albeit with 20-fold lower affinity compared with Kv1 subunits,[18] and stromatoxin also suppresses Kv4 channel gating.[19] Accordingly, direct molecular evidence is required to permit definitive conclusions regarding the role that VSM K_{DR} channels play in the control of the myogenic response.

Here we used an in vitro strategy involving expression of cDNAs encoding dominant-negative mutant or wild-type rabbit Kv1.5 in rat middle cerebral arteries. This approach uniquely provides for a direct and specific determination of the role of Kv1-containing K_{DR} in the myogenic response independent of channel subunit composition or stoichiometry. The findings of this study provide the first, direct molecular evidence that voltage-dependent Kv1-containing K_{DR} channels are critical for normal vasoregulation.

Materials and Methods

Male Sprague–Dawley rats (250 to 350 g; Charles River, Montréal, Québec, 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 whole brain was carefully removed and placed in cold (4°C) Krebs buffer containing (in mmol/L): NaCl 120, $NaHCO_3$ 25, KCl 4.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11, $CaCl_2$ 1.8. Left and right middle cerebral arteries were removed and cleaned of adherent fat, and the endothelium was removed (denuded) by passing a fine hair through the vessel lumen. Vessels were cannulated with fine glass pipettes mounted in a customized arteriograph chamber attached to a pressure myograph (Living Systems, Burlington, Vt) and superfused with warm (37°C) Krebs bath solution for measurement of arterial diameter with an automated edge detection system (IonOptix, Milton, Mass) (for additional details, see the online data supplement, available at http://circres.ahajournals.org). Reverse permeabilization and vessel culture were performed as in previous studies[20,21] and are described in detail in the online data supplement. RT-PCR, real-time PCR, immunoblotting, immunocytochemistry, myocyte isolation, and microelectrode recordings were as previously described[9,11,14] (see the online data supplement for additional details). Site-directed mutagenesis was performed using wild-type rabbit portal vein Kv1.5 (Kv1.5wt) subcloned into pcDNA3.1[22] to generate the dominant-negative Kv1.5W457F (Kv1.5DN) by replacing the native tryptophan at position 457 by phenylalanine. Ten residue c-myc or hemagluttinin (HA) epitope tags were added to the C terminus of Kv1.5wt and Kv1.5DN to facilitate immunoreactive detection of expressed protein (see the online data supplement for additional details). All data are shown as mean±SEM and were compared by paired Student's *t* test or repeated-measures ANOVA, followed by Bonferroni post hoc test. A level of $P<0.05$ was considered to be statistically significant.

Results

Figure 1 illustrates the myogenic response of freshly isolated arterial segments to increased intraluminal pressure and also shows the extent of passive dilation that occurs in the absence of external Ca^{2+} ($[Ca^{2+}]_o$; ie, no added Ca^{2+} and EGTA at 0.2 mmol/L in Krebs saline solution). The difference in diameter in the presence and absence of $[Ca^{2+}]_o$ represents the active, myogenic contractile response of the arteries to increased pressure. The potential involvement of Kv1-containing K_{DR} channels in the control of the cerebral arterial myogenic response is suggested by the significantly enhanced vasoconstriction to pressure following correolide[17] treatment (1 μmol/L; Figure 1).

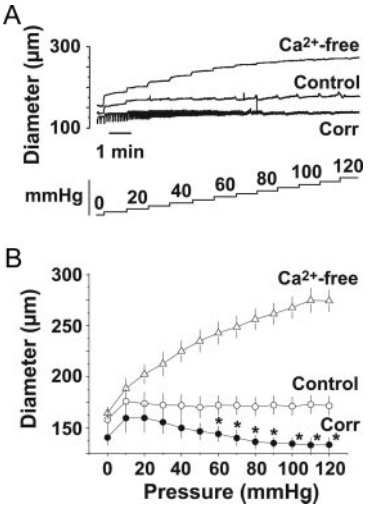

Figure 1. Suppression of Kv1-containing K_{DR} channels with correolide enhances cerebral arterial myogenic response. A, Representative active (1.8 mmol/L $[Ca^{2+}]_o$) and passive ($[Ca^{2+}]_o$-free) pressure-induced changes in diameter. B, Mean diameter (±SEM) vs pressure curves (n=5) for rat middle cerebral arteries before (Control) and after correolide (Corr) (1 μmol/L) treatment.*Significantly different from control ($P<0.05$) in this and subsequent figures.

Manipulation of functional Kv1-based K_{DR} expression level was accomplished using c-myc–tagged wild-type rabbit Kv1.5[22] (Kv1.5wt) and c-myc–tagged mutant Kv1.5W457F (Kv1.5DN) constructs. Mutation of a single tryptophan (W434) to a phenylalanine residue in the pore-forming loop of the Shaker K^+ channel sequence was previously shown to prevent K^+ permeation without affecting expression.[23] Mutation of this residue in rat Kv1.5 caused a specific dominant-negative inhibition of Kv1 but not Kv4 current in a heterologous expression system.[24] Control experiments were therefore performed in this study using human embryonic kidney 293 (HEK293) cells. These results confirmed that our constructs expressed normally, that Kv1.5DN coassembled with wild-type Kv1 subunits, and that Kv1.5DN expression specifically suppressed Kv1 but not Kv2 currents (see supplemental Figures I through III).

A reverse permeabilization protocol was used to permit transient transfection of short-term cultured segments of cerebral arteries.[20,21] Specifically, endothelium-denuded middle cerebral arteries were transfected with cDNAs encoding Kv1.5DN, Kv1.5wt, or empty plasmid (pcDNA3; ie, mock transfection) followed by 48 hours of culture in serum-free culture media to permit subunit protein expression.[20,21] Figure 2A and 2B shows that mock-transfected, cultured arteries

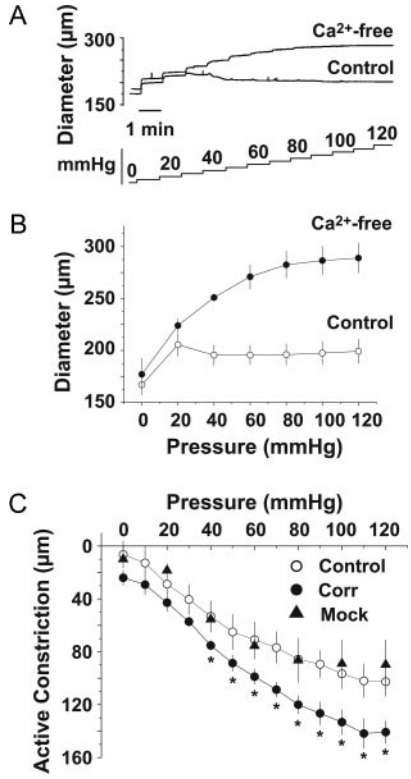

Figure 2. Mock-transfected and cultured vessels retain a myogenic response. A, Representative active (1.8 mmol/L $[Ca^{2+}]_o$) and passive ($[Ca^{2+}]_o$-free) pressure-induced changes in diameter. B, Mean diameter (±SEM) vs pressure curves (n=4) for mock-transfected, cultured arteries. C, Comparison of mean active constriction (±SEM) vs pressure curves for freshly isolated arteries before (Control) and after correolide (Corr) (1 μmol/L) treatment, as well as mock-transfected (Mock) (n=4) vessels based on data from Figures 1 and 2B.

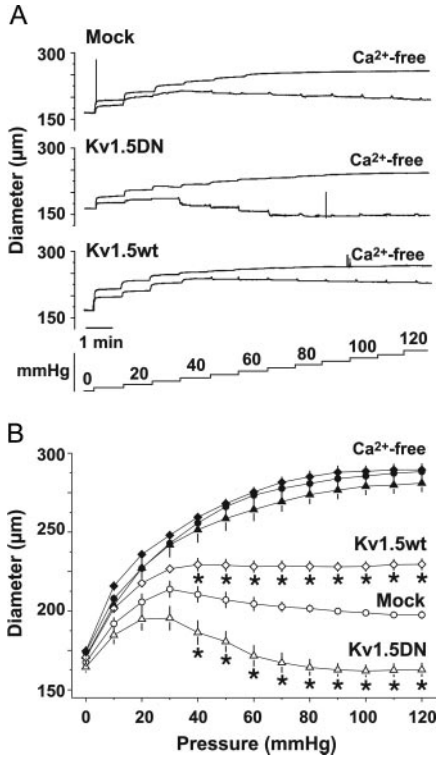

Figure 3. Expression of Kv1.5DN enhances, but Kv1.5wt suppresses, the myogenic response. A, Representative pressure-induced changes in diameter for mock-transfected arteries and vessels transfected with cDNAs encoding Kv1.5DN or Kv1.5wt. B, Mean diameter (±SEM) vs pressure curves for mock-transfected arteries (n=11) and vessels transfected with cDNAs encoding Kv1.5DN (n=9) or Kv1.5wt (n=9). Open and closed symbols correspond to the active and passive responses for each group of arteries, respectively.

were myogenic and demonstrated active constrictions in response to increased intraluminal pressure. Figure 2C shows a comparison of the active constriction of mock-transfected, cultured, and freshly isolated vessels. No difference between the active response to pressure was apparent, but correolide treatment caused a significant enhancement of the active constriction. Thus, the culture and transfection procedure did not alter arterial diameter or the extent of myogenic constriction to pressures between 0 and 120 mm Hg.

Representative effects of expression of Kv1.5wt, Kv1.5DN, or plasmid alone on the myogenic response of cerebral arteries are shown in Figure 3A. All vessels in each experimental group displayed a myogenic response to increased pressure, but differences in the magnitude of the response between the groups were readily apparent. Figure 3B shows mean diameter versus pressure curves for the 3 vessel groups. Vessel diameters were similar at pressures <40 mm Hg; however, arteries transfected with Kv1.5DN showed a significantly greater decrease in diameter, and the myogenic response of arteries transfected with Kv1.5wt was markedly suppressed, compared with mock-transfected vessels at pressures ≥40 mm Hg (Figure 3B). In contrast, the passive responses of the vessels in the absence of Ca^{2+} were not different over the entire range of intraluminal pressures tested (Figure 3B). Also, exposure to 60 mmol/L KCl-containing bath solution was found to evoke vasoconstrictions to similar minimal diameters in mock-transfected (162±4 μm), Kv1.5DN-transfected (159±2 μm), and Kv1.5wt-transfected (169±3) arteries (n=3 vessels in each group).

Comparison of vessel diameters in the presence and absence of $[Ca^{2+}]_o$ demonstrated the extent of active constriction as a function of intraluminal pressure for the 3 vessel groups, as shown in Figure 4A. The magnitude of active constriction increased proportionally with pressure in all vessels, but Kv1.5DN expression enhanced the active response compared with mock-transfected vessels, and wild-type Kv1.5 had the opposite effect (Figure 4A). Specifically, Kv1.5DN expression significantly enhanced active tone development at pressures ≥30 mm Hg, whereas Kv1.5wt suppressed the myogenic response at pressures ≥40 mm Hg compared with mock-transfected vessels. Figure 4B also shows that the levels of active constriction at 80 mm Hg were similar for freshly isolated and mock-transfected vessels but significantly greater for correolide-treated (1 μmol/L) and Kv1.5DN-transfected vessels and significantly less for Kv1.5wt-transfected arteries.

If the differences in active myogenic constriction shown in Figure 4 were owing to varied levels of Kv1-containing K_{DR} current activation, expression of mutant and wild-type Kv1.5

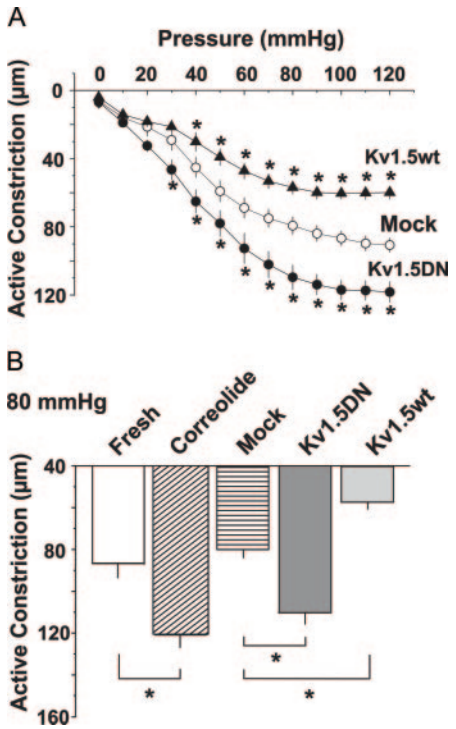

Figure 4. Alterations in active myogenic constriction by Kv1.5DN, Kv1.5wt, and correolide. A, Mean active constriction (±SEM) vs pressure curves for mock- (n=11), Kv1.5DN- (n=9), and Kv1.5wt-transfected (n=9) arteries derived from data in Figure 3. B, Mean active constriction (±SEM) at 80 mm Hg for freshly isolated arteries before (Fresh; n=5) and after correolide (1 μmol/L; n=5) treatment, as well as mock- (Mock; n=11), Kv1.5DN- (n=9), and Kv1.5wt-transfected (n=9) arteries.

subunits would be expected to alter the extent of myogenic depolarization, as well as the effect of correolide treatment on arterial diameter. Figure 5A and 5B shows representative sharp microelectrode recordings and average values of E_m for mock-, Kv1.5DN-, and Kv1.5wt-transfected arteries pressurized to 80 mm Hg. We found that E_m of VSM cells in vessels expressing Kv1.5DN was significantly more depolarized

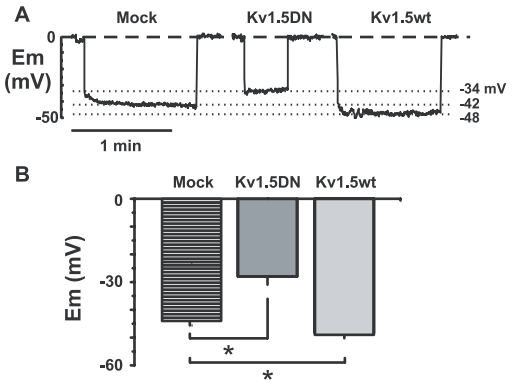

Figure 5. Expression of Kv1.5DN enhances, but Kv1.5wt suppresses, myogenic depolarization. A, Representative sharp microelectrode recordings of VSM cell E_m in mock-, Kv1.5DN-, and Kv1.5wt-transfected vessels at 80 mm Hg. Stable level of E_m in each record is shown on right. B, Mean value of E_m (±SEM) determined for mock- (n=3 vessels), Kv1.5DN- (n=4 vessels), and Kv1.5wt-transfected (n=3 vessels) arteries (2 to 3 impalements per vessel).

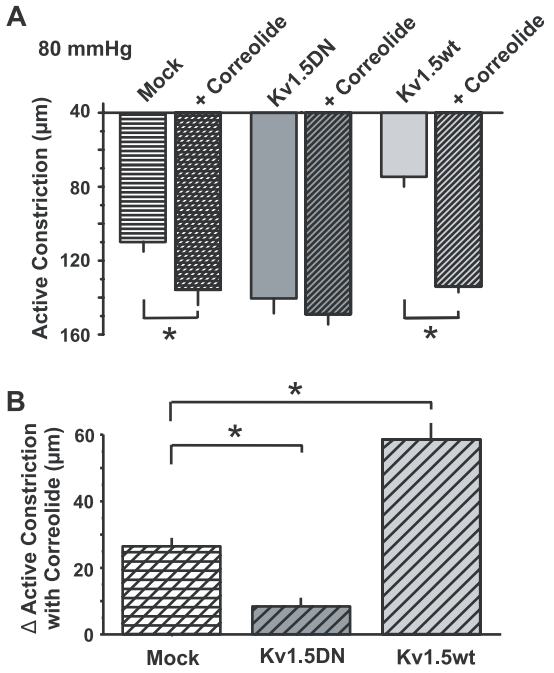

Figure 6. Expression of Kv1.5DN reduces, but Kv1.5wt increases, correolide-sensitive myogenic contraction. A, Mean active constriction (±SEM) before and after correolide (1 μmol/L) treatment of mock-, Kv1.5DN-, and Kv1.5wt-transfected arteries (n=3 for each group). B, Mean change in active constriction with correolide treatment of mock-, Kv1.5DN-, and Kv1.5wt-transfected arteries derived from data in A.

compared with mock-transfected arteries, whereas vessels transfected with Kv1.5wt were more hyperpolarized (Figure 5B). These data support the conclusion that manipulation of functional Kv1-containing K_{DR} channel expression affected vasoregulation by changing the extent of myogenic depolarization evoked by increased intraluminal pressure.

Figure 6 shows the divergent effect of correolide treatment on active constriction of mock-, Kv1.5DN-, and Kv1.5wt-transfected vessels at 80 mm Hg. Correolide (1 μmol/L) caused a significant increase in active constriction of mock- and Kv1.5wt-transfected arteries, but it did not affect vessels expressing Kv1.5DN (Figure 6A). Notably, the change in active constriction induced by correolide was significantly less in vessels expressing Kv1.5DN and significantly greater for Kv1.5wt compared with mock-transfected arteries (Figure 6B). These findings are consistent with the conclusion that the contribution of correolide-sensitive Kv1-containing K_{DR} channels to the control of the myogenic response was reduced and enhanced in Kv1.5DN and Kv1.5wt arteries, respectively, compared with mock-transfected vessels.

Confirmation of expression of c-myc–tagged mutant and wild-type Kv1.5 message and protein by arteries after 48 hours of in vitro culture was obtained by RT-PCR, immunoblotting, and immunocytochemistry. The primers used were specifically designed to amplify c-myc–tagged rabbit Kv1.5 but not endogenous rat Kv1.5. PCR reactions were also conducted before the RT reaction in 3 sets of experiments to control for contamination of DNase-treated RNA samples by the cDNAs used in the transfection procedure. No evidence of contamination was detected (Figure 7A, top). However,

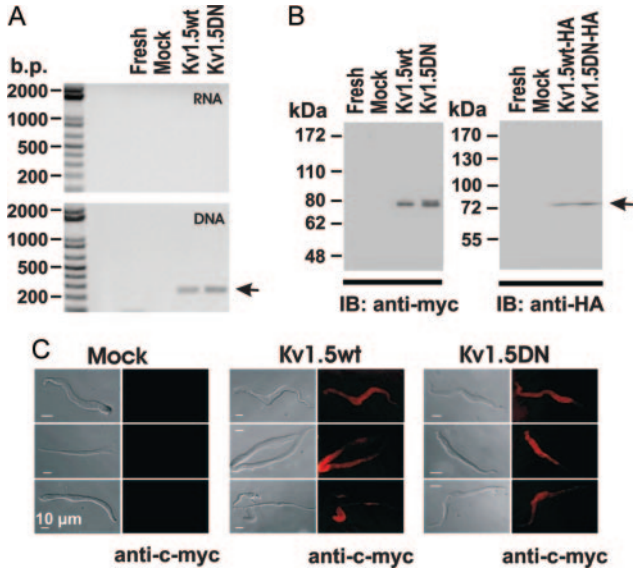

Figure 7. Rabbit Kv1.5DN and Kv1.5wt transcript and protein expression in rat middle cerebral arteries. A, Top, Lack of detection of amplicons for rabbit Kv1.5-c-myc in samples of DNase-treated RNA extracts of freshly isolated, mock-, Kv1.5wt-, and Kv1.5DN-transfected vessels when PCR was performed before a reverse-transcription reaction was completed. Similar results were obtained in 2 additional experiments. Bottom, Detection by RT-PCR of amplicons (224 bp) appropriate for rabbit Kv1.5-c-myc in RNA samples of Kv1.5wt- and Kv1.5DN-transfected arteries but not freshly isolated or mock-transfected vessels. Similar results were obtained in 2 additional experiments. B, Detection of anti–c-myc (left) and anti-HA (right) immunoreactive protein in lysates of c-myc– or HA-tagged Kv1.5wt- and Kv1.5DN-transfected arteries but not freshly isolated or mock-transfected vessels. C, Expression of c-myc immunoreactive protein by single smooth muscle cells isolated from Kv1.5DN- (right panels) and Kv1.5wt-transfected (middle panels) arteries, but not mock-transfected vessels (left panels), was detected with anti–c-myc and cy3-tagged secondary antibody.

amplicons of appropriate size (224 bp) were detected after RT-PCR in RNA samples from Kv1.5wt- and Kv1.5DN-transfected but not freshly isolated or mock-transfected arteries in 3 experiments (Figure 7A, bottom). The identity of the amplicons was confirmed by sequencing. We also detected anti–c-myc immunoreactive protein with an apparent molecular mass of ≈77 kDa in immunoblots of Kv1.5wt- and Kv1.5DN-transfected, but not freshly isolated or mock-transfected, vessels in 3 separate experiments (Figure 7B, left). Confirmation of these results was provided by 3 additional experiments in which the c-myc tag on both constructs was replaced with a hemagglutinin (HA) epitope and immunoblotting performed with an anti-HA (Figure 7B, right). Finally, 30% to 40% of single smooth muscle cells isolated from Kv1.5DN- and Kv1.5wt-transfected, but not mock-transfected, arteries (3 vessels per group) demonstrated immunofluorescence when exposed to anti–c-myc followed by cy3-tagged secondary antibody (Figure 7C). Taken together, these data confirm the expression of exogenous rabbit Kv1.5 message and protein by VSM cells of Kv1.5DN- and Kv1.5wt-transfected, but not control, arteries.

A final set of control experiments using quantitative PCR analysis was conducted to confirm that expression of

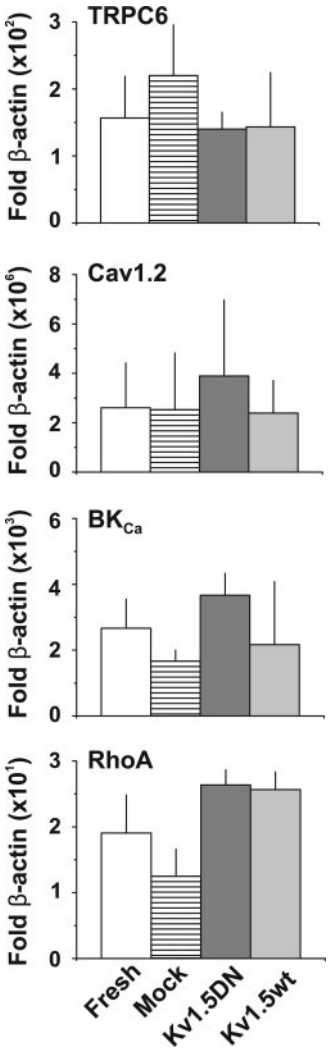

Figure 8. Lack of effect on expression of message encoding proteins relevant to myogenic response by vessels transfected with empty vector, Kv1.5DN, and Kv1.5wt. Message levels normalized to β-actin for TRPC6, Cav1.2, BK_{Ca}, and RhoA of freshly isolated middle cerebral arteries (Fresh) and vessels transfected with empty plasmid (Mock), Kv1.5DN, and Kv1.5wt (n=3 to 4 vessels in each group) as determined by real-time PCR.

Kv1.5DN, Kv1.5wt, and empty plasmid did not affect the levels of mRNAs encoding proteins previously implicated to be components of the myogenic mechanism of arterial smooth muscle. Figure 8 shows that the levels of transcripts encoding the transient receptor potential nonselective cation channel subunit TRPC6,[21] the voltage-dependent Ca^{2+} channel subunit, Cav1.2,[5] the pore-forming, Slo1 subunit of BK_{Ca},[7,8] and an element of the Ca^{2+} sensitization pathway, RhoA,[3,4] were not statistically different in mock-, Kv1.5DN-, and Kv1.5wt-transfected compared with freshly isolated middle cerebral arteries. These data are consistent with the view that the molecular strategy used specifically affected the Kv1-containing K_{DR} channel component of the myogenic mechanism.

Discussion

Our findings provide the first direct molecular evidence of a key role for voltage-dependent channel pore-forming Kv1

subunits in control of the arterial myogenic response. Thus, VSM K_{DR} channels containing Kv1 subunits are an important determinant of arterial diameter and, thereby, blood pressure regulation and organ-specific blood flow. Expression of Kv1.5DN-myc enhanced, whereas overexpression of wild-type Kv1.5 suppressed, myogenic contraction without affecting passive dilation to increased pressure. These findings provide compelling evidence that manipulating functional Kv1 channel expression by VSM cells alters vasoregulation. The validation of a simple molecular approach for specific determination of the contribution of heteromultimeric Kv1-containing K_{DR} in this study will facilitate direct analysis of their additional roles (eg, in the actions of vasoactive agonists) and via a similar approach, the roles of other K^+ channels (eg, Kv2 and inward rectifier K^+ channels), in control of arterial diameter in health and various models of vascular disease.

We used a dominant-negative strategy to evaluate the role of Kv1-containing K_{DR} channels in vasoregulation because they are thought to be heteromultimeric channels composed of multiple types of pore-forming Kv1 subunits. For example, rat mesenteric arterial K_{DR} were shown to contain 3 different Kv1 subunits, ie, Kv1.2, Kv1.5, and Kv1.6, as well as modulatory Kvβ subunits,[11] and coassembly of at least Kv1.2 and Kv1.5 has been demonstrated for rat cerebral arteries.[10] Application of alternative genetic/molecular approaches, such as knock-out, antisense, or siRNA strategies, was viewed to be limited by the potential need for independent suppression of each of the Kv1 subunits to manipulate the Kv1-based K_{DR} current. In contrast, the strategy of using Kv1.5DN is based on the assumption that the mutant subunits coassemble with endogenous, wild-type subunits and suppress K^+ permeation through the pore of the resultant heterotetrameric channels irrespective of Kv1 subunit composition or stoichiometry. Mutation of the tryptophan residue at position 434 to phenylalanine within the pore region of the Shaker K^+ channel was previously shown to prevent K^+ permeation (hence, ionic current flow through the channel pore) without affecting expression or gating currents.[23] Additionally, expression of mammalian Kv1.5 subunits with a similar mutation was shown to cause a dominant-negative suppression of currents attributable to heterologous expression of wild-type Kv1.5 but not Kv4 subunits in mammalian cells, as well as native K^+ current of rat cardiac myocytes.[24] We opted to use mutant and wild-type Kv1.5, rather than Kv1.2, based on previous observations indicating the presence of a C-terminal motif in Kv1.5 that promotes cell surface expression and a suboptimal motif in Kv1.2.[25] We confirmed that the Kv1.5DN used in this study coassembled with wild-type Kv1.2 and Kv1.5 subunits via coimmunoprecipitation experiments and that it suppressed ionic current attributable to Kv1.2 and Kv1.5, but not Kv2.1 channels expressed in human embryonic kidney cells by whole-cell voltage-clamp technique (see the online data supplement for these additional data). Moreover, expression of c-myc–tagged mutant and wild-type Kv1.5 subunit message and protein following transient transfection was confirmed by RT-PCR and immunoblotting using intact arteries and by immunocytochemistry using myocytes isolated from transfected arteries. Taken together, the data from these control experiments indicate the presence of exogenous subunit expression by smooth muscle cells of the transfected, cultured arteries and the ability of the mutant Kv1.5 subunit to coassemble with and specifically suppress K^+ current mediated by wild-type Kv1, but not Kv2 subunits.

We attribute the alterations in the myogenic response accompanying the expression of mutant or wild-type Kv1.5 subunits to differences in the contribution of Kv1-containing K_{DR} channels to control of pressure-induced myogenic depolarization and vasoconstriction. We do not believe that the alterations in the myogenic response were the result of nonspecific effects on the vessels resulting from the reverse-permeabilization, transfection, or culture procedures, or to non–K_{DR}-related variations in expression of other components of the myogenic mechanism. This view is based on the absence of any differences in the extent of: (1) passive dilation to pressure; (2) active myogenic contraction to 60 mmol/L KCl; and (3) expression of message encoding proteins previously implicated in the myogenic response, specifically TRPC6, Cav1.2, BK_{Ca}, and RhoA,[2–5,21,26] in mock-, Kv1.5DN-, and Kv1.5wt-transfected vessels compared with freshly isolated arteries.

On the other hand, we found that Kv1.5DN and Kv1.5wt expression affected the level of E_m and caused pressure-dependent alterations in arterial diameter. Specifically, E_m was more depolarized at approximately -29 ± 2 mV in vessels expressing Kv1.5DN compared with mock-transfected arteries at -44 ± 2 mV, whereas expression of Kv1.5wt had the opposite effect, and E_m was less depolarized at -48 ± 1 mV. These values are consistent with those previously reported for rat cerebral and skeletal arteries/arterioles in studies that indicate that the myogenic response is activated over a narrow range of E_m between approximately -55 and -30 mV and that small changes of E_m within this range are associated with substantial changes in intracellular Ca^{2+} concentration and arterial diameter.[6,26] The myogenic response is thought to involve VSM cell depolarization attributable to nonselective cation,[21,27] chloride,[28,29] and/or L-type Ca^{2+} channel activity,[30] with the amplitude of current activation and extent of depolarization increasing proportionally with increasing intraluminal pressure. The contribution of K_{DR} channels would also be expected to increase with increasing pressure if they are involved in negative-feedback regulation of myogenic depolarization. That Kv1-containing K_{DR} should activate more strongly with progressive depolarization to oppose myogenic changes in E_m may be predicted based on their voltage-dependent activation that overlaps with the E_m range of the myogenic response.[2,11] Direct experimental evidence of a pressure-dependent contribution to control of diameter is evident from our finding that the diameters of Kv1.5DN-, Kv1.5wt-, and mock-transfected arteries were similar at <40 mm Hg, but, at ≥40 mm Hg, the vasoconstriction of Kv1.5DN-transfected vessels was greater than that of mock-transfected vessels and significantly less than the mock-transfected vessels in arteries expressing myc-tagged wild-type Kv1.5 subunits. Thus, as we found for the level of E_m at 80 mm Hg, pressure-dependent alterations in control of arterial diameter were apparent regardless of whether functional Kv1 channel expression was manipulated

to decrease or increase K_{DR} activity. Taken together, these electrophysiological and pressure myography results provide a direct indication of the important role that Kv1 channels serve in providing a negative-feedback control mechanism that limits myogenic depolarization and opposes myogenic alterations in arterial diameter, vascular resistance and blood flow (see supplemental Figure IV for schematic illustration of the role of Kv1 channels). To place our observations in this physiological context, Poiseuille's Law predicts that the decrease in diameter at 100 mm Hg from 199±2 in mock- to 162±4 μm in Kv1.5DN-transfected vessels would be expected to cause a ≈2.4-fold increase in resistance and ≈60% reduction in blood flow and the increase in diameter of Kv1.5wt vessels to 227±3 μm an almost equivalent but opposite increase in flow. Clearly, altering the contribution of VSM Kv1-containing K_{DR} channels to the control of myogenic depolarization has the potential to exert a profound effect on blood flow in vivo.

The results of this study are consistent with the view that the level of Kv1 subunit expression and magnitude of functional K_{DR} current within a specific vessel bed may be an important determinant of the magnitude of the myogenic response. A lower relative contribution of Kv1-containing K_{DR} caused by reduced subunit expression or channel gating as a result of phosphorylation by protein kinase C (PKC) or Rho-associated kinase (see Cole et al[13]) would permit greater myogenic depolarization and tone development, whereas increased expression or gating (attributable to PKA[13]) would limit depolarization and myogenic constriction. This view is consistent with our previous findings that the relative abundance of Kv1 message detected by real-time PCR was greater in first-order conduit versus fourth-order resistance mesenteric arteries and that 4-AP or correolide converted apparently nonmyogenic first-order arteries into vessels that exhibited active tone development in response to increases in intraluminal pressure.[11] However, these data do not rule out the possibility that differences in functional contribution of other K^+ channels (eg, Kv2[15] and BK_{Ca}[26]) and/or inward currents act in concert with Kv1-containing K_{DR} in determining the pressure-dependence of VSM E_m. Defining the relative contributions of differences in ion channel expression and function, as well as varied levels of Ca^{2+} sensitization to vessel- and vascular bed-specific differences in the magnitude and pressure dependence of the myogenic response, as are apparent between cerebral and skeletal muscle arteries/arterioles,[6,26] will require considerable further study.

Previous findings provide compelling pharmacological and molecular evidence of a mechanism for control of arterial diameter involving BK_{Ca} of vascular myocytes.[7,8] For example, enhanced cerebral arterial myogenic depolarization and contraction were observed in the presence of the specific blocker of BK_{Ca} channels, iberiotoxin.[7] This BK_{Ca}-dependent mechanism is initiated by release of Ca^{2+} via ryanodine receptors in the sarcoplasmic reticulum (ie, "sparks")[8] as well as Ca^{2+} influx through L-type Ca^{2+} channels in close proximity to the BK_{Ca}.[31] Targeted deletion of the β_1 subunit to reduce the Ca^{2+} sensitivity of BK_{Ca} in mice increased peripheral blood pressure by 15 to 20 mm Hg,[32] and reduced β_1 expression is associated with the development of genetic hypertension in spontaneously hypertensive rats.[33] Reduced Kv1 and/or Kv2 subunit expression and/or K_{DR} current density were previously shown to be associated with pulmonary, cerebral, and/or peripheral hypertension,[15,34,35] and currently available pharmacological evidence is consistent with the view that suppression of Kv1- and/or Kv2-containing K_{DR} channels leads to vasoconstriction and an enhanced myogenic response.[10–15] However, until now, definitive molecular evidence that vascular K_{DR} contribute to the myogenic response has been lacking. Our results demonstrate that Kv1-based K_{DR} channels provide a key voltage-activated mechanism for control of myogenic constriction in cerebral arteries. The data do not exclude a parallel role for Kv2-containing channels in vasoregulation, as was recently suggested by Amberg and Santana[15] based on experiments using the tarantula venom component stromatoxin. According to our data and the findings of others, appropriate regulation of arterial diameter through the myogenic response requires the involvement of K_{DR} in addition to BK_{Ca} channels, and alterations in the behavior of negative-feedback mechanisms involving either channel type may be expected to contribute to dysfunctional control of arterial diameter, blood pressure, and organ-specific blood flow in disease.

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Disclosures

None.

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ONLINE SUPPLEMENTARY METHODS

Pressure myography: Arteries were allowed to equilibrate for 60 minutes at 37 °C and then subjected to a pressure step from 0 to 60 mmHg. Vessels that did not exhibit active constriction to the pressure step were discarded. Vessels were then subjected to increasing pressure steps from 0 to 120 mmHg in 10 mmHg increments. In some vessels, intra-luminal pressure was reduced to 5 mmHg and correolide (1 μmol/L) was added to the perfusate for 45 minutes, before the pressure steps were repeated or the vessels were exposed to 60 mmol/L KCl-containing bath solution (equimolar replacement of NaCl). The passive change in diameter for each pressure step was determined for each artery at the end of all experiments by replacing the control Krebs' solution with $[Ca^{2+}]_o$-free Krebs' solution containing EGTA (0.2 mmol/L) and repeating the series of pressure steps between 0 and 120 mmHg. Active myogenic constriction was defined as the difference between arterial diameter in 1.8 mmol/L $[Ca^{2+}]_o$-containing and $[Ca^{2+}]_o$-free/EGTA perfusate at each pressure step.

Reverse permeabilization and vessel culture: Artery segments were incubated for 20 minutes at 4 ºC in the following solution (in mmol/L): KCl 120, $MgCl_2$ 2, EGTA 10, Na_2ATP 5, TES 20, pH 6.8. The segments were then switched to solution containing cDNAs (Kv1.5DN, Kv1.5wt, Kv1.5DN-HA, Kv1.5wt-HA, or empty pcDNA3 at 10 mmol/L) overnight at 4 ºC and followed by cDNA–containing solution with elevated $[MgCl_2]$ (10 mmol/L). Permeabilization was reversed by placing the arteries for 30 minutes in nominally $[Ca^{2+}]_o$-free Krebs' solution and then sequentially increasing $[Ca^{2+}]_o$ in steps of 0.01, 0.1 and 1.8 mmol/L over a 60 minute period. Following the reversible permeabilization procedure, arteries were cultured for 48 h in D-MEM/F-12 culture media supplemented with L-glutamine (2 mmol/L), penicillin (50 units ml^{-1}), streptomycin (50 mg ml^{-1}), Na_2ATP (5 mmol/L) and 1x insulin-transferrin-selenium A (GIBCO; Invitrogen, Burlington, Canada) at a pH of 7.4.

RT-PCR analysis: Freshly isolated arteries and vessels transfected with empty plasmid, KV1.5DN or Kv1.5wt were placed in RNase- and DNase-free collection tubes. After total RNA extraction (RNeasy mini kit with DNase treatment; Qiagen, Valencia, USA), first-strand cDNA was synthesized with the Sensiscript RT kit (Qiagen). Subsequently, each first-strand cDNA reaction was used as the template in a PCR reaction containing 1.5 mmol/L $MgCl_2$, 0.25 mM forward and reverse primers (University of Calgary Core DNA Services), 0.2 mmol/L deoxynucleoside triphosphates and 2.5 units of recombinant *Taq* DNA polymerase. PCR reactions were hot started (94 ˚C for 3 min) and underwent 35 cycles of 94 ˚C for 0.5 min, 60 ˚C for 0.5 min, and 72 ˚C for 0.5 min before a final extension period at 72 ˚C for 10 min. Primers specific to rabbit Kv1.5DN-myc and Kv1.5WT-myc) were used:

Forward: 5'-GCACCTCCCTGGATGCAGGAGGC-3'

Reverse: 5'-GGGCCCTCTAGACTCGAGCAAGTCG-3'

The expected amplicon size was 224 bp and all reaction products were sequenced. Control experiments were performed to confirm the absence of genomic DNA contamination in the RNA samples by performing a PCR reaction on the RNA extract prior to first strand cDNA synthesis. No contamination was detected.

Immunoblotting: Western blotting was performed on protein extracts of freshly isolated, mock-transfected and transfected arteries as previously described,[1] using polyclonal anti-Kv1.5 (rabbit; Alomone Labs, Jerusalem, Israel), monoclonal anti-c-myc (mouse; Calbiochem, Darmstadt, Gremany), anti-HA (mouse; Roche Diagnostics, Laval, Canada), anti-rabbit IR800 (Rockland Immunochemica, VWR Canlab, Mississauga Canada) and anti-mouse IR680 (Molecular Probes, Burlington, Canada).

Myocyte isolation from rat middle cerebral arteries and immunocytochemistry: Arteries were placed in an ice-cold aerated dissection solution containing (in mmol/L): NaCl 120, $NaHCO_3$ 25,

KCl 4.2, KH_2PO_4 0.6, $MgCl_2$ 1.2, glucose 11, $CaCl_2$ 0.01. Single myocytes were dispersed from the arteries as previously described.[2] Immunocytochemistry was performed on isolated myocytes as previously described,[1,2] using anti-c-myc and anti-mouse IgG-cy3 (Jackson Labs, Bar Harbour, USA).

Generation of Kv1.5DN and tagged mutant and wild-type Kv1.5 subunits: PCR-based mutagenesis was performed to change the native codon (TGG) that encodes tryptophan (W) at position 457 in the highly conserved pore region to a codon (TTT) that encodes phenylalanine (F) of wild-type rabbit portal vein Kv1.5 (Kv1.5wt) in pcDNA3.1.[3] A three step PCR reaction was performed to amplify from the: 1) 5' (N-terminus) end to the point of mutation (~1450 bp), 2) point of mutation to the 3' (C-terminus) end (~500 bp) and 3) the full length mutant Kv1.5 PCR reaction using equimolar amounts of the products from reactions 1 and 2 as template (~1950 bp). Each PCR reaction contained 100 ng of template cDNA, 1.5 mmol/L $MgSO_4$, 0.25 mmol/L forward and reverse primers (University of Calgary), 0.2 mmol/L deoxynucleoside triphosphates, 1X PCRx Enhancer solution and 2.5 units of recombinant *Taq* DNA polymerase. PCR reactions were hot started (94 ºC for 3 min) and underwent 35 cycles of 94 ºC for 0.75 min, 55 ºC for 1 min, and 72 ºC for 2 min. PCR samples were then exposed to a final extension period at 72 ºC for 10 min. Amplicons of appropriate migration on an agarose gel were sequenced (University of Calgary Core DNA Services) to confirm the fidelity of each of the PCR reactions. All sequences were found to be error-free. The primer sequences used for each reaction were as follows with the altered nucleotides required to generate the W to F mutation shown in bold, underlined characters:

Reaction 1: 5' end to mutation:

Forward: 5'-CTCTGGTACCGAATGGAGATCGCCCTGGGGCCCCTG-3'

Reverse: 5'-CATGGTGACCACCGCCCA**<u>AA</u>**AGAAGGCATCGGGGATGC-3'

Reaction 2: Mutation to 3':

Forward: 5'-GCATCCCCGATGCCTTCT**TT**TGGGCGGTGGTCACCATG-3'

Reverse: 5'-ACCCTCACTAAAGGGAACAAAAGC-3'

Reaction 3: Full length:

Forward: 5'- CTCTGGTACCGAATGGAGATCGCCCTGGGGCCCCTG-3'

Reverse: 5'-ACCCTCACTAAAGGGAACAAAAGC-3'

Short (10 residue) antigenic c-myc or hemagluttinin (HA) epitope tags were added to the C-terminus of Kv1.5wt and Kv1.5DN in pcDNA3 to facilitate immunoreactive detection of the expressed channel proteins using epitope-specific antibodies. PCR-based mutagenesis reactions were performed on Kv1.5wt and Kv1.5DN in pcDNA3.1 to remove the stop codons and insert an Xho1 restriction sequence at the C-terminus. The forward reaction was primed downstream of the W457F mutation site at a BstEII restriction sequence and an amplicon of expected size (~500 bp) obtained. The amplicon was sequenced and was confirmed to be error-free. Wild-type Kv1.5 and Kv1.5DN in pcDNA3.1 were then subjected to restriction digest with Kpn1 and BamH1 to release the ~1.9 kb channel coding sequence. Myc-His(+) A pcDNA3.1 (Invitrogen) was subjected to restriction digest by Kpn1 and BamH1 to linearize the vector, which was then ligated (Rapid DNA Ligation Kit, Roche) to the ~1.9 kb channel coding sequence yielding Kv1.5DN-Myc-His(+) A pcDNA3.1. A similar approach was used to generate the HA-tagged Kv1.5DN.

Real-time PCR: Quantitative analysis of TRPC6, Cav1.2, BK_{Ca} and RhoA transcript abundance relative to β-actin message was performed by real-time PCR using a Bio-Rad iCycler as previously described.[2] Briefly, tissues were placed in RNase- and DNase-free collection tubes. Total RNA was extracted from rat cerebral vessels using an RNeasy Mini Kit (Qiagen) followed by an in-solution DNase treatment (Invitrogen DNase I). First-strand cDNA was synthesized using a Superscript RT Kit (Qiagen). To optimize 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. 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 Services to confirm the identity of the amplicon. Using the identified ideal annealing temperature for each reaction to be performed (58.0 °C for BK_{Ca}, TRPC6 and RhoA, 54.7 °C for Cav1.2), 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. Real-time PCR was performed using 1.0 μL first-strand cDNA in a final volume of 25 μL containing Quanti-Tect SYBR Green Supermix (Qiagen) and 0.25μmol/L forward and reverse primers. The reactions were as follows: hot-start 95 °C 30 s, and 40x (60 cycles for Cav1.2) 94 °C for 15 s, annealing temperature for 30 s, 72 °C for 30 s. Quantification of transcript abundance was accomplished using the $2^{\Delta\Delta Ct}$ method[4] and β-actin expression as a reference for cDNA level normalization. The primers employed were as follows:

β-actin Forward: 5'-ACGGCCAGGTCATCACTATTG-3'

Reverse: 5'-CCAAGAAGGAAGGCTGGAAAAGA-3'

TRPC6 Forward: 5'-ATGCGGATGTGGAGTGGAAG-3'

Reverse: 5'-AGCAGGGACTTTGGACTTGG-3'

Cav1.2 Quanti-Tect Primer Assay (Qiagen)

BK_{Ca} Forward: 5'-TGGAACTGGAAAGAGGGTGATG-3'

Reverse: 5'-GATTGGCAAGCATTGTGGAGAG-3'

RhoA Forward: 5'-AAGGACCAGTTCCCAGAGGT-3'

Reverse: 5'-TGTCCAGCTGTGTCCCATAA-3'

ONLINE SUPPLEMENT REFERENCES

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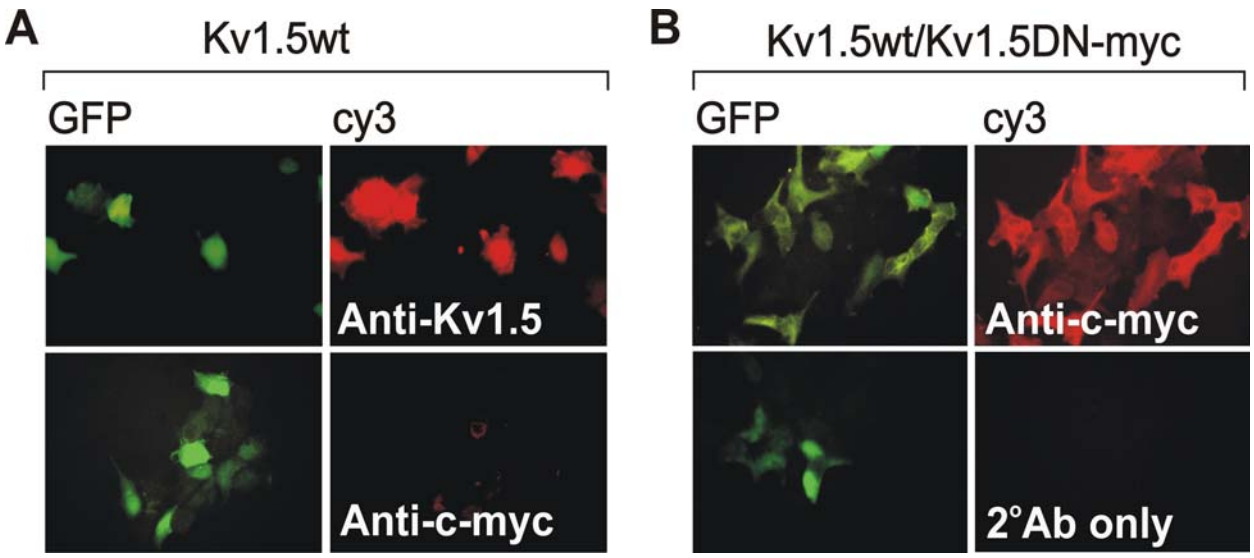
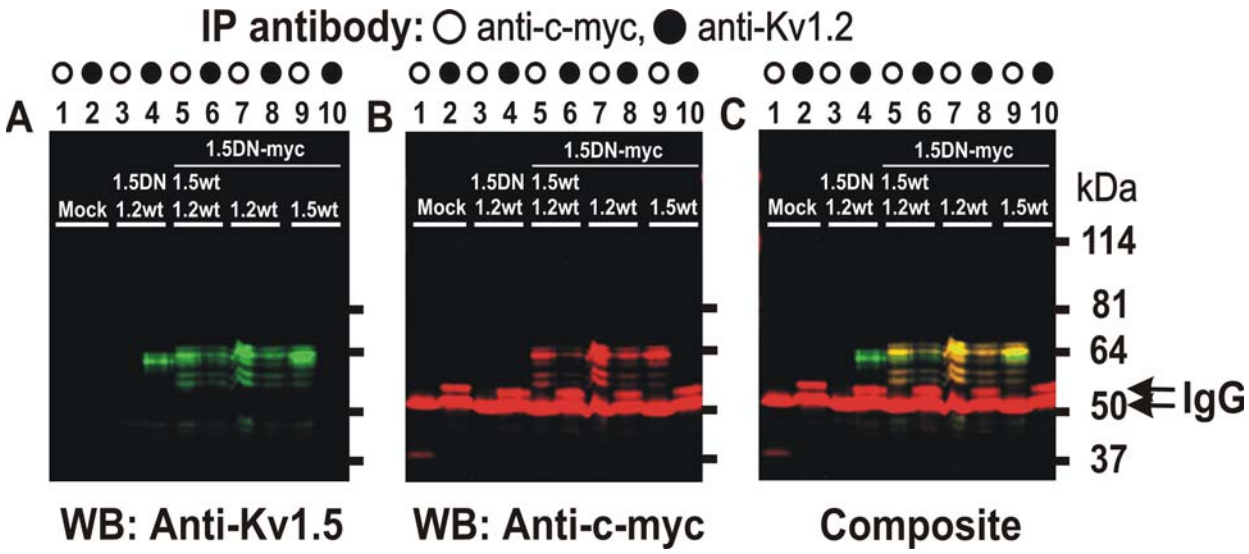
Online Supplementary Figure 1: Immunocytochemical detection of Kv1.5 expression in HEK cells.



Control experiments were performed to ensure that the c-myc-tagged dominant-negative mutant Kv1.5 subunit (Kv1.5DN-myc) construct used in this study was expressed by human embryonic kidney 293 (HEK) cells. **Panel A:** Two pairs of light micrographs depict fluorescence due to the expression of green fluorescent protein (GFP) and corresponding Kv1.5- or c-myc-immunoreactive protein expression detected using a cy3-coupled secondary antibody (2° Ab) in the same HEK cells 24 h post-transfection with cDNAs encoding GFP and non-tagged Kv1.5wt using FuGENE-6™ (Boehringer Mannheim). **Panel B:** The upper pair of micrographs shows the presence of c-myc immunoreactive protein expression in GFP fluorescent HEK cells 24 h after transfection with cDNAs encoding GFP, Kv1.5wt and Kv1.5DN-myc. The lower pair of micrographs indicates the lack of c-myc-immunofluorescent signal in identical HEK cells exposed to 2° antibody only. Taken together, these data indicate the successful expression of the engineered Kv1.5DN-myc construct at the protein level by the heterologous cell type.

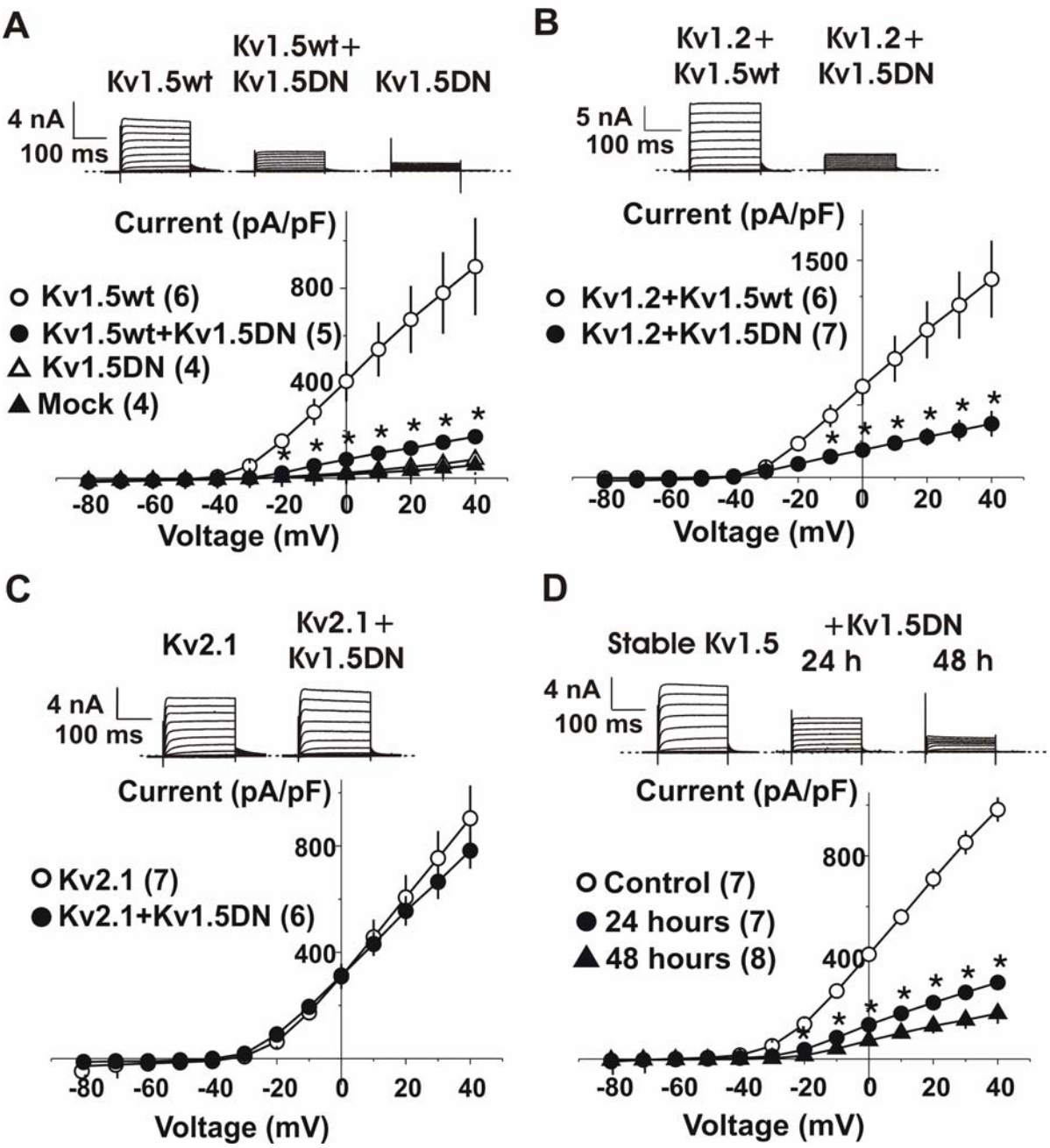
Online Supplementary Figure 2: Co-assembly of Kv1.5DN-myc with wild-type Kv1.5 and Kv1.2 subunits in HEK cells.



The strategy of employing Kv1.5DN-myc is based on the assumption that the mutant subunits co-assemble with wild-type subunits and suppress K^+ permeation through the pore of the resultant functional, heteromultimeric channels. The assembly of Kv1.5DN-myc with wild-type Kv1.5 and Kv1.2 was assessed by immunoprecipitation with anti-Kv1.2 or anti-c-myc and subsequent immunoblotting with a monoclonal mouse anti-c-myc and polyclonal rabbit anti-Kv1.5. Secondary antibodies specific for mouse or rabbit IgGs and coupled respectively to red and green fluorophores were employed to permit simultaneous detection of the c-myc epitope and Kv1.5. The yellow signal in the composite panel C indicates dual labelling of protein by both antibodies. Lysates of HEK cells (48 h post-transfection) expressing: a) empty plasmid (Mock; lanes 1 & 2), b) non-tagged Kv1.5DN (1.5DN) and Kv1.2wt (lanes 3 & 4), c) Kv1.5DN-myc, Kv1.5wt and Kv1.2wt (lanes 5 & 6), d) Kv1.5DN-myc and Kv1.2wt (lanes 7 & 8) and e) Kv1.5DN-myc and Kv1.5wt (lanes 9 & 10) were immunoprecipitated using anti-c-myc (lanes 1, 3, 5, 7, 9) or anti-Kv1.2 (lanes 2, 4, 6, 8, 10). **Lanes 1 & 2 of panels A-C** show that no immunoreactive, non-IgG protein was detected in

immunoprecipitates of mock (empty plasmid)-transfected cells probed with anti-c-myc or anti-Kv1.5. **Lanes 3 & 4 of panel A** show that Kv1.5-immunoreactive protein at an apparent molecular weight appropriate for rabbit Kv1.5 (~65 kDa) was detected in the anti-Kv1.2, but not the c-myc immunoprecipitates of HEK cells expressing Kv1.2wt and non-tagged Kv1.5DN. **Lanes 3 & 4 of panel B** show the corresponding absence of anti-c-myc immunoreactivity from both anti-c-myc and anti-Kv1.2 immunopreciptates of these cells. These data indicate the specificity of the anti-c-myc for c-myc-tagged Kv1.5DN. **Lanes 5 to 8 of panels A and B** show the presence of Kv1.5- (note slightly greater apparent molecular weight owing to the c-myc epitope) and c-myc-immunoreactive protein in anti-c-myc and anti-Kv1.2 immunoprecipitates of cells expressing Kv1.5DN-myc, Kv1.2wt and Kv1.5wt (lanes 5 & 6) or Kv1.5DN-myc and Kv1.2wt (lanes 7 & 8). Note the yellow signal indicative of dual labelling of the protein by anti-Kv1.5 and anti-c-myc in lanes 5 to 8 of panel C. The detection in lanes 7 & 8 of Kv1.5 and the c-myc epitope confirms the ability of the dominant-negative subunit to co-assemble with wild-type subunits. **Lanes 9 & 10 of panels A and B** show Kv1.5- and c-myc-immunoreactive protein in anti-c-myc, but not anti-Kv1.2 immunoprecipitates of cells expressing Kv1.5DN-myc and Kv1.5wt (i.e. no Kv1.2 expression). These data indicate lack of cross-reactivity of anti-Kv1.2 with Kv1.5DN or Kv1.5wt proteins. Taken together these data establish the specificity of the antibodies employed and the ability of Kv1.5DN to co-assemble with wild-type Kv1 subunits.

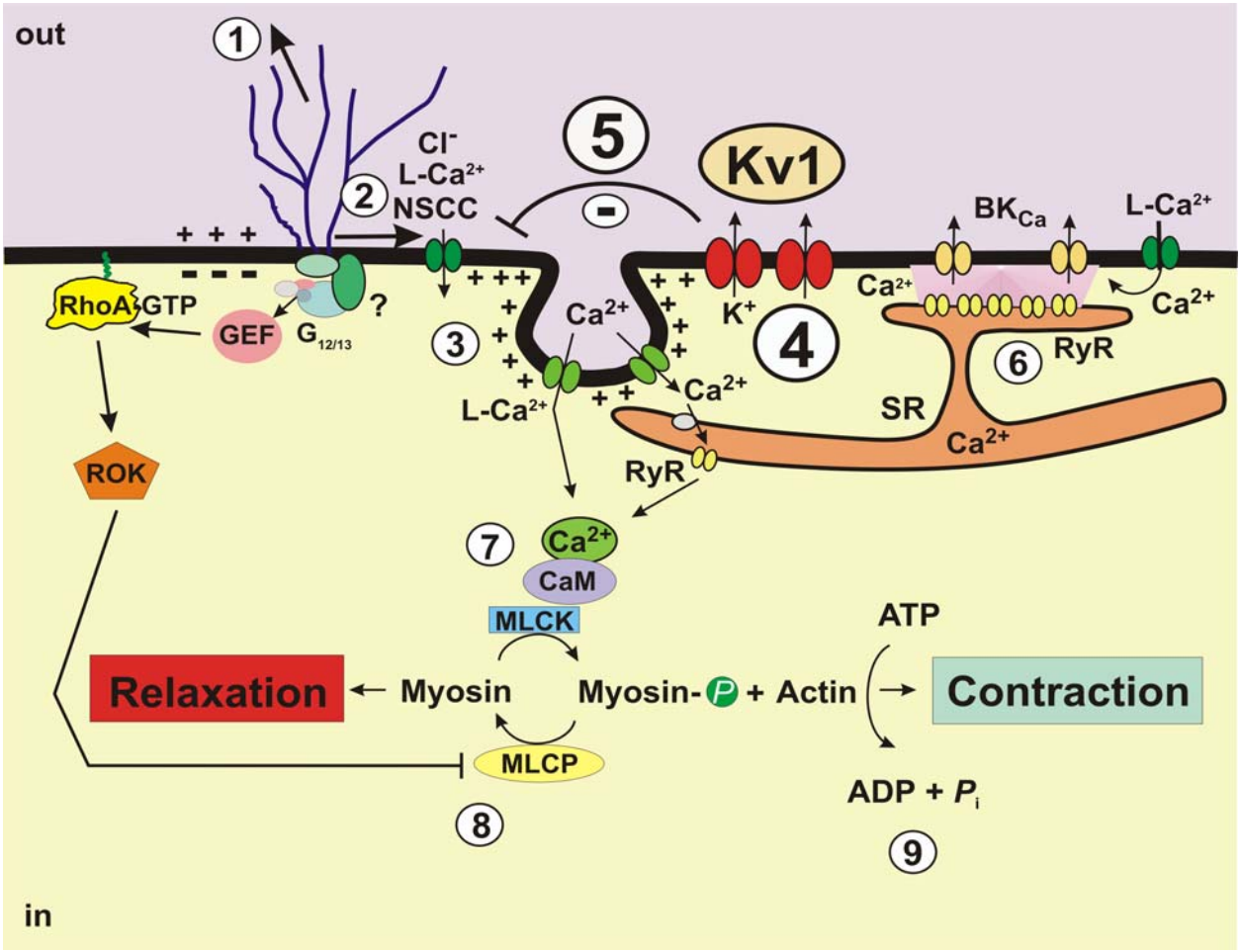
Online Supplementary Figure 3: Suppression of Kv1, but not Kv2 current by Kv1.5DN.



The ability of Kv1.5DN-myc to suppress whole-cell currents due to Kv1-family channels was determined by whole-cell voltage clamp (pipette solution contained (in mM): potassium gluconate 110, KCl 30, Na_2ATP 5, $MgCl_2$ 0.5, BAPTA 10, HEPES 5 and GTP 1 (pH 7.2) and recordings were made using HEPES-buffered (in mM; NaCl 120, $NaHCO_3$ 3, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 0.5,

glucose 10, HEPES 10 and $CaCl_2$ 1.8) external saline solution. Cell capacitance (pF) was determined and current amplitudes normalised for differences in cell size by conversion to current density (pA/pF). Data were compared by repeated measures ANOVA followed by Bonferroni's post hoc test. A level of $P<0.05$ was considered to be statistically significant. **Panel A** shows representative families of whole-cell K_{DR} currents (evoked by 250 ms voltage clamp steps from a holding potential of -60 mV to between -80 and +40 mV in 10 mV increments, followed by a step to -50 mV to evoke deactivating tail currents) and current density versus voltage plots for HEK cells transiently expressing Kv1.5wt alone, Kv1.5wt and Kv1.5DN, and Kv1.5DN-myc alone. A current density plot for HEK cells expressing empty plasmid (Mock-transfected) is also included. Note the significant suppression of Kv1.5wt current density by Kv1.5DN and lack of time-dependent current in cells transfected with Kv1.5DN alone. * indicates significantly different from control ($P<0.05$) here and in subsequent panels. **Panel B** shows representative families of whole-cell K_{DR} currents and current density versus voltage plots for HEK cells transiently expressing Kv1.2wt and Kv1.5wt or Kv1.2wt and Kv1.5DN-myc. Note suppression of Kv1.2wt current by Kv1.5DN. **Panel C** shows representative families of whole-cell currents and current density versus voltage plots for HEK cells transiently expressing Kv2.1 alone or Kv2.1 and Kv1.5DN-myc. Note the lack of effect of Kv1.5DN-myc on Kv2.1 current. **Panel D** shows representative families of whole-cell currents and current density versus voltage plots for HEK cells stably expressing Kv1.5wt in the absence (Control) and at 24 and 48 h after transfection with Kv1.5DN-myc. Note time-dependent suppression of Kv1.5 current by Kv1.5DN-myc. These data establish that Kv1.5DN-myc caused a specific, dominant-negative suppression of Kv1, but not Kv2 current.

Online Supplementary Figure 4: Role of Kv1-containing K_{DR} channels in the myogenic control of arterial diameter.



Increased intra-luminal/transmural pressure causes deformation of the extracellular matrix **(Step 1)** and/or cytoskeleton of vascular smooth muscle cells that evokes (by an unknown mechanism (?)) an increase in inward current due to enhanced non-selective cation (NSCC), L-type Ca^{2+} ($L\text{-}Ca^{2+}$) and/or Cl^- channel activity **(Step 2)**. This increase in inward current provokes depolarisation of the smooth muscle membrane potential **(Step 3)**, leading to an activation of L-type Ca^{2+} channels. If unopposed, this would lead to regenerative Ca^{2+} entry, action potential initiation and phasic contraction. This study provides molecular evidence that vascular smooth muscle K_{DR} channels

containing Kv1 subunits contribute to control of the myogenic response of resistance arteries by providing a voltage-dependent "brake" **(Step 4)** owing to hyperpolarising, outward K^+ current that opposes and limits the extent of myogenic depolarisation **(Step 5)**. This mechanism acts in parallel to a Ca^{2+}-dependent "brake" previously shown to be provided by large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) activated by focal Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR) or Ca^{2+} influx through nearby L-type Ca^{2+} channels **(Step 6)**. These two mechanisms for control of membrane potential, acting in concert, limit Ca^{2+} channel activation and provide for accurate regulation of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that stimulate myosin light chain kinase (MLCK) activity following binding of Ca^{2+} to calmodulin (CaM) **(Step 7)**. The balance between MLCK and myosin light chain phosphatase (MLCP) activity determines the level of phosphorylated myosin. Increased pressure also activates (via an unknown mechanism) a signalling pathway involving $G_{12/13}$, guanine nucleotide exchange factor (GEF), RhoA, and Rho-associated kinase (ROK) that inhibits MLCP activity **(Step 8)** and induces Ca^{2+} sensitisation of the contractile machinery (i.e. increased myosin phosphorylation and tone development at a given $[Ca^{2+}]_i$). Increased myosin phosphorylation results in an increase in actomyosin-P which, in the presence of ATP, results in cross-bridge cycling and contraction **(Step 9)**. Thus, the activation of Kv1-containing K_{DR} channels of vascular smooth muscle cells is a fundamental mechanism for precise control of vascular smooth muscle membrane potential depolarisation and, thereby, arterial diameter, blood pressure and organ-specific blood flow.