Protein Kinase A–Phosphorylated Kv1 Channels in PSD95 Signaling Complex Contribute to the Resting Membrane Potential and Diameter of Cerebral Arteries

Christopher L. Moore, Piper L. Nelson, Nikhil K. Parelkar, Nancy J. Rusch, Sung W. Rhee

**Rationale:** Postsynaptic density-95 (PSD95) is a scaffolding protein that associates with voltage-gated, Shaker-type K⁺ (Kv1) channels and promotes the expression of Kv1 channels in vascular smooth muscle cells of the cerebral (cVSMCs) circulation. However, the physiological role of PSD95 in mediating molecular signaling in cVSMCs is unknown.

**Objective:** We explored whether a specific interaction between PSD95 and Kv1 channels enables protein kinase A phosphorylation of Kv1 channels in cVSMCs to promote vasodilatation.

**Methods and Results:** Rat cerebral arteries were used for analyses. A membrane-permeable peptide (Kv1-C peptide) corresponding to the postsynaptic density-95, discs large, zonula occludens-1 binding motif in the C terminus of Kv1.2α was designed as a dominant-negative peptide to disrupt the association of Kv1 channels with PSD95. Application of Kv1-C peptide to cannulated, pressurized cerebral arteries rapidly induced vasoconstriction and depolarized cVSMCs. These events corresponded to reduced communoprecipitation of the PSD95 and Kv1 proteins without altering surface expression. Middle cerebral arterioles imaged in situ through cranial window also constricted rapidly in response to local application of Kv1-C peptide. Patch-clamp recordings confirmed that Kv1-C peptide attenuates Kv1 channel blocker (5-(4-phenylalkoxypsoralen)—sensitive current in cVSMCs. Western blots using a phospho-protein kinase A substrate antibody revealed that cerebral arteries exposed to Kv1-C peptide showed markedly less phosphorylation of Kv1.2α subunits. Finally, phosphatase inhibitors blunted both Kv1-C peptide–mediated and protein kinase A inhibitor peptide–mediated vasoconstriction.

**Conclusions:** These findings provide initial evidence that protein kinase A phosphorylation of Kv1 channels is enabled by a dynamic association with PSD95 in cerebral arteries and suggest that a disruption of such association may compromise cerebral vasodilatation and blood flow. (Circ Res. 2014;114:1258-1267.)

**Key Words:** cerebral arteries ■ muscle, smooth, vascular ■ PDZ domains ■ potassium channels

**Shaker-type** voltage-gated K⁺ (Kv1) channels composed of Kv1.2 and Kv1.5 α-subunits are expressed in cerebral vascular smooth muscle cells (cVSMCs), where they contribute to the resting diameter and vasodilatation of cerebral arteries (CAs).12 Kv1 channels are multiprotein structures composed of 4 Kvα pore-forming subunits coassembled with intracellular Kvβ subunits, which may affect channel trafficking and kinetics.3–8 In addition, post-translational modifications, such as glycosylation and protein kinase A (PKA)–mediated phosphorylation of the Kvα subunits, may increase protein expression and activity of Kv1 channels.9–15

Recently, we reported the expression of a scaffolding protein, postsynaptic density protein-95 (PSD95), in rat CA.16 Previously, PSD95 was studied primarily in neurons, where it provides an assembly platform at the plasma membrane for macromolecular signaling complexes including ion channels.17–22 However, we reported that PSD95 serves as a molecular scaffold for Kv1 channels in cVSMCs, and this interaction is required for the proper expression of Kv1 channels that exerts a tonic vasodilator influence.16 Accordingly, antisense-mediated knockdown of PSD95 in rat CA resulted in a loss of Kv1 channel expression and caused vasoconstriction, inferring that PSD95 promotes the expression of Kv1 channels in cVSMCs.16

Notably, the C terminus of the Kv1.2α subunit contains a structural motif that permits the channel to interact with...
PSD95.16–20 Collectively, the interactions of signaling proteins with PSD95 are enabled by 3 postsynaptic density-95, discs large, zonula occludens-1 (PDZ) domains, which act as docking sites for signaling molecules and show preference for distinct binding partners (Figure 1A). PDZ1 and PDZ2 preferentially bind to the C terminus of the Kᵥ1.2α subunit via an association that is intrinsically unstable, thereby permitting a dynamic and reversible interaction.17,19,23,24 Src-homology and guanylate kinase domains also interact with other scaffolding proteins, such as guanylate kinase–associated protein or A-kinase anchoring protein (AKAP), providing a platform for macromolecular complexes.21,25,26

Because the 3 PDZ domains of PSD95 can form interactions with several signaling molecules, the design of interfering peptides that disrupt the interaction between PSD95 and a specific molecular partner has emerged as an important strategy to pinpoint the physiological impact of a single scaffolding interaction.27,28 In this approach, a dominant-negative peptide of identical sequence to the PDZ-binding motif of a molecular partner is overexpressed to disrupt this PDZ interaction only. The importance of PSD95 scaffolding of N-methyl-D-aspartate receptors (NMDARs) and neuronal nitric oxide synthase in neurons was revealed using this strategy.27,28 A similar dominant-negative peptide was administered to rodents and nonhuman primates in vivo to reduce neuronal damage after experimental stroke by disrupting PSD95-dependent excitotoxic signaling between NMDAR and neuronal nitric oxide synthase. To achieve optimal cell penetration in these studies, an HIV-tat sequence was coupled to the C terminus peptide sequence of the NMDAR-NR2B subunit that binds to PDZ domains.29–32

In the present study, we adopted this general strategy to evaluate whether association with PSD95 is required for the proper function of Kᵥ1 channels in rat CA and to identify other components in the PSD95 complex that also may be required to confer cerebral vasodilation. We designed a cell-permeable dominant-negative peptide corresponding to the C terminus PDZ motif of the Kᵥ1.2-subunit (Kᵥ1-C peptide) to disrupt Kᵥ1 scaffolding by PSD95. Our findings draw attention to PSD95 as a key scaffolding protein in cVSMCs that enables the basal phosphorylation and opening of Kᵥ1 channels to contribute to the resting diameter of CA and infer that conditions that interrupt the PSD95 complex may compromise cerebral vasodilation and blood flow.

Methods

CAs were isolated from 10- to 14-week-old male Sprague–Dawley rats as approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. A dominant-negative peptide (Kᵥ1-C) was used to disrupt the association of Kᵥ1 and PSD95 (Figure 1B). Kᵥ1-C consists of the final 10 amino acids of the C terminus of Kᵥ1.2α attached to an N terminus HIV-tat sequence (NH3-YGRKKRRQRRR) to confer membrane permeability. An N terminus fluorescein label was attached to some peptides for

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**Nonstandard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
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<tr>
<td>CA</td>
<td>cerebral artery</td>
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<tr>
<td>cVSMC</td>
<td>cerebral vascular smooth muscle cell</td>
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<tr>
<td>Kᵥ1</td>
<td>Shaker-type voltage-gated K⁺ channel</td>
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<tr>
<td>Kᵥ1-C</td>
<td>membrane-permeable peptide mimicking Kᵥ1.2α C-terminus</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<tr>
<td>PDZ</td>
<td>postsynaptic density-95, discs large, zonula occludens-1</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>Psora4</td>
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**Figure 1.** Membrane-permeable peptide mimicking Kᵥ1.2α C-terminus (Kᵥ1-C) disrupts associations of postsynaptic density-95 (PSD95) and Kᵥ1.2α. A. Schematic of the association of Kᵥ1.2α channels with the PSD95 scaffolding protein via the postsynaptic density-95, discs large, zonula occludens-1 (PDZ1)–binding domain. PSD95 contains 3 PDZ-binding domains (PDZ1-3), Src-homology (SH3), and guanylate kinase (GK) domains. B. The Kᵥ1-C dominant-negative peptide was designed to compete for the PDZ-binding domain on PSD95. The last 10 amino acids of the Kᵥ1.2α C terminus were conjugated to HIV-tat (YGRKKRRQRRR) to confer cell-permeability. P is a spacer. LTDV is a class-1 PDZ binding motif on Kᵥ1.2α. A peptide with same amino acid composition but in a scrambled order (Scm) was used as control. C. Immunoprecipitation using anti-Kᵥ1.2α of cerebral artery (CA) lysate treated with Scm or Kᵥ1-C peptide for 30 minutes. Elation (Kᵥ1.2α immunoprecipitate [IP]) and column flow-through (flow-through) were probed for PSD95 on a Western blot. Depicted is a representative scan from 3 similar experiments. D. Biotinylation of CA treated with Scm or Kᵥ1-C peptide for 30 minutes. Cytosolic and surface fractions were probed for Kᵥ1.2α. Control lysate from freshly isolated CA was loaded for size comparison. Depicted is a representative blot from 5 similar experiments.
Visualization. Two scrambled variations of the peptide were used as negative controls (21st Century Biochemicals). Peptide disruption of K\(_\text{v}1\) channel-PSD95 association was determined by coimmunoprecipitation.\(^{36}\) Protein surface expression was determined by biotinylation.\(^{33}\) CA diameter was measured using a pressure myograph and software (Danish Myo Technology). In situ response of middle cerebral arterioles to local application of peptides was measured by suffused cranial window imaging using a Sony HDR-PJ580 camera and an automated IPLab script. Membrane potential was measured by glass microelectrodes connected to a preamplifier (Dagan) and analyzed with WinDaq Lite software (DataQ). Whole-cell cVSMC patch-clamp was performed with an EPC 7 amplifier (HEKA) and pCLAMP 6 software (Molecular Devices).\(^{16}\) Nonpermeable peptides were targeted,\(^{16}\) only the full form of PSD95 (upper band at 95 kDa) and subunits was not different between arteries exposed to K\(_{\text{V1}}\)-C compared with Scm, indicating that K\(_{\text{V1}}\)-C did not cause a loss of surface K\(_{\text{V1}}\) channels (Figure 1D). We also confirmed that K\(_{\text{V1}}\)-C peptide penetrated cVSMCs of intact arteries using confocal imaging (Figure 2). After 1 minute of incubation, fluorescein-labeled K\(_{\text{V1}}\)-C peptide appeared at the cell surface and a strong fluorescent signal was observed intracellularly by 3 minutes that persisted until 30 minutes. Similar penetration patterns were observed for fluorescein-labeled Scm peptide (Online Figures I and II). Compared with smooth muscle cells, neurons in the adventitia and the endothelial cells absorbed the peptides sooner, within the first minute (Online Figure I). Collectively, these findings indicate that our cell-permeable dominant-negative peptide successfully competes with the native K\(_{\text{V1}}\)-2 subunit to prevent its binding to the PDZ domains of PSD95, and it provides a tool to disrupt the association between K\(_{\text{V1}}\) channels and PSD95 in cVSMCs of intact arteries.

**K\(_{\text{V1}}\)-C Peptide Constricts and Depolarizes Isolated CA**

Diameter recordings of isolated, pressurized rat CA exposed to K\(_{\text{V1}}\)-1-C peptide revealed a rapid constriction of CA in response to disruption of the K\(_{\text{V1}}\) channel–PSD95 complex, whereas Scm peptide did not affect vessel diameter (Figure 3A). Increasing concentrations of 1-, 3-, and 10-μmol/L K\(_{\text{V1}}\)-C peptide progressively reduced CA diameter by −7.4±2.0%, −19.3±2.5%, and −20.3±2.1% (Figure 3B). The corresponding values for Scm peptide were −4.2±2.8%, −3.7±0.6%, and −3.9±1.9%. A second scrambled control peptide (Scm2) also did not significantly constrict CA (Online Figure III). Bath application of the K\(_{\text{V1}}\) channel blocker, 5-(4-phenylalkoxyxyporalen) (Psora4)\(^{2,16,34,35}\) did not further reduce the diameter of arteries already constricted by K\(_{\text{V1}}\)-C peptide (Figure 3C and 3D). Notably, the constrictor response to 100-nmol/L Psora4 was not different from the constriction caused by 10-μmol/L K\(_{\text{V1}}\)-C peptide (Figure 3D) or the diameter change induced by combined Psora4 and K\(_{\text{V1}}\)-C peptide (Figure 3D). Absolute diameter values are provided in the Online Figure IV. The constriction induced by K\(_{\text{V1}}\)-C peptide was not significantly

![Figure 2. Membrane-permeable peptide mimicking K\(_{\text{V1}}\)-1,2α C-terminus (K\(_{\text{V1}}\)-C) rapidly penetrates cerebral vascular smooth muscle cells (cVSMCs) in isolated cerebral arteries (CAs). A. Confocal images of isolated rat CA incubated with fluorescein-labeled K\(_{\text{V1}}\)-C peptide (top row) for 0, 1, 3, 5, 10, and 30 minutes at 37°C. Alexa350-labeled wheat germ agglutinin (bottom row, WGA) was used as a cell surface marker for cVSMCs. Individual cVSMCs are visible vertically wrapping around the CA circumferentially because the artery was placed horizontally for imaging. The brightness settings for the green channel in 10- and 30-minute treatments were reduced to display individual cells. Representative images from 3 similar experiments. B. Merged images from 1-, 3-, and 10-minute time points.](http://circres.ahajournals.org/doi/10.1161/CIRCRESAHA.114.303008)
altered by the presence of 100-μmol/L Nω-Nitro-l-arginine methyl ester and 10-μmol/L indomethacin in the bath solution to block nitric oxide synthase and cyclooxygenase, respectively (Online Figure V). Constrictor responses to 1-μmol/L linopirdine, a K<sub>v</sub>7 channel blocker, or 30-μmol/L BaCl<sub>2</sub>, a K<sub>ir</sub> channel blocker, were not significantly altered by the presence of 3-μmol/L K<sub>v</sub>1-C peptide (Online Figure VI). In addition, 1-μmol/L glibenclamide, a K<sub>ATP</sub> channel blocker, or 30-nmol/L stromatoxin, a K<sub>V2.1</sub> channel blocker, did not constrict pressurized CA significantly (n=5 each, data not shown).

Importantly, microelectrode recordings indicated that cVSMCs of pressurized CA depolarized from a resting membrane potential (E<sub>m</sub>) of −47.6±2.1 to −35.2±3.2 mV in response to 10-μmol/L K<sub>v</sub>1-C peptide (Figure 4A and 4C), a finding consistent with a loss of hyperpolarizing K<sup>+</sup> current. In contrast, cVSMCs in arteries treated with Scm peptide showed no significant change in resting E<sub>m</sub> (Figure 4B and 4C). The addition of 60-mmol/L KCl to bath solutions already containing K<sub>v</sub>1-C or Scm peptide further depolarized arteries of both groups to similar values (Figure 4). These findings indicate that the specific disruption of K<sub>v</sub>1.2 channel–PSD95 association in pressurized CA leads to depolarization and constriction from the resting tone. These effects seem to correspond selectively to the blockade of K<sub>v</sub>1.2 channels and imply a loss

Figure 3. Membrane-permeable peptide mimicking K<sub>v</sub>1.2α C-terminus (K<sub>v</sub>1-C) disrupts association of postsynaptic density-95 (PSD95) and K<sub>v</sub>1.2 and constricts cerebral arteries (CA).

A. Representative recording of outer diameter in a cannulated, pressurized (80 mmHg) rat CA. The artery was initially exposed to 10-μmol/L scrambled (Scm) peptide, and then after extensive washes, exposed to 10-μmol/L K<sub>v</sub>1-C peptide.

B. CA constricted in response to K<sub>v</sub>1-C peptide in a concentration-dependent manner, but did not constrict to Scm peptide (n=5 each). Significant different from Scm, P<0.05.

C. Diameter recordings from a CA exposed sequentially to 10-μmol/L K<sub>v</sub>1-C peptide and the specific K<sub>v</sub>1 channel antagonist, 100-nmol/L 5-(4-phenylalkoxy)soralen (Psora4).

D. Percentage change in diameter from baseline in response to 10-μmol/L K<sub>v</sub>1-C peptide and 100-nmol/L Psora4 (n=6 each). *Significantly different from Scm, P<0.05.

Figure 4. Membrane-permeable peptide mimicking K<sub>v</sub>1.2α C-terminus (K<sub>v</sub>1-C) causes depolarization of in situ cerebral vascular smooth muscle cells (cVSMCs).

A and B. Recordings of cerebral artery (CA) diameter and membrane potential (E<sub>m</sub>) in rat CA exposed to K<sub>v</sub>1-C (A) or scrambled (Scm; B) peptide. After recording baseline E<sub>m</sub> (1, 4), 10-μmol/L K<sub>v</sub>1-C (2), or Scm (5) peptide was added to the bath followed by 60-mmol/L KCl (3, 6) to elicit maximal depolarization. E<sub>m</sub> was recorded in each step for ≈5 minutes as indicated by the numbered boxes in the diameter trace. C. Average E<sub>m</sub> values in the presence of K<sub>v</sub>1-C peptide or Scm peptide only, and after the further addition of 60-mmol/L KCl (n=6 each). a to c, Significantly different, P<0.05.
of function of K_1,2 channels in cVSMCs when dissociated from the PSD95 complex.

**K_1,1-C Peptide Constricts CA In Vivo**

After confirming the selective disruption of K_1,1–PSD95 interaction ex vivo by K_1,1-C peptide, we evaluated the impact of K_1,1-C peptide on CA in vivo. A partial craniectomy of the right parietal plate of anesthetized rats immobilized in a stereotaxic frame, followed by mounting of a cranial window, exposed branches of the middle CAs for imaging and topical peptide treatment (Figure 5A). Suffusion of the cranial window with 30-μmol/L K_1,1-C peptide resulted in a rapid constriction of CA, which was sustained for ≥20 minutes (Figure 5B and 5C). Average values indicated that middle CAs treated with K_1,1-C peptide showed a significant reduction in diameter of −16.1±3.9% by 5 minutes with maximum constriction at 15 minutes averaging −16.8±3.5% (Figure 5D). In contrast, the same concentration of Scm peptide did not affect vessel diameter (Figure 5B and 5C), although the same arteries responded to 60-mmol/L KCl by strongly constricting as evidence of viability (Figure 5B). Arteries in physiological salt solution or Scm peptide showed a small decrease in diameter by 15 minutes, which were not statistically significant from each other (Figure 5D). These results provide initial evidence that K_1,1 channels in PSD95 signaling complexes contribute to the resting diameter of cerebral resistance arteries in vivo and disruption of such association may have a profound effect on cerebral blood flow.

**K_1,1-C Peptide Suppresses Psora4-Sensitive K^+ Currents in cVSMCs**

Next, we used patch-clamp techniques to confirm that K_1,1-C peptide directly reduces K_1 channel function in cVSMCs. In these studies, nonpermeable peptides were added to the dialyzing pipette solution after extensive attempts to add permeable HIV-tat–containing peptides extracellularly resulted in disruption of high-resistance gigaohm seals. Accordingly, control K^+ currents were recorded from cVSMCs dialyzed with either 3-μmol/L Scm nonpermeable (Figure 6A) or 3-μmol/L K_1,1-C nonpermeable (Figure 6B) before addition of the selective K_1 channel blocker, 100 nmol/L Psora4.2,16,34,35 Digital subtraction of post-Psora4 (+Psora4) from pre-Psora4 (−Psora4) recordings provided an estimation of Psora4-sensitive K_1 current (Figure 6C, I_Psora). The residual currents were regarded as Psora4-insensitive (Figure 6D, I_other). The peak I_Psora density at +58 mV was 8.47±1.29 pA/pF for Scm nonpermeable-treated cells. This value decreased by 45% to 4.65±0.71 pA/pF for cells dialyzed with K_1,1-C nonpermeable (Figure 6C). In contrast, the peak density of residual Psora4-insensitive current (I_other) was not significantly different between Scm and K_1,1-C–treated cells (Figure 6D). These findings concur with reports that only K_1,1α subunits have PDZ-binding motifs to enable PSD95 interaction.16,17 In this regard, Shab-type K_2 channels and KCNQ (K_v7) channels likely contribute to the Psora4-insensitive residual current. Stomatoxin-sensitive K_2 channels are not blocked by Psora4 in cVSMCs16,35 and Psora4 does not compromise vasoconstrictor responses of CA to the K_v7 channel blocker, linopirdine (Online Figure VI).

**K_1,1-C Peptide Reduces PKA Phosphorylation of K_1 Channels**

After demonstrating that PSD95 is the critical scaffold required for K_1 channel function and dilation of CA, we searched for the molecular component(s) of the PSD95 signaling complex responsible for the basal opening of K_1 channels. One candidate binding partner of PSD95 is AKAP150, which is

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**Figure 5. Membrane-permeable peptide mimicking K_1,2α C-terminus (K_1,1-C) constricts rat cerebral arteries (CAs) in vivo.** A, An overview of the cranial window. A branch of middle cerebral artery (arrowhead) is analyzed for diameter changes. B, Representative images reveal the constrictor response of a middle cerebral artery branch to 30-μmol/L K_1,1-C peptide, but not to the same concentration of scrambled (Scm) peptide. C, Diameter responses of single arterial branches to Scm or K_1,1-C peptide. D, Percentage change in diameter from baseline in response to 30-μmol/L Scm or K_1,1-C peptides or physiological salt solution (PSS). Scale bar, 5 minutes. **Significantly different from PSS and Scm, P<0.01, n=5 to 7.
known to act as a focal point in multiprotein signaling complexes to facilitate PKA-dependent phosphorylation of target proteins. Huang et al first reported that direct application of PKA catalytic subunits lead to the activation of cloned cardiac Kv1 channels in oocytes. Later, PKA-dependent phosphorylation and opening of Kv1 channels were reported in native smooth muscle cells from rabbit portal vein and canine colon. However, many later studies using cAMP analogues or forskolin on recombinant Kv1.2 channels in heterologous cells only revealed an increase in Kv1.2 protein levels by PKA phosphorylation with small to no increase in gating of channels. Therefore, we examined whether the association of Kv1 channels with PSD95 signaling complex could explain this apparent discrepancy.

Previously, Connors et al successfully used an antibody that detects PKA-phosphorylation of serine or threonine residues to identify PKA phosphorylation of rat Kv1.2α subunits overexpressed in a heterologous expression system. We used the same antibody to explore whether Kv1.2α subunit–PSD95 interaction is required for PKA-phosphorylation of Kv1 channels in cVSMCs, thereby inferring colocalization of Kv1 channels with AKAP150 in the vascular PSD95 signaling complex. CAs treated with 10-μmol/L Scm or Kv1-C peptide for 30 minutes were lysed and loaded equally (30 μg) into lanes of a Western blot probed with anti–phospho-PKA substrate. Compared with CA treated with Scm peptide, Kv1-C–treated CA showed a marked loss in the phosphorylation of the ~75 kDa band (Figure 7A, left blot), which is the fully glycosylated form of Kv1.2α1 required for PKA phosphorylation. Average densitometric values from 5 similar preparations demonstrated a ~50% reduction in PKA phosphorylation of Kv1.2α, whereas the expression level of total Kv1.2α protein did not change significantly (Figure 7A, left panel and Figure 7B). These results suggest that PSD95 scaffolding promotes PKA-mediated phosphorylation of Kv1 channels, an event associated with increased Kv1 channel opening.

To confirm that reduced PKA-mediated phosphorylation of Kv1 channels contributed to the constriction of CA by Kv1-C peptide, we intervened to preserve channel phosphorylation using low concentrations of the protein serine/threonine phosphatase inhibitors, okadaic acid (OA, 300 nmol/L) and calyculin-A (100 nmol/L). Neither phosphatase inhibitor significantly altered the resting arterial diameter of isolated CA (Figure 8B), but both significantly blunted the constrictor response to Kv1-C peptide (Figure 8A and 8B). In drug-free physiological salt solution, Kv1-C peptide reduced the diameter of CA by −19.5±1.6% compared with −9.3±3.3% and 914.1±1.1% in the presence of okadaic acid and calyculin-A, respectively (Figure 8B). Constriction in response to KCl was not altered by the presence of okadaic acid or calyculin-A (Figure 8C), indicating that the ability of CA to constrict was not nonspecifically reduced by the presence of low concentrations of phosphatase inhibitors. In a final set of experiments we verified that a loss of PKA activity results in constriction of rat CA because earlier studies defining mechanisms of PKA modulation of Kv1 channel properties were mostly performed in cell systems. Indeed, 1-μmol/L myristoylated protein kinase A inhibitor peptide rapidly constricted isolated rat CA by ~27.3±0.7%, and this constrictor response was significantly reduced in the presence of 100-nmol/L calyculin-A (Figure 8D and 8E). These findings highlight that PKA phosphorylation of Kv1 channels is an important contributor to the

Figure 6. Membrane-permeable peptide mimicking Kv1.2α C-terminus (Kv1-C) reduces 5-(4-phenylalkoxyxypсорalen) (Psora4)-sensitive K+ current. A and B, Representative whole-cell K+ currents recorded with 3-μmol/L scrambled (Scm; A) or Kv1-C peptide (B) in the pipette solution. Current densities before (−Psora) and after (+Psora) the bath addition of 100-nmol/L Psora4 in are plotted. Psora4-sensitive current density I_{P4} was calculated by digitally subtracting post-Psora (±Psora) from pre-Psora (+Psora). C and D, Psora4-sensitive (C) and Psora4-insensitive (D) current densities (n=6 each). *Significant difference from Scm, P<0.05. NP indicates nonpermeable.
basal dilation of CA and that a dynamic association with the PSD95 signaling complex is critically required for PKA phosphorylation of KV1 channels in CA.

Discussion

PSD95 was originally regarded as an exclusive feature of the postsynaptic density in neurons, and it was only rarely detected in nonneural tissues.37,38 However, two recent reports identified PSD95 as a potentially important scaffold of KV1 channels in lymphocytes and in cVSMCs.16,39 First, mutation of the KV1.3 subunit PDZ-binding motif or knockdown of PSD95 in T lymphocytes inhibited the recruitment of KV1.3 into contact points on the cell surface, implying an important role of PSD95–KV1 channel interaction in T-cell activation.39 Second, we demonstrated in cVSMCs that PSD95 is expressed and antisense knockdown of PSD95 in rat CA for 72 hours results in a concomitant loss of KV1 channel protein and vasodilator function. These findings suggested a critical role of PSD95 in maintaining the expression of KV1 channels in the cerebral circulation.16 However, because KV1 channels were downregulated by PSD95 knockdown in our previous study, the impact of PSD95 signaling complex on KV1 channel function could not be assessed. Furthermore, knockdown of PSD95 effectively limits interaction with all of its binding partners, potentially disrupting multiple signaling pathways in addition to KV1 channels involved in regulating the diameter of CA. For example, β1-adrenergic receptors,40 serotonin receptors,41 inward-rectifying potassium channels,42 and neuronal nitric oxide synthase43 are known binding partners of PSD95 in neurons that could similarly interact with PSD95 in arteries to alter vascular tone. Thus, the present study designed a dominant-negative peptide to specifically disrupt the scaffolding interaction between KV1 channels and PSD95 in cVSMCs. Our results provide initial evidence that the PSD95 signaling complex is critically required for KV1 channel dilator function in rat CA in situ and further suggest that PSD95

Figure 7. Protein kinase A (PKA) phosphorylation of KV1,2α is reduced in arteries exposed to membrane-permeable peptide mimicking KV1,2α C-terminus (KV1-C). A, Western blot using a phospho-PKA substrate antibody (PKApH, left blot) detected a ≈75-kDa phospho-corresponding to KV1,2α in cerebral artery (CA) protein lysates. OA from age-matched rats were incubated in either scramble (Scm) or KV1-C peptide, and then lysates (30 μg) were loaded into adjacent lanes. Lanes 1 to 2 and lanes 3 to 4 represent separate preparations. The blot was stripped and reprobed with anti-KV1,2α antibody (KV1,2α, right blot). B, Densitometry measurement of PKApH and KV1,2α bands relative to Scm treatment (n=5). *Significantly different from Scm, P<0.05.

Figure 8. Phosphatase inhibitors blunt the vasoconstriction caused by membrane-permeable peptide mimicking KV1 channel C terminus (KV1-C). A, Representative traces show that the phosphatase inhibitors, 300-nmol/L okadaic acid (OA) and 100-nmol/L calyculin A (CalA), blunt the vasoconstrictor response of rat cerebral artery (CA) to KV1-C peptide. B, Average percentage change in diameter in response to vehicle (veh), OA, CalA, 3-μmol/L KV1,1-C peptide, or KV1,1-C peptide in the presence of veh, OA, or CalA (n=5–10). The phosphatase inhibitors did not affect the resting diameter of CA, but blunted the vasoconstrictor response to KV1,1-C peptide. a to c: statistical significance, P<0.05. C, Average percentage change in diameter caused by 60-nmol/L KCl in the presence and absence of a phosphatase inhibitor (n=4–10). No significant difference between groups. D, Representative traces show that 100-nmol/L CA plus PKI blunts the vasoconstrictor response to 1-μmol/L protein kinase A inhibitor peptide (PKI). E, Average percentage change in diameter caused by 1-μmol/L PKI in the absence or presence of CalA (n=5). *Significantly different from PKI alone, P<0.05.
provides a platform for interaction between PKA and Kv1 channels that enables PKA-mediated phosphorylation and opening of Kv1 channels to promote hyperpolarization and relaxation of cVSMCs.

Our initial experiments explored whether a dynamic association with PSD95 was required for the proper vasodilator function of Kv1 channels in CA. After directly disrupting the Kv1–PSD95 association by application of Kv1-C peptide, we observed a rapid and profound vasoconstriction ex vivo and in vivo. Ex vivo Kv1-C peptide–induced constriction was equivalent to constriction caused by the Kv1 channel blocker Psora4 and there were no additive effects of concomitant treatment, whereas vasoconstriction caused by Kv7 channel blocker linopirdine or KIR channel blocker BaCl2 was not altered by Kv1-C peptide. Psora4 is a highly selective blocker of Kv1 channels and does not significantly block other voltage-gated K+ channels or large-conductance, Ca2+-activated K+ channels. A recent report identified a secondary binding site in the unique side pocket of Kv1 channels that strengthens the selectivity of Psora4. In our patch-clamp study, Kv1-C peptide caused a significant reduction in Psora4-sensitive K+ currents, whereas Psora4-insensitive K+ currents that may contain K2.1 or K7 currents remained identical to Scm-treated cells. Considering the diversity of K+ channels in CA, the effects caused by disruption by Kv1-C peptide is remarkably specific to the blockade of Kv1 channels and strengthens the idea of selective PDZ binding of Kv1 channels to the PSD95 complex.

Application of Kv1-C peptide to isolated CA also depolarized the cVSMC membrane without an apparent change in Kv1 channel expression, implying that basal Kv1 channel opening relies on PSD95 scaffolding to mediate hyperpolarizing K+ efflux. The rapid onset of Kv1-C peptide–induced effects was consistent with the idea that the intrinsic instability of the C terminus of the Kv1.2α subunit allows for a dynamic interaction with the PDZ-binding domains of PSD95. In general, PDZ binding seems to be reversible and similar competing peptides have been applied to cultured neuronal cells and to neurons in vivo to disrupt the binding of NMDAR and PSD95 and break the excitotoxic signaling pathway mediated by PSD95. The rapid intracellular delivery of peptides conferred by the HIV-tat sequence has been reported in many cell types and now successfully used in the present study to explore ion channel signaling pathways in arteries. Considering our finding that an HIV-tat–conjugated peptide targeting PSD95 can markedly alter cerebrovascular tone, it is possible that the vasoconstrictor effects of an HIV-tat peptide delivered intravenously by Bach et al and Cook et al to interrupt NMDAR–PSD95 interaction in neurons and ameliorate stroke may have a direct action on cVSMCs as an unexpected side effect. Because Kv1.2α and NMDAR share the PDZ1 and PDZ2-binding domains of PSD95, C terminus peptides designed for NMDAR can potentially cross-compete with Kv1 channels for binding to PSD95. Considering this therapy is already in phase 1 and 2 clinical trials in Canada and the United States, the need to fully define the physiological role of PSD95 in CA is even more urgent. In this respect, the present study provides initial proof for mechanism by which disruption of PSD95 scaffolding could adversely affect cerebral circulation.

We also observed that Kv1-C peptide reduced PKA phosphorylation of Kv1 channels without altering cell surface expression. Our study indicates that association with the PSD95 complex is required for the direct PKA-mediated phosphorylation of Kv1.2α subunit and increased channel function independent of expression level. Our observation may explain the dichotomy between early reports of PKA-induced increase in Kv1.2 channel activity and the lack of such increase in Kv1.2 channel activity in many later studies in heterologous expression systems. This apparent discrepancy may have resulted from the lack of PSD95 signaling complex in heterologous cells, which is necessary to hold PKA in proximity to Kv1.2 channels for proper phosphorylation. In seeming contrast to the present study, we previously reported that antisense-knockdown of PSD95 in rat CA for 72 hours resulted in marked loss of the Kv1.2α protein. However, in the present study, CAs were only exposed to the Kv1-C peptide for 30 minutes, a time frame that apparently was too short to cause a loss of Kv1 channels and only their vasodilator function was significantly blunted. The finding that PSD95 is required for PKA-mediated phosphorylation of the Kv1.2α subunit in cVSMCs raises the possibility that PSD95–AKAP interaction exists as unrecognized scaffolding machinery in the vasculature. Colledge et al reported that PSD95–AKAP interaction is required for PKA-dependent phosphorylation of AMPA receptors in neurons, and our data suggest that the PSD95–AKAP complex also may be required for PKA-dependent phosphorylation of vascular Kv1 channels. Dissociation of Kv1.2α-containing channels from this complex by the Kv1-C peptide may reduce subunit phosphorylation leading to phosphatase-dependent dephosphorylation, less channel opening, depolarization of the cell, and constriction of CA.

Several limitations of our study should be acknowledged. First, we have used nonpermeable peptides in patch-clamp studies to verify that Kv1-C peptide disruption of PSD95–Kv1 interaction reduces Kv1 channel–mediated K+ current. Standard whole-cell and perforated-patch studies were attempted with the cell-permeable peptides, but application of the HIV-tat peptide even in low concentrations consistently disrupted gigaohm seals. Thus, apparently the HIV-tat sequence that contains several positive charges interferes with seal integrity. For this reason, we also performed membrane potential measurements to provide strong evidence that K+ efflux through Kv1 channels is attenuated by Kv1-C peptide disruption of the Kv1 channel–PSD95 complex. Second, we used a higher concentration of Kv1-C peptide for in vivo than ex vivo studies of vascular reactivity. Isolated CA constricted maximally to 10-μmol/L Kv1-C peptide, but 30-μmol/L Kv1-C was required to elicit sustained constriction in vivo (Online Figure VII). In contrast to isolated perfused arteries, a reduction in peptide potency and efficacy may be expected in vivo, because the cranial window preparation unavoidably has adventitia and neural tissue as barriers or sinks of peptide, potential peptide loss through cerebrospinal fluid or metabolism may occur, and compensatory mechanisms may exist to normalize arterial diameter in response to disruption of normal homeostatic mechanisms. Third, we did not identify which serine or threonine residues of Kv1.2α subunit are targets of PSD95-mediated PKA phosphorylation in cVSMCs and left this line...
of inquiry for future studies. Finally, K1-C peptide may have unidentified nonspecific effects in CA. Our K1-C peptide corresponding to a PDZ-binding motif on the K1.2α subunit was designed to act as a dominant-negative peptide to compete for the PDZ1 and PDZ2 domains on PSD95. Of the known voltage-gated K+ channel subunits in CA, K1.2α is uniquely equipped with a PDZ-binding motif,16 which may enable the K1 channels to be downstream targets of PKA-dependent signaling on the PSD95 scaffold. However, the PDZ domains of PSD95 also could bind unidentified vasoactive proteins in CA that contain a PDZ-binding motif. In addition to K1.2α, these proteins also could be displaced by the K1-C peptide, thereby potentially modifying arterial diameter.

In summary, we propose that the scaffolding of K1 channels by PSD95 in CA is a dynamic interaction required for the efficient vasodilator function of K1 channels through PKA phosphorylation. Dissociation of K1 channels from this complex by a dominant-negative peptide causes rapid vasconstriction of CA ex vivo and in vivo. Our findings emphasize that therapeutic targeting of PSD95 to reduce cytotoxic injury caused by stroke or other ischemic insults should consider that manipulation of PSD95 also may critically alter cerebral arterial diameter and blood flow independently of neuronal function. Similarly, disease-based alterations of PSD95 structure or function may potentially contribute to cerebral blood flow abnormalities by disrupting K1 channel dilator function or interfering with the roles of other PSD95-binding partners in VSMCs.

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Disclosures
None.

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Novelty and Significance

What Is Known?

- Postsynaptic density-95 (PSD95), once considered a neuronal marker, is a scaffolding protein that assembles ion channels and signaling molecules at the plasma membrane of several cell types.
- PSD95 is expressed and coassembles with Shaker-type potassium (Kv1) channels in the vascular smooth muscle cells (cVSMCs) of cerebral arteries (CAs).
- The opening of Kv1 channels contributes to the resting diameter of CA, which determines the level of blood flow to distal neurons.

What New Information Does This Article Contribute?

- Selective disruption of the association between PSD95 and Kv1 channels results in a severe constriction of rat CA ex vivo and in vivo.
- Protein kinase A–mediated phosphorylation and opening of Kv1 channels in CA requires the PSD95 scaffolding function.
- Protein kinase A–mediated opening of Kv1 channels enabled by PSD95 maintains the resting diameter of CA and prevents abnormal vasoconstriction.

PSD95, a scaffolding protein, is expressed near the plasma membrane of cVSMCs and interacts with Kv1 channels in rat CA. However, the physiological role of PSD95 in cVSMCs is unknown. Here, we demonstrate that the PSD95 scaffold is required for protein kinase A–mediated phosphorylation and opening of Kv1 channels, which, in turn, significantly contribute to the resting diameter of rat CA. Our results reveal a vasodilator signaling complex on the PSD95 scaffold in cVSMCs, which regulate cerebral perfusion. Our results suggest that conditions that disrupt the PSD95 scaffolding of Kv1 channels in cVSMCs may cause a loss of vasodilator function and impaired cerebral blood flow. Further studies are warranted to investigate the role of the PSD95 complex in pathological conditions, such as hypertension, a disease in which vasodilator function is compromised.
Protein Kinase A–Phosphorylated Kv1 Channels in PSD95 Signaling Complex Contribute to the Resting Membrane Potential and Diameter of Cerebral Arteries
Christopher L. Moore, Piper L. Nelson, Nikhil K. Parelkar, Nancy J. Rusch and Sung W. Rhee

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**Supplemental Material**

**Detailed Methods:**

**Dominant-negative peptides**

KV1-C peptide (Figure 1B) consisted of the final 10 amino acids of the C-terminus of Kv1.2α, which represents a PDZ binding motif (VNITKMLTDV). It was attached by a spacer (P) to an N-terminus HIV-tat sequence (NH3-YGRKKRRQRRR) to confer membrane permeability. A fluorescein label was attached to the N-terminus of some peptides for visualization. Two scrambled variations of the peptide (Scm: DVNMTKLVIT; Scm2: TLMKVDTVNI) attached by the same spacer to HIV-tat were used as negative controls (21st Century Biochemicals). For patch-clamp experiments, non-permeable (NP) 10 amino-acid peptides without the HIV-tat (Kv1-C NP and Scm NP) were used (GenScript). Myristoylated PKA inhibitor peptide 14-22 (PKI) was purchased from Tocris. All peptides were prepared as 1 mmol/L aqueous stocks, aliquoted and kept at -20°C until use.

**Animals**

All protocols complied with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Cerebral arteries (CA) were dissected from ten- to fourteen-week-old male Sprague–Dawley rats (Harlan). Rats were euthanized by decapitation under isoflurane anesthesia.

**Protein lysate**

CA were homogenized in lysis buffer of the following composition (mmol/L): 150 NaCl; 1 EGTA; 1.5 MgCl2; 50 HEPES; 1% Triton X-100; 10% glycerol; pH 7.3 plus protease inhibitor cocktail (Thermo Scientific). Phosphatase inhibitor cocktail (Thermo Scientific) was included when appropriate. A plastic pestle or a tissue homogenizer (Bullet Blender, Next Advance) and 0.9-2.0 mm stainless steel beads were used to homogenize the arteries.

**Co-immunoprecipitation**

Co-immunoprecipitation (co-IP) was performed as previously described with the Pierce Co-IP Kit (Thermo Scientific) per the manufacturer's instructions with Modified Dulbecco's phosphate buffered saline (PBS) as the binding/wash buffer. Briefly, columns were initially prepared by immobilizing 150 µg of Kv1.2 antibody (Neuromab) on to 50 µL of AminoLink Plus Coupling Resin with a 2 hour incubation. After washing with the supplied coupling buffer and wash solution, the columns were loaded with 125 µg cerebral artery lysate and incubated overnight at 4°C. Dissected CA were incubated with Kv1-C or Scm peptide (10 µmol/L) in PBS for 30 min at 37°C then rinsed in PBS prior to lysing. To preserve the dynamic association of Kv1.2 and PSD95, the centrifugation speed for lysate preparation was reduced to 3000g for co-IP studies (Online Figure VIII-A). Antibody-bound proteins were eluted with 60 µL of elution buffer provided with the kit after incubating for 8 min at room temperature.

**Biotinylation**

CA dissected from a single rat were incubated with Kv1-C or Scm peptide (10 µmol/L) in PBS for 30 min at 37°C. Subsequently, arteries were centrifuged at 2,300g for 5 min at 4°C, rinsed with chilled PBS and incubated for 1 hour at room temperature in biotin solution (EZ-Link Melamide-PEG-Biotin and Sulfo-NHS-LC-LC-Biotin, 1 mg/mL each, Thermo Scientific) before quenching with 100 mmol/L glycine. An ample amount of avidin beads (Pierce Monomeric Avidin Agarose, Thermo Scientific) were used to separate the biotinylated surface fraction and the cytosolic fraction. The surface fraction was eluted from the avidin beads by heating for 3 min in sodium dodecyl sulfate loading buffer containing 10% 2-mercaptoethanol in near-boiling water.

**Western blots**

As previously described, cerebral arterial protein samples were separated by electrophoresis using 4–15% gradient gels (Invitrogen) and transferred to PVDF membranes. Membranes were blocked in 10% milk or 10% bovine serum albumin (to preserve phosphorylation) and incubated sequentially in primary and secondary antibodies. Primary antibodies were Kv1.2 (1:100, NeuroMab), PSD95 (1:100, NeuroMab) or phospho-PKA substrate (1:1000, Cell Signaling). Antibody against α-actin (Sigma) was used to normalize for loading variability. Secondary antibodies were horseradish peroxidase-linked sheep anti-mouse antibody (GE...
Healthcare, 1:3000 dilution) for K\textsubscript{v}1.2 and PSD95 and donkey anti-rabbit antibody (GE Healthcare, 1:5000 dilution) for phospho-PKA substrate. Immunoreactive bands were detected using enhanced chemiluminescence reagents (Thermoscientific) along with Hyblot CL film (Denville Scientific) and quantified using ImageJ software (NIH). The density of immunoreactive bands was first normalized to \(\alpha\)-actin density for each lane and the resulting values for K\textsubscript{v}1-C treatment group were represented as a fraction of Scm treatment. The unglycosylated K\textsubscript{v}1.2\(\alpha\) band at ~60 kD was proportional to the fully-glycosylated K\textsubscript{v}1.2\(\alpha\) band at ~75 kD in each sample lane but was not included in the quantification. The lower band is more prominent in lysates prepared from 3000g centrifugation compared to 100,000g spin\(^3\) and also becomes dominant when the sample is heated in 1% SDS as needed for biotinylation (Online Figure VIII-B).

**Confocal imaging of fluorescently-labeled peptide**

Dissected CA were incubated with fluorescin-labeled K\textsubscript{v}1-C or Scm peptide (3 \(\mu\)mol/L) in phosphate buffered saline (PBS) for varying times (0 – 30 min) and then immediately fixed in 4% formaldehyde for 20 min. After a PBS wash, arteries were incubated in 10 \(\mu\)g/mL Alexa350-labeled wheat germ agglutinin (Invitrogen) for 30 min for cell surface counter staining or were mounted directly in Prolong Gold with DAPI (Invitrogen) for nuclear staining and placed on a glass-bottom dish (Mattek) for imaging. The samples were imaged with appropriate filter sets with an Orca EM (Hamamatsu) camera and a 63x 1.4 N.A. oil-immersion objective on an Axiovert 200M microscope (Zeiss) with a CARV II spinning-disk confocal unit (CrEST). Images were acquired and processed with IPLab version 4.04 (BD Biosciences).

**Diameter and membrane potential recording in pressurized arteries**

Cerebellar arteries were isolated, cannulated and pressurized to 80 mm Hg and equilibrated at 37°C for 1 hour while being superfused with physiological salt solution (PSS) containing (in mmol/L): 119 NaCl, 4.7 KCl, 24 NaHCO\(_3\), 1.18 NaH\(_2\)PO\(_4\), 1.17 MgSO\(_4\)\(\cdot\)7H\(_2\)O, 5.5 glucose, 0.03 EDTA, and 1.6 CaCl\(_2\) and bubbled with a 7% CO\(_2\) / 93% O\(_2\) gas mixture to obtain pH 7.4. Any arteries that did not develop spontaneous tone after 1 hour equilibration or lacked a vasoconstrictive respond to 60 mM KCl were not used for study. External diameters were automatically measured and recorded by DMTVAS software (DMT). Diameter changes were represented as a percent of the resting diameter before treatment. Drugs were added directly to the superfusate bath away from the artery and allowed to diffuse. Membrane potential (\(E_m\)) was measured using glass microelectrodes filled with 3 mol/L KCl fitted over a silver chloride wire mounted on a micromanipulator and connected to a preamplifier (DAGAN). The readings were recorded and analyzed by WinDaq Lite software (DATAQ). A successful measurement of \(E_m\) was defined as an abrupt drop in voltage upon impalement, maintenance of a stable value for a minimum of 20 s and an immediate return to baseline upon withdrawal of the microelectrode. Three such measurements were averaged to yield the final value for each \(E_m\) recording (Online Figure IX).

**Cranectomy and in situ imaging**

Rats were anesthetized with isoflurane (2% at 1.0 L/min O\(_2\)), mounted in a stereotaxic frame (KOPF) and positioned under a dissecting microscope. Body temperature was monitored with a rectal probe and maintained between 37-38°C with a heating pad. The skull was exposed, cleaned of periosteum, flushed with saline and dried. A 3x7-mm section of bone was cut with a high speed micro-drill and removed from the right parietal plate. The dura mater was retracted and excised using a 30 gauge needle and a micro-knife. A custom-made ported cranial window was fitted to the skull using bone wax, dental acrylic and cyanoacrylate gel. The window was suffused with 37°C PSS bubbled (7% CO\(_2\), 93% O\(_2\)) to physiological pH. Middle cerebral artery branches were imaged using an HDR-PJ580 camera (Sony) and analysis was accomplished using an automated script in IPLab.

**Patch-Clamp**

Patch-clamp recordings were performed in the standard whole-cell configuration on an EPC amplifier (List) and pCLAMP 6 software (Molecular Devices), and data was analyzed with pCLAMP 10. Gigaohm seals and cell access were acquired in a drug-free bath solution composed of (in mmol/L): NaCl 140, KCl 4, CaCl\(_2\) 0.1, MgCl\(_2\) 1, HEPES 10, glucose 20, pH 7.4 adjusted with NaOH. After initial capacitance recording confirming cell access, bath solution containing 100nmol/L Iberiotoxin was superfused to eliminate any contaminating BK channel currents. The recording electrodes were dipped for 5-10 sec in regular pipette solution consisting of (in mmol/L): K-glutamate 145, MgCl\(_2\) 1, HEPES 10, EGTA 4, Na\(_2\)ATP 1, pH 7.2 adjusted with KOH and then backfilled with pipette solution containing either 3 \(\mu\)mol/L Scm peptide or 3 \(\mu\)mol/L K\textsubscript{v}1-C peptide. Currents
were recorded using a voltage-step protocol that applies 500 ms voltage steps from -70 mV to +58 mV in 8 mV increments before and after superfusion with bath solution containing 100 nmol/L Psora4. Psora4-depedent currents were obtained from digitally subtracting post-Psora4 currents from pre-Psora4 currents. Current densities were calculated by normalizing all currents to the cell capacitance.

Drugs
The following drugs were prepared as stock solutions in DMSO: 5-(4-phenylalkoxypsoralen) (Psora4; Sigma), 10 mmol/L; calyculin-A (CalA; Tocris), 500 µmol/L; okadaic acid (OA; Tocris), 100 µmol/L; and indomethacin (Sigma), 100 mmol/L. Stock solutions for other drugs were prepared in water: Nω-nitro-L-arginine methyl ester (L-NAME; Sigma), 100mmol/L; Linopirdine (Lino; Tocris), 10mmol/L; and BaCl2 (Sigma), 30mmol/L. For pressurized vessel experiments, drugs were diluted first in PSS when appropriate and added directly to the chamber at either 100x or 1000x dilution for the final bath concentrations referred to in the text. The maximum amount of DMSO used in drug delivery (0.6 µL in 1 mL of PSS) was tested as the vehicle control which did not produce significant changes to the diameter (Figure 8B, veh).

Statistical analysis
Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using unpaired t tests or when appropriate with one-way ANOVA with Newman-Keuls post-hoc or two-way ANOVA with Bonferroni post-hoc for cranial window measurements. P<0.05 was considered statistically significant.

Online Supplement References:

Online Figure Legend

Online Figure I. Representative confocal images of isolated rat CA incubated with 3 µmol/L fluorescein-labeled Scm peptide for 1 min at 37°C. Three layers - adventitia (Ad), smooth muscle (SM), and endothelial cell (EC) – were imaged with DAPI as nuclear counter staining. Individual cVSMCs are vertically wrapping around the cerebral artery circumferentially while endothelial cells run horizontally.

Online Figure II. Representative confocal images of isolated rat CA incubated with 3 µmol/L fluorescein-labeled Scm peptide for 20 min at 37°C. Three layers - adventitia (Ad), smooth muscle (SM), and endothelial cell (EC) – were imaged with DAPI as nuclear counter staining. Individual cVSMCs are vertically wrapping around the cerebral artery circumferentially while endothelial cells run horizontally.

Online Figure III. A) Two different control peptides (Scm and Scm2) do not constrict CA significantly up to 10 µmol/L whereas Kv1-C peptide constricts CA at 3 µmol/L and 10 µmol/L concentrations (n=5 each). Both baseline (base) and peptide-treated (pep) diameters are shown in raw numbers. a, b: significant difference, P<0.05. B) KCl-induced constriction is not significantly different between Scm, Scm2, and Kv1-C peptide treatment groups. Replot of 10 µmol/L group from pane A along with diameter recordings after 60 mM KCl application. NS: not significantly different. *: significant difference from Scm and Scm2, P<0.05.

Online Figure IV. Kv1-C peptide disrupts association of PSD95 and Kv1.2 and constricts CA. Data from Figure 3D expressed in actual diameters. Diameter recordings from CA in response to 10 µmol/L peptide and 100 nmol/L of the specific KV1 channel antagonist Psora4 (n=6 each). a, b: significant difference, P<0.05.

Online Figure V. Average % change in diameter of rat CA in response to 3 µmol/L Scm or Kv1-C peptide with or without pretreatment with 100 µmol/L L-NAME (L) and 10 µmol/L indomethacin (I) (n=6 each). a, b: significant difference, P<0.05.

Online Figure VI. A) An example trace of a rat CA constricting in response to 1 µmol/L linopirdine in the presence of 3 µmol/L Kv1-C peptide. B) Average % change in diameter of rat CA in response to 1 µmol/L linopirdine (Lino) or 30 µmol/L Ba²⁺ in the absence (Without Kv1-C) or presence (After Kv1-C) of 3 µmol/L Kv1-C peptide (n=5-9). NS: no statistical significance.

Online Figure VII. Time course of arterial diameter changes in response to Kv1-C peptide administered at either a concentration of 10 µmol/L (n=4) or 30 µmol/L (n=7) through a cranial window. Percent change in diameter from baseline. *: Significant difference from 10 µmol/L, P<0.05.

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Online Figure IX. An example trace of E_m measurements recorded from CA pressurized to 80 mmHg in control PSS before the application of Kv1-C peptides. Three good measurements (a, b and c) were made along with one unstable recording marked by X. The mean of three good measurements were averaged to yield the final E_m value of -47.5 mV (red line) for this particular CA.
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