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PKA-Phosphorylated K_V1 Channels in PSD95 Signaling Complex Contribute to the Resting Membrane Potential and Diameter of Cerebral Arteries

Christopher L. Moore[1], **Piper L. Nelson**[1], **Nikhil K. Parelkar**[2], **Nancy J. Rusch**[1], and **Sung W. Rhee**[1]

[1]Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

[2]University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City, Kansas 66160

Abstract

Rationale—Postsynaptic density-95 (PSD95) is a scaffolding protein that associates with voltage-gated, *Shaker*-type K^+ (K_V1) channels and promotes the expression of K_V1 channels in vascular smooth muscle cells (cVSMCs) of the cerebral 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 K_V1 channels enables PKA phosphorylation of K_V1 channels in cVSMCs to promote vasodilation

Methods and Results—Rat cerebral arteries (CA) were used for analyses. A membrane-permeable peptide (K_V1-C peptide) corresponding to the PDZ binding motif in the C-terminus of $K_V1.2\alpha$ was designed as a dominant negative peptide to disrupt the association of K_V1 channels with PSD95. Application of K_V1-C peptide to cannulated, pressurized CA rapidly induced vasoconstriction and depolarized cVSMCs. These events corresponded to reduced co-immunoprecipitation of the PSD95 and K_V1 proteins without altering surface expression. Middle cerebral arterioles imaged *in situ* through cranial window also constricted rapidly in response to local application of K_V1-C peptide. Patch-clamp recordings confirmed that K_V1-C peptide attenuates K_V1 channel blocker (Psora4)-sensitive current in cVSMCs. Western blots employing a phospho-PKA substrate antibody revealed CA exposed to K_V1-C peptide showed markedly less phosphorylation of $K_V1.2\alpha$ subunits. Finally, phosphatase inhibitors blunted both K_V1-C peptide-mediated and PKA inhibitor peptide-mediated vasoconstriction.

Conclusions—These findings provide initial evidence that PKA phosphorylation of K_V1 channels is enabled by a dynamic association with PSD95 in CA, and suggest that a disruption of such association may compromise cerebral vasodilation and blood flow.

Address correspondence to: Dr. Sung W. Rhee, Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham Street, #611, Little Rock, AR 72205, Tel: 501-686-5467, Fax: 501-686-5510, RheeSung@uams.edu.

DISCLOSURES
None

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cerebral arteries; PDZ domains; potassium channels; vascular smooth muscle

INTRODUCTION

Shaker-type voltage-gated K^+ (K_V1) channels composed of $K_V1.2$ and $K_V1.5$ α-subunits are expressed in cerebral vascular smooth muscle cells (cVSMCs), where they contribute to the resting diameter and vasodilation of cerebral arteries (CA).[1, 2] K_V1 channels are multi-protein structures composed of four $K_V\alpha$ pore-forming subunits co-assembled with intracellular $K_V\beta$ 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 $K_V\alpha$ subunits may increase protein expression and activity of K_V1 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 K_V1 channels in cVSMCs, and this interaction is required for the proper expression of K_V1 channels that exerts a tonic vasodilator influence.[16] Accordingly, antisense-mediated knockdown of PSD95 in rat CA resulted in a loss of K_V1 channel expression and caused vasoconstriction, inferring that PSD95 promotes the expression of K_V1 channels in cVSMCs.[16]

Notably, the C-terminus of the $K_V1.2\alpha$ 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 three PDZ (post synaptic density-95, discs large, zonula occludens-1) 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 $K_V1.2\alpha$ subunit via an association that is intrinsically unstable, thereby permitting a dynamic and reversible interaction.[17, 19, 23, 24] Src-homology (SH3) and guanylate kinase (GK) 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]

Since the three 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 (NMDAR) and neuronal nitric oxide synthase (nNOS) in neurons was revealed using this strategy.[27, 28] A similar dominant-negative peptide was administered to rodents and non-human primates in vivo to reduce neuronal damage after experimental stroke by disrupting PSD95-dependent excitotoxic signaling between NMDAR

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and nNOS. In order 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 if association with PSD95 is required for the proper function of K_V1 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 $K_V1.2\alpha$-subunit (K_V1-C peptide) to disrupt K_V1 scaffolding by PSD95. Our findings draw attention to PSD95 as a key scaffolding protein in cVSMCs that enables the basal phosphorylation and opening of K_V1 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

Cerebral arteries were isolated from ten- to fourteen-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 (K_V1-C) was used to disrupt the association of K_V1 and PSD95 (Figure 1B). K_V1-C consists of the final 10 amino acids of the C-terminus of $K_V1.2\alpha$ attached to an N-terminus HIV-tat sequence (NH_3-YGRKKRRQRRR) to confer membrane permeability. An N-terminus fluorescein label was attached to some peptides for visualization. Two scrambled variations of the peptide were used as negative controls (21st Century Biochemicals). Peptide disruption of K_V1 channel-PSD95 association was determined by co-immunoprecipitation.[16] 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 by WinDaq Lite software (DATAQ). Whole-cell cVSMC patch-clamp was performed with an EPC 7 amplifier (HEKA) and pCLAMP 6 software (Molecular Devices).[16] Non-permeable peptides (NP) without the HIV-tat (GenScript) were used for patch-clamp experiments. Images were obtained using a confocal microscope.[16] Data are presented as mean ± SEM. $P<0.05$ was considered statistically significant. An expanded Methods section is available in the Online Data Supplement.

RESULTS

Cell-permeable K_V1-C peptide disrupts the interaction of $K_V1.2\alpha$ and PSD95 in cVSMCs

K_V1 channel and PSD95 association (Figure 1A) was targeted for disruption using a peptide (K_V1-C), which couples an N-terminus HIV-tat sequence to the last 10 amino acid sequence of the $K_V1.2\alpha$ C-terminus that contains a class I PDZ binding motif, *LTDV* (Figure 1B). To ensure specificity of the K_V1-C peptide, a scrambled peptide (Scm) sharing the same amino acid composition as K_V1-C but randomly ordered was used as control (Figure 1B). In isolated CA, incubation for 30 min with K_V1-C peptide disrupted the $K_V1.2\alpha$-PSD95

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interaction as evidenced by a loss of immunoreactive signal for PSD95 in anti-$K_V1.2\alpha$ immunoprecipitate (IP) compared to arteries incubated with Scm peptide (Figure 1C, lanes 1-2). A corresponding increase in the PSD95 band was observed in the flow-through fraction that did not bind to $K_V1.2\alpha$ antibody (Figure 1C, lanes 3-4). As previously reported,[16] only the full form of PSD95 (upper band at 95 kD) associates with the $K_V1.2\alpha$ subunit. In order to examine the effect of K_V1-C peptide on the plasma membrane expression of K_V1 channels, isolated arteries were treated with Scm or K_V1-C peptides, biotinylated and analyzed by Western blot.[33] In the resulting Western blot, the bulk of $K_V1.2\alpha$–containing channels were found in the biotinylated surface fraction compared to the non-biotinylated cytosolic fraction (Figure 1D). The surface expression of $K_V1.2\alpha$ subunits was not different between arteries exposed to K_V1-C compared to Scm, indicating that K_V1-C did not cause a loss of surface K_V1 channels (Figure 1D). We also confirmed that K_V1-C peptide penetrated cVSMCs of intact arteries using confocal imaging (Figure 2). After 1 min of incubation, fluorescein-labeled K_V1-C peptide appeared at the cell surface and a strong fluorescent signal was observed intracellularly by 3 min that persisted until 30 min. Similar penetration patterns were observed for fluorescein-labeled Scm peptide (Online Figures I and II). Compared to 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 $K_V1.2\alpha$ subunit to prevent its binding to the PDZ domains of PSD95, and it provides a tool to disrupt the association between K_V1 channels and PSD95 in cVSMCs of intact arteries.

K_V1-C peptide constricts and depolarizes isolated CA

Diameter recordings of isolated, pressurized rat CA exposed to K_V1-C peptide revealed a rapid constriction of CA in response to disruption of the K_V1 channel-PSD95 complex, whereas Scm peptide did not affect vessel diameter (Figure 3A). Increasing concentrations of 1, 3 and 10 μmol/L K_V1-C peptide progressively reduced CA diameter by $-7.4 \pm 2.0\%$, $-19.3 \pm 2.5\%$ and $-20.3 \pm 2.1\%$ (Figure 3B). The corresponding values for Scm peptide were $-4.2 \pm 2.8\%$, $-3.7 \pm 0.6\%$ and $-3.9 \pm 1.9\%$. A second scrambled control peptide (Scm2) also did not significantly constrict CA (Online Figure III). Bath application of the K_V1 channel blocker, 5-(4-phenylalkoxypsoralen) (Psora4)[2, 16, 34, 35] did not further reduce the diameter of arteries already constricted by K_V1-C peptide (Figures 3C-D). Notably, the constrictor response to 100 nmol/L Psora4 was not different from the constriction caused by 10 μmol/L K_V1-C peptide (Figure 3D) or the diameter change induced by combined Psora4 and K_V1-C peptide (Figure 3D). Absolute diameter values are provided in Online Figure IV. The constriction induced by K_V1-C peptide was not significantly altered by the presence of 100 μmol/L Nω-Nitro-L-arginine methyl ester (L-NAME) 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_V7 channel blocker, or 30 μmol/L $BaCl_2$, a K_{IR} channel blocker, were not significantly altered by the presence of 3 μmol/L K_V1-C peptide (Online Figure VI). Additionally, 1 μmol/L glibenclamide, a K_{ATP} channel blocker, or 30 nmol/L stromatoxin, a $K_V2.1$ channel blocker, did not constrict pressurized CA significantly (n=5 each, data not shown).

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Importantly, microelectrode recordings indicated that cVSMCs of pressurized CA depolarized from a resting membrane potential (E_m) of −47.6 ± 2.1 mV to −35.2 ± 3.2 mV in response to 10 μmol/L K_V1-C peptide (Figures 4A and 4C), a finding consistent with a loss of hyperpolarizing K^+ current. In contrast, cVSMCs in arteries treated with Scm peptide showed no significant change in resting E_m (Figures 4B-C). The addition of 60 mmol/L KCl to bath solutions already containing K_V1-C or Scm peptide further depolarized arteries of both groups to similar values (Figure 4). These findings indicate that the specific disruption of K_V1.2 channel-PSD95 association in pressurized CA leads to depolarization and constriction from the resting tone. These effects appear to correspond selectively to the blockade of K_V1.2 channels and imply a loss of function of K_V1.2 channels in cVSMCs when dissociated from the PSD95 complex.

K_V1-C peptide constricts CA in vivo

After confirming the selective disruption of K_V1-PSD95 interaction ex vivo by K_V1-C peptide, we evaluated the impact of K_V1-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 cerebral arteries for imaging and topical peptide treatment (Figure 5A). Suffusion of the cranial window with 30 μmol/L K_V1-C peptide resulted in a rapid constriction of CA, which was sustained for at least 20 min (Figures 5B-C). Average values indicated that middle cerebral arteries treated with K_V1-C peptide showed a significant reduction in diameter of −16.1 ± 3.9% by 5 min with maximum constriction at 15 min averaging −16.8 ± 3.5% (Figure 5D). In contrast, the same concentration of Scm peptide did not affect vessel diameter (Figures 5B-C), although the same arteries responded to 60 mmol/L KCl by strongly constricting as evidence of viability (Figure 5B). Arteries in physiological salt solution (PSS) or Scm peptide showed a small decrease in diameter by 15 min, which were not statistically significant from each other (Figure 5D). These results provide initial evidence that K_V1 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_V1-C peptide suppresses Psora4-sensitive K^+ currents in cVSMCs

Next we used patch-clamp techniques to confirm that K_V1-C peptide directly reduces K_V1 channel function in cVSMCs. In these studies, non-permeable (NP) 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 NP (Figure 6A) or 3 μmol/L K_V1-C NP (Figure 6B) before addition of the selective K_V1 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_V1 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 NP-treated cells. This value decreased by 45% to 4.65 ± 0.71 pA/pF for cells dialyzed with K_V1-C NP (Figure 6C). In contrast, the peak density of residual Psora4-insensitive current (I_{other}) was not significantly different between Scm and K_V1-C treated cells (Figure 6D). These findings concur with reports that only $K_V1\alpha$-subunits have PDZ binding motifs to enable PSD95

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interaction.[16, 17] In this regard, *Shab*-type K_V2 channels and *KCNQ* (K_v7) channels likely contribute to the Psora4-insensitive residual current. Stromatoxin-sensitive K_V2 channels are not blocked by Psora4 in cVSMCs[16, 35] and Psora4 does not compromise vasoconstrictor responses of CA to the K_V7 channel blocker, linopirdine (Online Figure VI).

K_V1-C peptide reduces PKA phosphorylation of K_V1 channels

After demonstrating that PSD95 is the critical scaffold required for K_V1 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_V1 channels. One candidate binding partner of PSD95 is AKAP150, which is known to act as a focal point in multi-protein signaling complexes to facilitate PKA-dependent phosphorylation of target proteins.[26] Huang *et al* first reported that direct application of PKA catalytic subunits lead to the activation of cloned cardiac K_V1 channels in oocytes.[10] Later, PKA-dependent phosphorylation and opening of K_V1 channels were reported in native smooth muscle cells from rabbit portal vein[11] and canine colon.[12] However, many later studies using cAMP analogues or forskolin on recombinant $K_V1.2$ channels in heterologous cells only revealed an increase in $K_V1.2$ protein levels by PKA phosphorylation with small to no increase in gating of channels.[13–15, 36] Therefore we examined if the association of K_V1 channels with PSD95 signaling complex could explain this apparent discrepancy.

Previously, Connors *et al*[14] successfully used an antibody that detects PKA-phosphorylation of serine or threonine residues to identify PKA phosphorylation of rat $K_V1.2\alpha$ subunits overexpressed in a heterologous expression system. We used the same antibody to explore if $K_V1.2\alpha$ subunit-PSD95 interaction is required for PKA-phosphorylation of K_V1 channels in cVSMCs, thereby inferring co-localization of K_V1 channels with AKAP150 in the vascular PSD95 signaling complex. CA treated with 10 μmol/L Scm or K_V1-C peptide for 30 min were lysed and loaded equally (30 μg) into lanes of a Western blot probed with anti-phospho PKA substrate.[14] Compared to CA treated with Scm peptide, K_V1-C treated CA showed a marked loss in the phosphorylation of the ~75 kD band (Figure 7A, left blot), which is the fully-glycosylated form of $K_V1.2\alpha$[1] required for PKA phosphorylation.[9] Average densitometric values from five similar preparations demonstrated a ~50% reduction in PKA phosphorylation of $K_V1.2\alpha$, whereas the expression level of total $K_V1.2\alpha$ protein did not change significantly (Figures 7A, left panel and 7B). These results suggest that PSD95 scaffolding promotes PKA-mediated phosphorylation of K_V1 channels, an event associated with increased K_V1 channel opening.

In order to confirm that reduced PKA-mediated phosphorylation of K_V1 channels contributed to the constriction of CA by K_V1-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 (CalA, 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 K_V1-C peptide (Figures 8A-B). In drug-free PSS, K_V1-C peptide reduced the diameter of CA by $-19.5 \pm 1.6\%$ compared to $-9.3 \pm 3.3\%$ and $-14.1 \pm 1.1\%$ in the presence of OA and CalA, respectively (Figure 8B). Constriction in response to KCl was not altered by the presence of

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OA or CalA (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, since earlier studies defining mechanisms of PKA modulation of K_V1 channel properties were mostly performed in cell systems.[11, 12] Indeed, 1 μmol/L PKI 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 CalA (Figures 8D-E). These findings highlight that PKA phosphorylation of K_V1 channels is an important contributor to the basal dilation of CA and that a dynamic association with the PSD95 signaling complex is critically required for PKA phosphorylation of K_V1 channels in CA.

DISCUSSION

PSD95 was originally regarded as an exclusive feature of the post-synaptic 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 K_V1 channels in lymphocytes and in cVSMCs.[16, 39] First, mutation of the $K_V1.3$ subunit PDZ binding motif or knockdown of PSD95 in T lymphocytes inhibited the recruitment of $K_V1.3$ into contact points on the cell surface, implying an important role of PSD95-K_V1 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 K_V1 channel protein and vasodilator function. These findings suggested a critical role of PSD95 in maintaining the expression of K_V1 channels in the cerebral circulation.[16] However, because K_V1 channels were down-regulated by PSD95 knockdown in our previous study, the impact of PSD95 signaling complex on K_V1 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 K_V1 channels involved in regulating the diameter of CA. For example, β1 adrenergic receptors[40], serotonin receptors[41], inward-rectifying potassium channels[42], and nNOS[43] 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 K_V1 channels and PSD95 in cVSMCs. Our results provide initial evidence that the PSD95 signaling complex is critically required for K_V1 channel dilator function in rat CA *in situ* and further suggest that PSD95 provides a platform for interaction between PKA and K_V1 channels that enables PKA–mediated phosphorylation and opening of K_V1 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 K_V1 channels in CA. After directly disrupting the K_V1-PSD95 association by application of K_V1-C peptide, we observed a rapid and profound vasoconstriction ex vivo and in vivo. Ex vivo K_V1-C peptide-induced constriction was equivalent to constriction caused by the K_V1 channel blocker Psora4 and there were no additive effects of concomitant treatment whereas vasoconstriction caused by K_V7 channel blocker linopirdine or K_{IR} channel blocker $BaCl_2$ was not altered by K_V1-C peptide. Psora4 is a highly selective blocker of K_V1 channels and does not significantly block other voltage-

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gated K^+ channels or large-conductance, Ca^{2+}-activated K^+ channels.[2, 16, 34, 35] A recent report identified a secondary binding site in the unique side pocket of K_V1 channels that strengthens the selectivity of Psora4.[35] In our patch clamp study K_V1-C peptide caused a significant reduction in Psora4-sensitive K^+ currents while Psroa4-insensitive K^+ currents that may contain $K_V2.1$ or K_V7 currents remained identical to Scm-treated cells. Considering the diversity of K^+ channels in CA, the effects caused by disruption by K_V1-C peptide is remarkably specific to the blockade of K_V1 channels and strengthens the idea of selective PDZ binding of K_V1 channels to the PSD95 complex.

Application of K_V1-C peptide to isolated CA also depolarized the cVSMC membrane without an apparent change in K_V1 channel expression, implying that basal K_V1 channel opening relies on PSD95 scaffolding to mediate hyperpolarizing K^+ efflux. The rapid onset of K_V1-C peptide-induced effects was consistent with the idea that the intrinsic instability of the C-terminus of the $K_V1.2\alpha$ subunit allows for a dynamic interaction with the PDZ binding domains of PSD95.[24] In general, PDZ binding appears to be reversible and similar competing peptides have been applied to cultured neuronal cells[28] and to neurons in vivo[30–32] 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[44] 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 cerebral vascular tone, it is possible that the vasoconstrictor effects of an HIV-tat peptide delivered intravenously by Bach *et al*[30] and Cook *et al*[31] to interrupt NMDAR-PSD95 interaction in neurons and ameliorate stroke may have a direct action on cVSMCs as an unexpected side effect. Since $K_V1.2\alpha$ and NMDAR share the PDZ1 and PDZ2 binding domains of PSD95[30], C-terminus peptides designed for NMDAR can potentially cross-compete with K_V1 channels for binding to PSD95. Considering this therapy is already in Phase 1 and 2 clinical trials in Canada and the U.S., 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 K_V1-C peptide reduced PKA phosphorylation of K_V1 channels without altering cell surface expression. Our study indicates that association with the PSD95 complex is required for the direct PKA-mediated phosphorylation of $K_V1.2\alpha$ subunit and increased channel function independent of expression level. Our observation may explain the dichotomy between early reports of PKA-induced increase in $K_V1.2$ channel activity[10–12] and the lack of such increase in $K_V1.2$ channel activity in many later studies in heterologous expression systems.[13–15] 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 $K_V1.2$ 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 results in marked loss of the $K_V1.2\alpha$ protein.[16] However, in the present study, CA were only exposed to the K_V1-C peptide for 30 min, a time frame that apparently was too short to cause a loss of K_V1 channels and only their vasodilator function was significantly blunted. The finding that PSD95 is required for PKA–mediated phosphorylation of the

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$K_V1.2\alpha$ subunit in cVSMCs raises the possibility that PSD95-AKAP interaction exists as unrecognized scaffolding machinery in the vasculature. Colledge *et al*[26] 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 K_V1 channels. Dissociation of $K_V1.2\alpha$–containing channels from this complex by the K_V1-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 non-permeable peptides in patch clamp studies to verify that K_V1-C peptide disruption of PSD95-K_V1 interaction reduces K_V1 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 carried out membrane potential measurements to provide strong evidence that K^+ efflux through K_V1 channels is attenuated by K_V1-C peptide disruption of the K_V1 channel-PSD95 complex. Second, we used a higher concentration of K_V1-C peptide for in vivo than ex vivo studies of vascular reactivity. Isolated CA constricted maximally to 10 μmol/L K_V1-C peptide, but 30 μmol/L K_V1-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, since 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 $K_V1.2\alpha$ subunit are targets of PSD95-mediated PKA phosphorylation in cVSMCs and left this line of inquiry for future studies. Finally, K_V1-C peptide may have unidentified non-specific effects in CA. Our K_V1-C peptide corresponding to a PDZ-binding motif on the $K_V1.2\alpha$ 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, $K_V1.2\alpha$ is uniquely equipped with a PDZ binding motif[16], which may enable the K_V1 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 $K_V1.2\alpha$, these proteins also could be displaced by the K_V1-C peptide, thereby potentially modifying arterial diameter.

In summary, we propose that the scaffolding of K_V1 channels by PSD95 in CA is a dynamic interaction required for the efficient vasodilator function of K_V1 channels through PKA phosphorylation. Dissociation of K_V1 channels from this complex by a dominant negative peptide causes rapid vasoconstriction 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

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disease-based alterations of PSD95 structure or function may potentially contribute to cerebral blood flow abnormalities by disrupting K_V1 channel dilator function or interfering with the roles of other PSD95 binding partners in vascular smooth muscle cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

AKAP	A-kinase anchoring protein
CA	cerebral arteries
CalA	calyculin-A
cVSMCs	cerebral vascular smooth muscle cells
E_m	membrane potential
GK	guanylate kinase
IP	immunoprecipitate
K_V1	*Shaker*-type voltage-gated K^+ channel
K_V1-C	membrane-permeable peptide mimicking K_V1 channel C-terminus
L-NAME	Nω-Nitro-L-arginine methyl ester
NMDAR	N-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NP	non-permeable
OA	okadaic acid
PDZ	post synaptic density-95, discs large, zonula occludens-1
PKA	protein kinase A
PKI	myristoylated protein kinase A inhibitor peptide
Psora4	5-(4-phenylalkoxypsoralen)
PSD95	postsynaptic density protein-95
Scm	scrambled control peptide

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SH3	Src homology 3

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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 co-assembles with *Shaker*-type potassium (K_V1) channels in the vascular smooth muscle cells (cVSMCs) of cerebral arteries (CA).
- The opening of K_V1 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 K_V1 channels results in a severe constriction of rat CA ex vivo and in vivo.
- Protein kinase A (PKA)-phosphorylation and opening of K_V1 channels in CA requires the PSD95 scaffolding function.
- PKA-mediated opening of K_V1 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 K_V1 channels in rat CA. However, the physiological function of PSD95 in cVSMCs is unknown. Here, we demonstrate that the PSD95 scaffold is required for PKA-mediated phosphorylation and opening of K_V1 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 K_V1 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.

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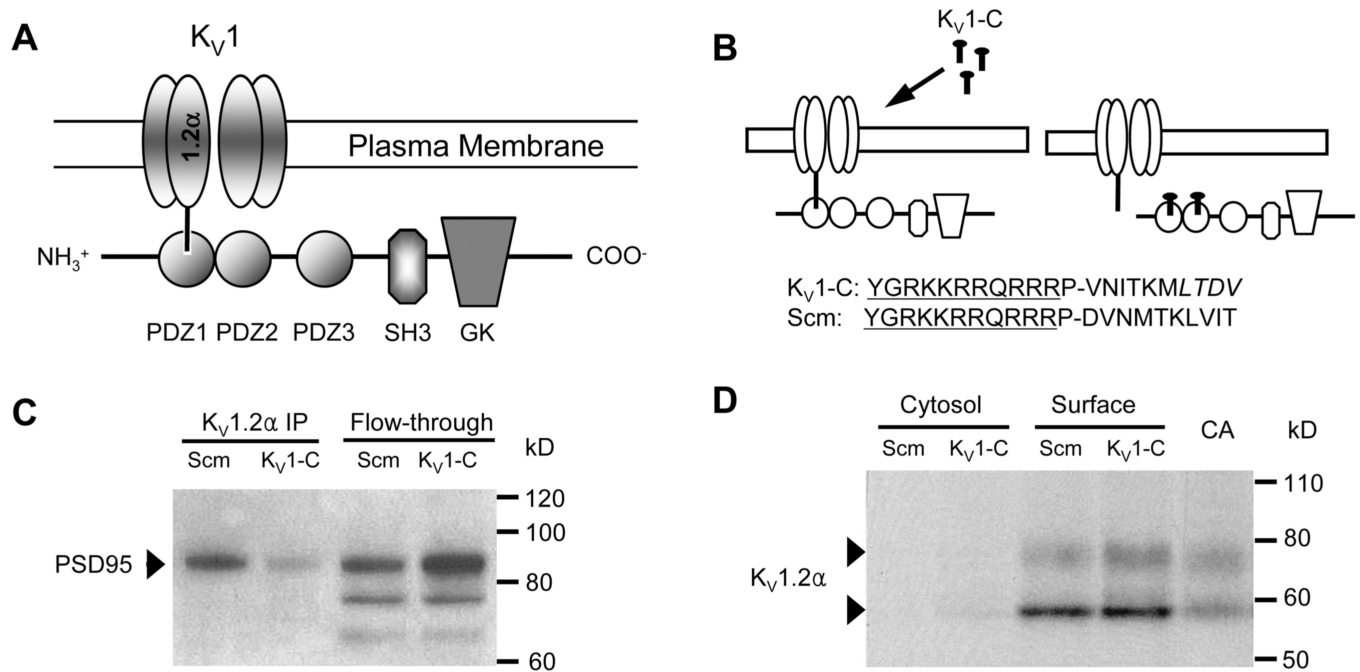

Figure 1. K_V1-C peptide disrupts association of PSD95 and $K_V1.2\alpha$

A) Schematic of the association of $K_V1.2\alpha$ channels with the PSD95 scaffolding protein via the PDZ1 binding domain. PSD95 contains three PDZ binding domains (PDZ1-3), Src-homology (SH3) and guanylate kinase (GK) domains. **B)** The K_V1-C dominant negative peptide was designed to compete for the PDZ binding domain on PSD95. The last 10 amino acids of the $K_V1.2\alpha$ 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 $K_V1.2\alpha$. A peptide with same amino acid composition but in a scrambled order (Scm) was used as control. **C)** Immunoprecipitation using anti-$K_V1.2\alpha$ of CA lysate treated with Scm or K_V1-C peptide for 30 min. Elution ($K_V1.2\alpha$ IP) and column flow-through (Flow-through) were probed for PSD95 on a Western blot. Depicted is a representative scan from three similar experiments. **D)** Biotinylation of CA treated with Scm or K_V1-C peptide for 30 min. Cytosolic and surface fractions were probed for $K_V1.2\alpha$. Control lysate from freshly isolated CA was loaded for size comparison. Depicted is a representative blot from five similar experiments.

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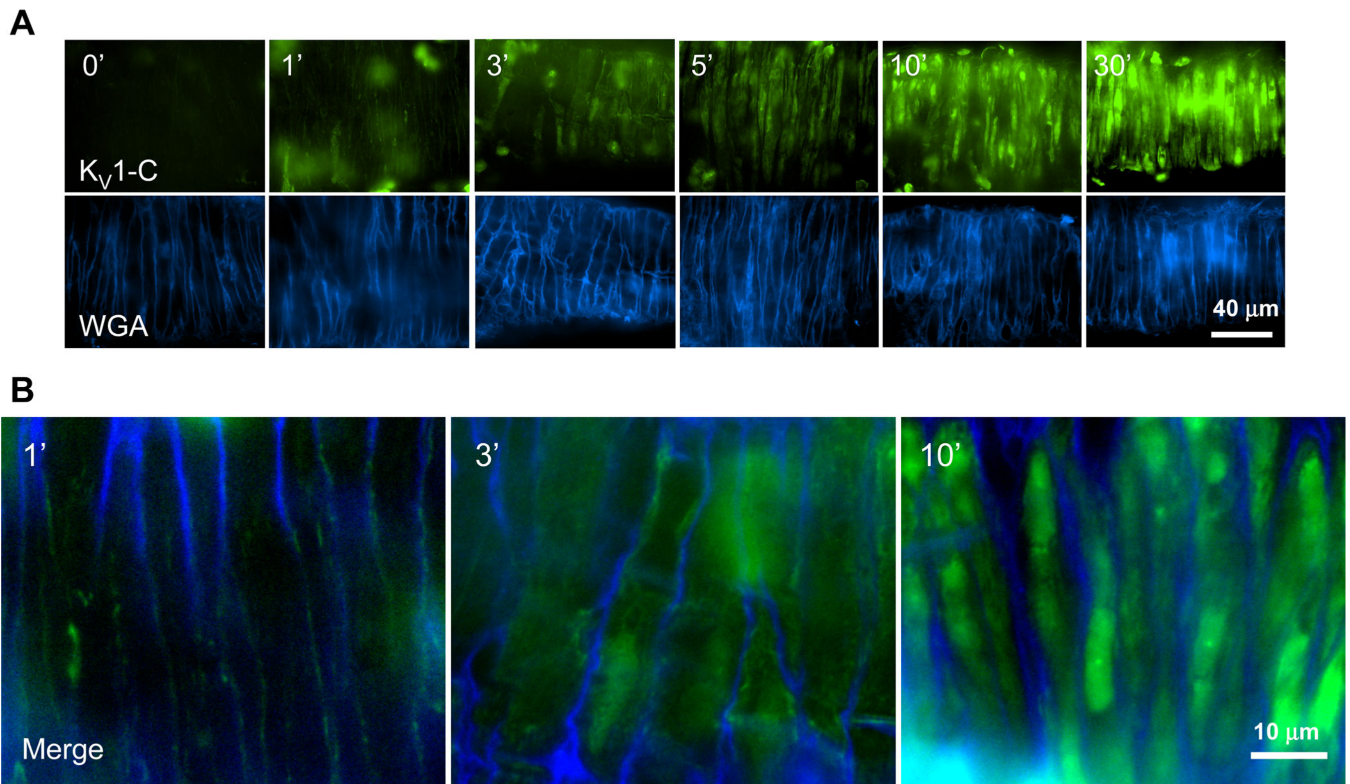

Figure 2. K_V1-C peptide rapidly penetrates cVSMCs in CA

A) Confocal images of isolated rat CA incubated with fluorescein-labeled K_V1-C peptide (top row) for 0, 1, 3, 5, 10, and 30 min 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, since the artery was placed horizontally for imaging. The brightness settings for the green channel in 10- and 30-minute treatments were reduced in order to display individual cells. Representative images from three similar experiments. **B)** Merged images from 1, 3, and 10-minute time points.

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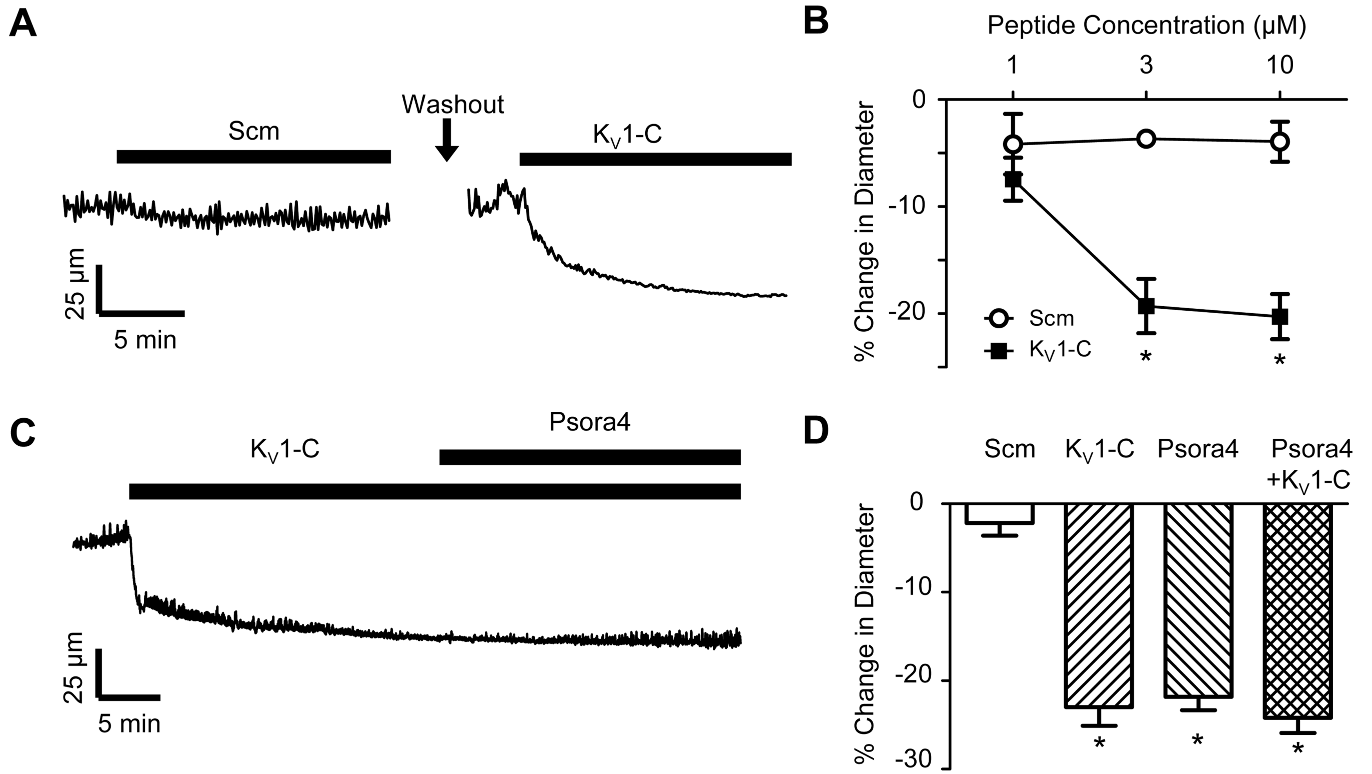

Figure 3. K_V1-C peptide disrupts association of PSD95 and K_V1.2 and constricts CA

A) Representative recording of outer diameter in a cannulated, pressurized (80 mmHg) rat CA. The artery was initially exposed to 10 μmol/L Scm peptide, and then after extensive washes, exposed to 10 μmol/L K_V1-C peptide. **B)** CA constricted in response to K_V1-C peptide in a concentration-dependent manner, but did not constrict to Scm peptide (n=5 each). * indicates significant difference from Scm, P<0.05. **C)** Diameter recordings from a CA exposed sequentially to 10 μmol/L K_V1-C peptide and the specific K_V1 channel antagonist, 100 nmol/L Psora4. **D)** Percent change in diameter from baseline in response to 10 μmol/L K_V1-C peptide and 100 nmol/L Psora4 (n=6 each). *: significant difference from Scm, P<0.05.

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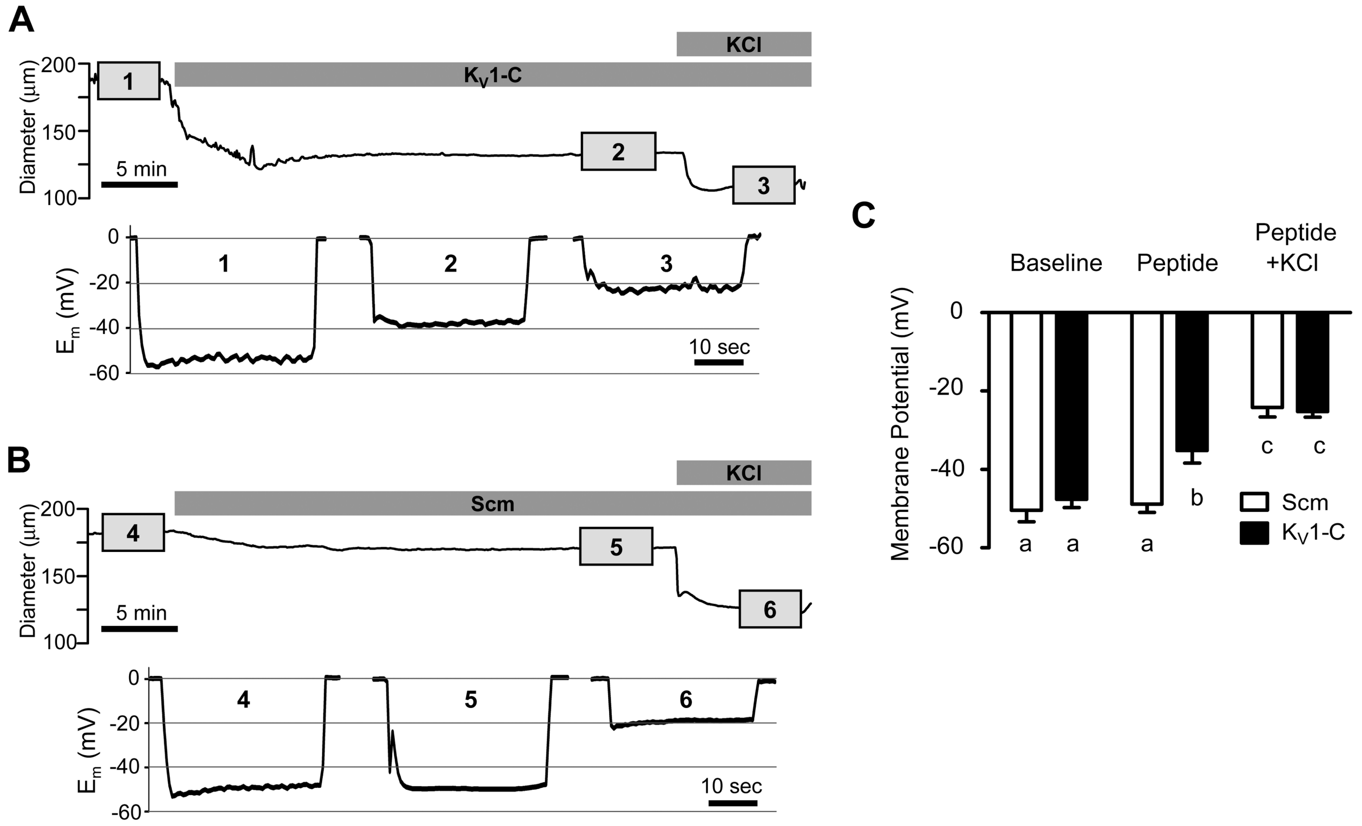

Figure 4. K_V1-C peptide causes depolarization of *in situ* cVSMCs

A, B) Recordings of CA diameter and membrane potential (E_m) in rat CA exposed to K_V1-C (A) or Scm (B) peptide. After recording baseline E_m (1, 4), 10 µmol/L K_V1-C (2) or Scm (5) peptide was added to the bath followed by 60 mmol/L KCl (3, 6) to elicit maximal depolarization. E_m was recorded in each step for ~5 min as indicated by the numbered boxes in the diameter trace. **C)** Average E_m values in the presence of K_V1-C peptide or Scm peptide only, and after the further addition of 60 mmol/L KCl (n=6 each). a,b,c: significant difference, P <0.05.

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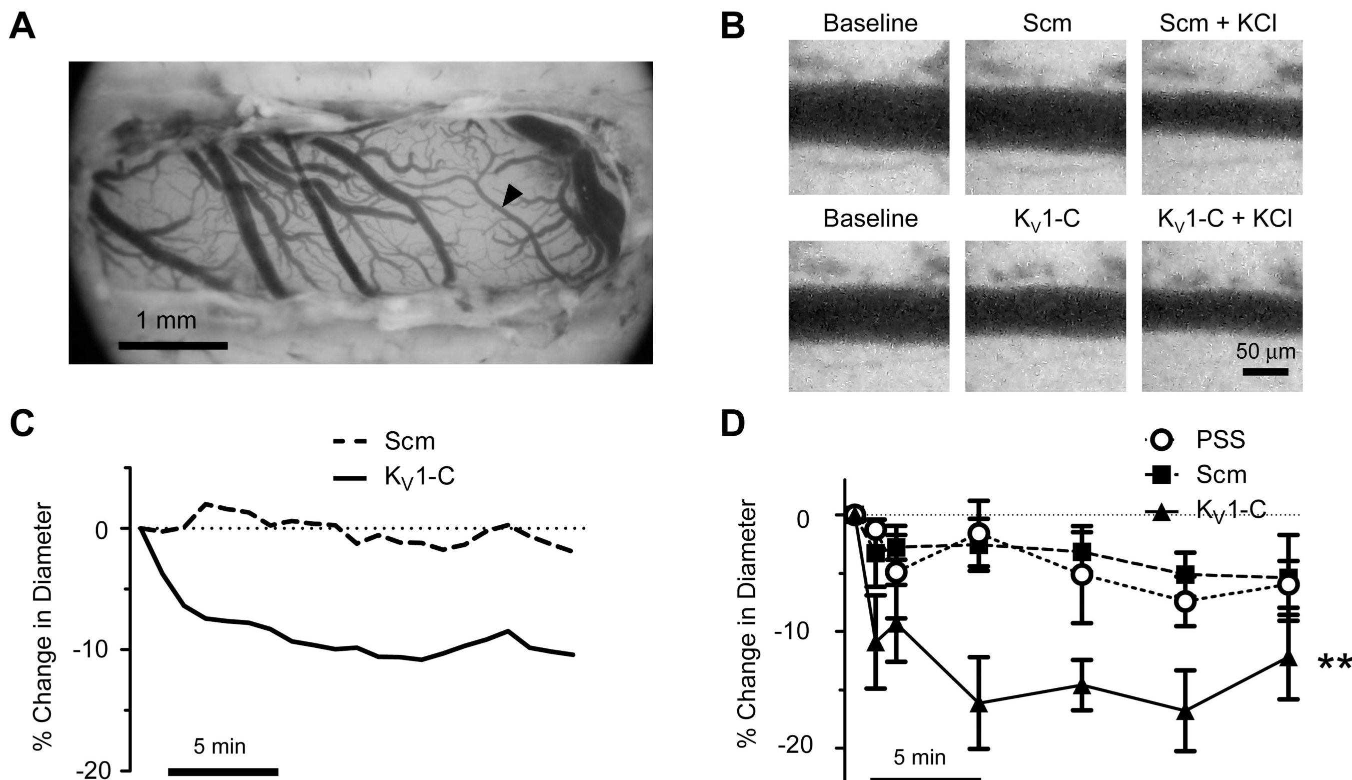

Figure 5. K_V1-C peptide constricts rat CA in vivo

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_V1-C peptide, but not to the same concentration of Scm peptide. **C)** Diameter responses of single arterial branches to Scm or K_V1-C peptide. **D)** Percent change in diameter from baseline in response to 30 μmol/L Scm or K_V1-C peptides or physiological salt solution (PSS). Scale bar, 5 min. **: Significant difference from PSS and Scm, $P<0.01$, n=5–7.

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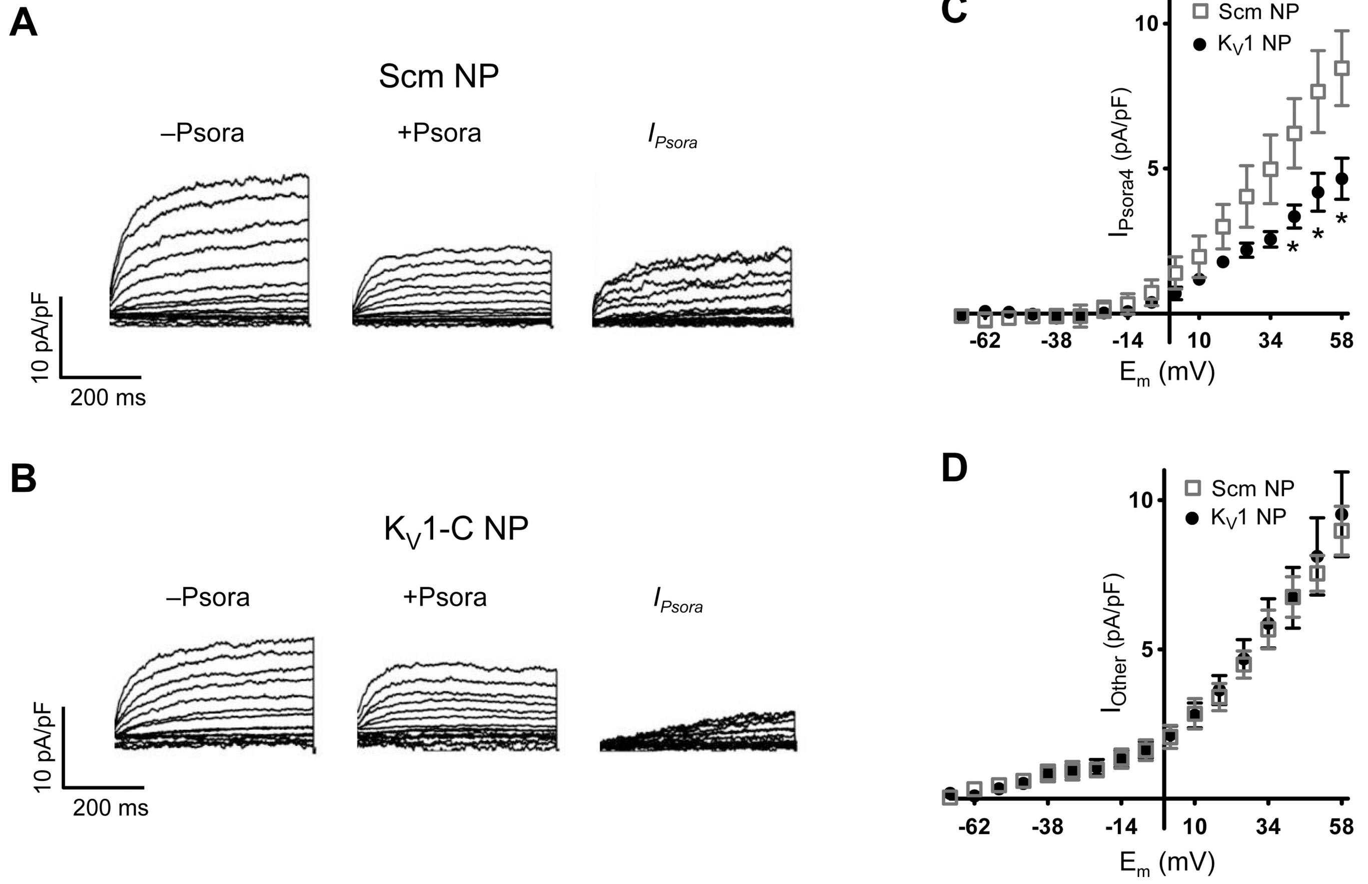

Figure 6. K_V1-C peptide reduces Psora4 -sensitive K^+ current

A, B) Representative whole-cell K^+ currents recorded with 3 μmol/L Scm (A) or K_V1-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_{Psora} was calculated by digitally subtracting post-Psora4 (+Psora) from pre-Psora4 (–Psora). **C, D**) Psora4-sensitive (C) and Psora4-insensitive (D) current densities (n=6 each). * indicates significant difference from Scm, P<0.05.

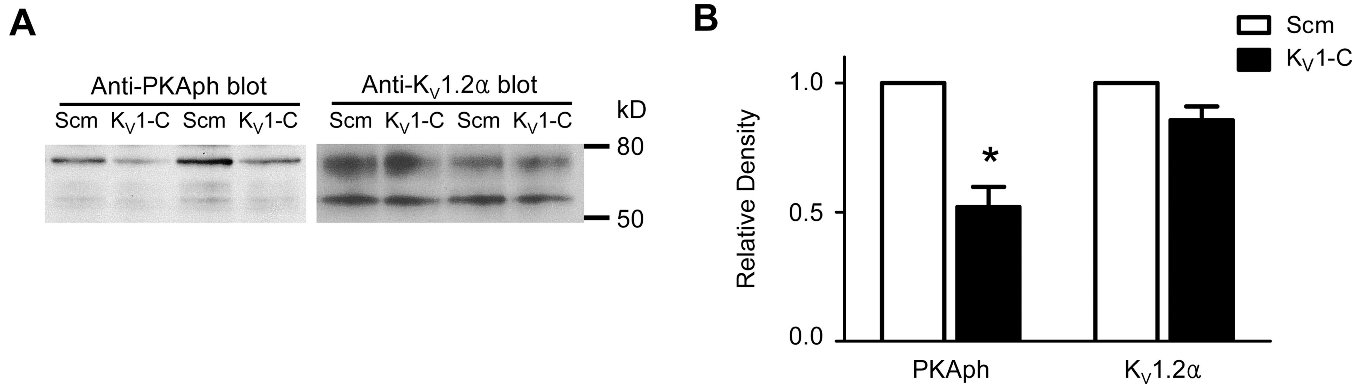

Figure 7. PKA phosphorylation of $K_V1.2\alpha$ is reduced in arteries exposed to K_V1-C peptide

A) Western blot using a phospho-PKA substrate antibody (PKAph, left blot) detected a ~75 kD protein corresponding to $K_V1.2\alpha$ in CA protein lysates. CA from age-matched rats were incubated in either Scm or K_V1-C peptide, and then lysates (30 μg) were loaded into adjacent lanes. Lanes 1-2 and lanes 3-4 represent separate preparations. The blot was stripped and reprobed with anti-$K_V1.2\alpha$ antibody ($K_V1.2\alpha$, right blot). **B)** Densitometry measurement of PKAph and $K_V1.2\alpha$ bands relative to Scm treatment (n=5). *: significant difference from Scm, $P<0.05$.

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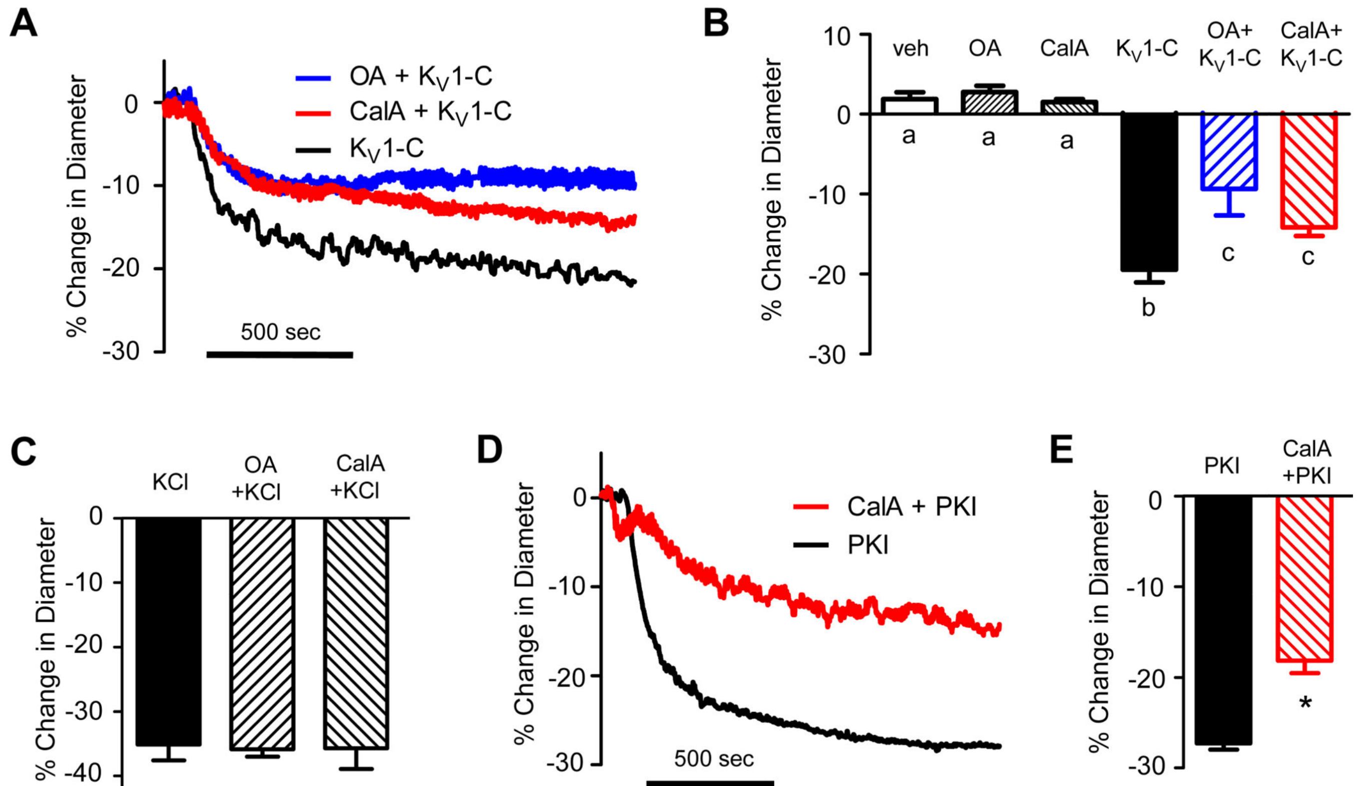

Figure 8. Phosphatase inhibitors blunt the vasoconstriction caused by K_V1-C

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 CA to K_V1–C peptide. **B)** Average % change in diameter in response to vehicle (veh), OA, CalA, 3 μmol/L K_V1–C peptide, or K_V1–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 K_V1-C peptide. a, b, c: statistical significance, P<0.05. **C)** Average % change in diameter caused by 60 mmol/L KCl in the presence and absence of a phosphatase inhibitors (n=4-10). No significant difference between groups. **D)** Representative traces show that 100 nmol/L CalA blunts the vasoconstrictor response to 1 μmol/L PKA inhibitor peptide (PKI). **E)** Average % 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.