Smooth Muscle Cell $\alpha_2\delta$-1 Subunits Are Essential for Vasoregulation by CaV1.2 Channels

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Rationale: Voltage-dependent L-type ($\text{CaV}_1.2$) Ca$^{2+}$ channels are a heteromeric complex formed from pore-forming $\alpha_1$ and auxiliary $\alpha_2\delta$ and $\beta$ subunits. CaV1.2 channels are the principal Ca$^{2+}$ influx pathway in arterial myocytes and regulate multiple physiological functions, including contraction. The macromolecular composition of arterial myocyte CaV1.2 channels remains poorly understood, with no studies having examined the molecular identity or physiological functions of $\alpha_2\delta$ subunits.

Objective: We investigated the functional significance of $\alpha_2\delta$ subunits in myocytes of resistance-size (100 to 200 $\mu$m diameter) cerebral arteries.

Methods and Results: $\alpha_2\delta$-1 was the only $\alpha_2\delta$ isoform expressed in cerebral arterial myocytes. Pregabalin, an $\alpha_2\delta$-1/-2 ligand, and an $\alpha_2\delta$-1 antibody, inhibited CaV1.2 currents in isolated myocytes. Acute pregabalin application reversibly dilated pressurized arteries. Using a novel application of surface biotinylation, data indicated that >95% of CaV1.2 $\alpha_1$ and $\alpha_2\delta$-1 subunits were present in the arterial myocyte plasma membrane. $\alpha_2\delta$-1 knockdown using short hairpin RNA reduced plasma membrane-localized CaV1.2 $\alpha_1$ subunits, caused a corresponding elevation in cytosolic CaV1.2 $\alpha_1$ subunits, decreased intracellular Ca$^{2+}$ concentration, inhibited pressure-induced vasoconstriction (“myogenic tone”), and attenuated pregabalin-induced vasodilation. Prolonged (24-hour) pregabalin exposure did not alter total $\alpha_2\delta$-1 or CaV1.2 $\alpha_1$ proteins but decreased plasma membrane expression of each subunit, which reduced myogenic tone.

Conclusions: $\alpha_2\delta$-1 is essential for plasma membrane expression of arterial myocyte CaV1.2 $\alpha_1$ subunits. $\alpha_2\delta$-1 targeting can block CaV1.2 channels directly and inhibit surface expression of CaV1.2 $\alpha_1$ subunits, leading to vasodilation. These data identify $\alpha_2\delta$-1 as a novel molecular target in arterial myocytes, the manipulation of which regulates contractility. (Circ Res. 2009;105:00-00.)

Key Words: L-type Ca$^{2+}$ channels $\bullet$ arterial contractility

Voltage-dependent calcium (Ca$^{2+}$) channels are expressed in multiple cell types, including neurons, cardiac myocytes, skeletal muscle, and smooth muscle.$^1$ Voltage-dependent Ca$^{2+}$ channels are heteromeric complexes comprising a pore-forming $\alpha_1$ subunit and auxiliary $\alpha_2\delta$, $\beta$, and $\gamma$ subunits.$^1$ Four major $\beta$ subunit isoforms ($\beta_1$ to $\beta_3$) have been described, which are the products of different genes.$^{1,2}$ Four genes that encode different $\alpha_2\delta$ isoforms ($\alpha_2\delta$-1 to $\alpha_2\delta$-4) have also been cloned.$^{3,4}$ Each $\alpha_2\delta$ isoform is the product of a single gene, which is posttranslationally cleaved into a highly glycosylated extracellular $\alpha_2$ and a smaller membrane-spanning $\delta$. $\alpha_2$ and $\delta$ subunits reassociate via a disulfide bond to form a single functional protein.$^{3,5,6}$

In arterial smooth muscle cells, voltage-dependent Ca$^{2+}$ channels are the major Ca$^{2+}$ entry pathway and regulate numerous cellular functions, including contractility and gene expression.$^{7-9}$ CaV1.2 $\alpha_1$ is generally considered to be the principal pore-forming Ca$^{2+}$ subunit that is expressed in arterial smooth muscle cells.$^{9-11}$ In contrast, expression and physiological functions of Ca$^{2+}$ auxiliary subunits in the vasculature are poorly understood. Several $\beta$ subunit isoforms ($\beta_1\delta$, $\beta_2$, and $\beta_3$) have been identified in smooth muscle cells, although physiological functions of these subunits are uncertain.$^{7,12-15}$ Furthermore, there are no studies that have examined the molecular identity and physiological functions of $\alpha_2\delta$ subunits that are expressed in arterial smooth muscle cells. Investigating Ca$^{2+}$ channel auxiliary subunits in arterial smooth muscle cells is important given the relevance of these channels to vascular physiology.

Cellular functions of $\alpha_2\delta$ subunits have previously been studied primarily through heterologous overexpression of recombinant proteins. $\alpha_2\delta$ subunits promote plasma membrane trafficking of recombinant Ca$^{2+}$ channel $\alpha_1$ subunits and modify the biophysical properties of currents generated by...
recombinant Cav channels. However, recent studies performed in native cell types have suggested that physiological functions of α,β subunits may differ from those in heterologous expression systems. In dysgenic myotubes, α,β-1 did not alter membrane targeting of Cavα,1.1 α subunit or modify Ca\(^{2+}\) current amplitude and was not essential for excitation–contraction, but knockdown accelerated current activation.\(^{17,18}\) In cardiac myocytes, α,β-1 knockdown did not alter Cavα,1.2 α subunit membrane targeting but modified current voltage dependence and inhibited excitation–contraction coupling.\(^{19}\) Thus, evidence suggests that physiological functions of native α,β subunits can differ from those of recombinant proteins.

Here, we investigated the molecular identity and physiological functions of α,β subunits that are expressed in arterial smooth muscle cells. Our study was performed using resistance-size cerebral arteries that regulate brain regional blood pressure and flow. Our data indicate that cerebral artery smooth muscle cells express only the α,β isoform that is expressed in arterial smooth muscle cells. Targeting of membrane resident α,β-1 subunits inhibits Cavα,1.2 currents and causes vasodilation. We also show that α,β-1 stimulates membrane insertion of Cavα,1.2 α subunits and that inhibition of this process causes prolonged vasodilation. These data indicate that α,β-1 subunits are an essential regulator of arterial contractility and identify α,β-1 targeting as a novel approach to cause vasodilation.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Tissue Preparation
Male Sprague–Dawley rats (\(\sim 250\) g) were euthanized by intraperitoneal injection of sodium pentobarbital solution (150 mg/kg). The brain was removed and placed into physiological saline solution containing (in mmol/L): KCl 6, NaCl\(^2\) 112, NaHCO\(_3\) 24, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, CaCl\(_2\) 1.8, and glucose 10. Middle cerebral, posterior cerebral, and cerebellar arteries (100 to 200 \(\mu\)m diameter) were dissected from the brain and used for this study. Smooth muscle cells were enzymatically dissociated from cerebral arteries, as previously described.\(^{20}\)

Reverse Transcription and Polymerase Chain Reaction
For RT-PCR, acutely isolated arterial smooth muscle cells were manually collected under a microscope using an enlarged patch-clamp pipette to prevent message contamination from other vascular wall cell types, as we have done previously.\(^{21}\) RT-PCR was also performed on whole brain and intact cerebral arteries.

α,β-1 Knockdown
Three gene-specific short hairpin RNA sequences were designed to the α,β and δ-1 regions of the α,β-1 gene and inserted into the pRNA-U6.1/Neo vector (Genscript). All 3 α,β-1 suppression vectors (α,δ-1shV) were inserted (10 \(\mu\)g/mL) into rat cerebral arteries using reverse permeabilization.\(^{22}\) Suppression vectors that encode scrambled sequences (α,δ-1scr) were used as a control. Arteries were then placed into serum-free DMEM F12 supplemented with 1% penicillin–streptomycin (Sigma) for 4 days at 37°C in a sterile incubator (21% O\(_2\)/5% CO\(_2\)), as we have described previously.\(^{23}\)

Biotinylation
Arteries were incubated with EZ-Link Sulfo-NHS-LC-LC-Biotin and Maleimide-PEG2-Biotin reagents (Pierce). Unbound biotin was removed by quenching and washing. For fluorescence measurement, biotinylated arteries or control arteries were incubated with Texas red–conjugated streptavidin. Texas red was excited at 561 nm, and emission was collected at 575 to 632 nm using a Zeiss 5-lane scanning confocal microscope.

For protein determination, biotinylated arteries were homogenized in radioimmunoprecipitation assay buffer (Sigma), and cellular debris was removed by centrifugation. Total protein was determined\(^{24}\) to allow normalization for Avidin (Monomeric Avidin, Pierce) pull-down of biotinylated surface proteins. Following pull-down, the supernatant comprised the nonbiotinylated (cytosolic) protein fraction, whereas surface proteins remained bound to the Avidin beads. Proteins were eluted from beads by boiling in Laemmli buffer containing 2-mercaptoethanol. Total, surface, and cytosolic proteins were analyzed using Western blotting. Band intensity was determined usingQuantity One software (Bio-Rad). Surface and cytosolic proteins were calculated as a percentage of total protein.

Patch-Clamp Electrophysiology
Whole cell patch-clamp recordings were carried out on isolated cerebral artery smooth muscle cells, as previously described.\(^{14}\)

Pressurized Artery Myography
Where required, endothelium was denuded as previously described.\(^{21}\) Pressurized cerebral artery diameter was measured using edge-detection myography. Myogenic tone (%) was calculated as 100 \(\times\) (active diameter/passive diameter).

Statistical Analysis
Summary data are presented as means±SEM. Significance was determined using ANOVA for multiple groups, or paired or unpaired t-tests with Welsh correction. \(P<0.05\) was considered significant. Power analysis was carried out where \(P>0.05\) to verify that sample size was sufficient to give a value of \(>0.8\).

Results
Rat Cerebral Artery Smooth Muscle Cells Express Only One α,δ Isoform, α,δ-1
To measure α,δ isoform expression, RT-PCR was performed on RNA extracted from rat brain, cerebral arteries, and pure isolated cerebral artery smooth muscle cells. RT-PCR amplified transcripts for all four α,δ isoforms in brain and cerebral arteries, which contain multiple cell types (Figure 1A and Online Figure I). In contrast, only α,δ-1 message was detected in isolated arterial smooth muscle cells (Figure 1A). Western blotting indicated that α,δ-1 protein was expressed in cerebral arteries (Figure 1B). The α,δ-1 antibody used for Western blotting recognizes an extracellular epitope on α,δ. Immunofluorescence, performed by applying the α,δ-1 primary antibody to live isolated smooth muscle cells via the bath solution, produced plasma membrane–localized fluorescence that was abolished by antigenic peptide or removal of the primary antibody (Figure 1C). These data indicate that cerebral artery smooth muscle cells express only the α,δ-1 isoform.
isoform of α,β and that α,δ-1 subunits are present in the smooth muscle cell plasma membrane.

**Pregabalin, an α,β Ligand, and an α,δ-1 Antibody Inhibit Arterial Smooth Muscle Cell Cav1.2 Currents**

Regulation of arterial smooth muscle cell Cav1.2 currents by α,δ-1 was examined using patch-clamp electrophysiology. Pregabalin, an α,δ-1/2 ligand, at 10 and 100 μmol/L reduced mean voltage-dependent Ba2+ currents by ≈10% and ≈33%, respectively (at +20 mV; Figure 2A and B; Online Figure II, A). In contrast, pregabalin did not shift the current–voltage (I-V) relationship, alter steady-state inactivation, or modify the rate of current inactivation (Figure 2B through 2D; Online Figure II, B).

Bath application of the α,δ-1 antibody that was used for Western blotting and immunofluorescence experiments also reduced arterial smooth muscle cell voltage-dependent Ba2+ currents to ≈65% of control (at +20 mV) (Figure 2E). Similarly to pregabalin, α,δ-1 antibody did not shift the I-V relationship (Figure 2F). Boiled (95°C for 15 minutes) antibody did not inhibit voltage-dependent Ba2+ currents, when compared to untreated control (Figure 2E and 2F). These data indicate that α,δ-1 ligands target membrane-resident α,δ-1 subunits in arterial smooth muscle cells, leading to Ca1.2 current inhibition.

**Pregabalin Dilates Pressurized Cerebral Arteries**

Ca1.2 channels are essential for pressure-induced vasoconstriction; termed the “myogenic response.” To examine physiological functions of smooth muscle cell α,δ-1 subunits, we studied diameter responses to α,δ-1 manipulation in pressurized cerebral arteries.

Acute pregabalin caused a concentration-dependent, reversible vasodilation in arteries pressurized to 60 mm Hg (Figure 3A and 3B). Endothelium denudation did not alter pregabalin-induced vasodilation (Figure 3B). GABA, which is molecularly similar to pregabalin but does not bind to α,δ subunits, did not alter the diameter of endothelium-intact or -denuded pressurized arteries (Figure 3B).


**Figure 3.** Pregabalin dilates cerebral arteries. A, Exemplary traces illustrating changes in diameter in response to acute pregabalin application. B, Bar graph summarizing mean data for acute pregabalin-induced vasodilation in endothelium-intact (n=5 to 8 for each) or -denuded (n=7 for each) arteries. GABA concentration was 100 μmol/L (n=4 for each). Mean myogenic tone in endothelium-intact and -denuded arteries was 28.0±3.2% and 29.2±5.3%, respectively (P>0.05).

α₂δ-1 Knockdown Reduces Surface Expression of Cav1.2 α₁ Subunits

To further examine physiological functions of α₂δ-1 subunits in cerebral artery smooth muscle cells, vectors encoding short hairpin RNA (α₂δ-1shV) were constructed and inserted intracellularly into cerebral arteries using reverse permeabilization. Vectors encoding scrambled short hairpin RNA (α₂δ-1scrm) were used as a control. Total protein for α₂δ-1, Cav1.2 α₁, and β₁ subunits were quantified using Western blotting. α₂δ-1shV reduced cerebral artery α₂δ-1 protein to ≈57% of α₂δ-1scrm (Figure 3A and 3B). α₂δ-1 knockdown did not alter β₁ subunit protein but elevated Cav1.2 α₁ protein to ≈134% of α₂δ-1scrm (Figure 3A and 3B).

Surface biotinylation was used to study the regulation of Cav1.2 subunit membrane expression by α₂δ-1 in cerebral arteries. To our knowledge, this is the first time that biotinylation has been used to measure the proportion of plasma membrane–localized ion channel proteins in native arterial smooth muscle cells. We confirmed that biotin effectively binds to extracellular proteins on smooth muscle cells by imaging biotinylated arteries that had been exposed to Texas red–conjugated streptavidin. Scale bar=20 μm. D, Representative Western blot illustrating detection of heat shock protein 90 (HSP90) in arterial lysate (total) or in the nonbiotinylated (cytosolic) or biotinylated (surface) fractions isolated from biotin-treated arteries. E, Representative Western blots showing cytosolic and surface protein levels of α₂δ-1 and α₁ subunits in arteries treated with α₂δ-1shV or α₂δ-1scrm. F, Mean relative cytosolic and surface Cav1.2 α₁ and α₂δ-1 protein in arteries treated with α₂δ-1shV (n=4 to 5) relative to α₂δ-1scrm (n=4 to 5) control. *P<0.05 vs cytosol.

**Figure 4.** α₂δ-1 knockdown reduces surface expression of Cav1.2 α₁ and α₂δ-1 subunits in cerebral arteries. A, Representative blot illustrating the effect of α₂δ-1shV and α₂δ-1scrm on total Cav1.2 α₁, α₂δ-1, and β₁ subunit total protein. B, Mean effect of α₂δ-1shV on total Cav1.2 α₁ (n=9), α₂δ-1 (n=7), and β₁ (n=5) subunit protein. C, Fluorescent images of control arteries (left) or arteries treated with biotin reagents (right) followed by exposure to Texas red–conjugated streptavidin. Scale bar=20 μm. D, Representative Western blot illustrating detection of heat shock protein 90 (HSP90) in arterial lysate (total) or in the nonbiotinylated (cytosolic) or biotinylated (surface) fractions isolated from biotin-treated arteries. E, Representative Western blots showing cytosolic and surface protein levels of α₂δ-1 and α₁ subunits in arteries treated with α₂δ-1shV or α₂δ-1scrm. F, Mean relative cytosolic and surface Cav1.2 α₁ and α₂δ-1 protein in arteries treated with α₂δ-1shV (n=4 to 5) relative to α₂δ-1scrm (n=4 to 5) control. *P<0.05 vs cytosol.

α₂δ-1 Knockdown Reduces [Ca²⁺]₀ and Dilates Cerebral Arteries

α₂δ-1 knockdown reduced mean [Ca²⁺]₀ in endothelium-denuded arteries from ≈129 to 115 nmol/L (Figure 5A). α₂δ-1 knockdown also attenuated depolarization-induced (60 mmol/L K⁺) [Ca²⁺]₀, from ≈263 to 192 nmol/L (Figure 5A). Nimodipine, a voltage-dependent Ca²⁺ channel blocker, reduced [Ca²⁺]₀ to a similar level in arteries treated with α₂δ-1shV or α₂δ-1scrm, and removal of extracellular Ca²⁺ in the presence of nimodipine had no further effect on [Ca²⁺]₀. α₂δ-1 knockdown reduced myogenic tone to between 43% and 63% of α₂δ-1scrm over a range of intravascular pressures between 20 and 100 mm Hg (Figure 5B and 5C). α₂δ-1 knockdown also reduced 60 mmol/L K⁺–induced vasocon-
striction (at 60 mm Hg) to \(\approx 72\%\) of \(\alpha_2\delta\)-1shV (Figure 5C). Nimodipine fully dilated pressurized arteries that had been treated with \(\alpha_2\delta\)-1shV or \(\alpha_2\delta\)-1scrm, indicating that myogenic tone in these vessels occurred because of Ca\(_{1.2}\) channel activation (Figure 5B and 5C). \(\alpha_2\delta\)-1 knockdown also reduced pregabalin-induced vasodilation to \(\approx 41\%\) of that in \(\alpha_2\delta\)-1scrm control arteries (Figure 5D). These data indicate that \(\alpha_2\delta\)-1 controls Ca\(^{2+}\) influx through Ca\(_{1.2}\) channels in myocytes, thereby regulating arterial diameter.

**Figure 5.** \(\alpha_2\delta\)-1 knockdown reduces [Ca\(^{2+}\)] and dilatates cerebral arteries. A, Mean [Ca\(^{2+}\)] in endothelium-denuded arteries treated with \(\alpha_2\delta\)-1scrm (n=5) or \(\alpha_2\delta\)-1shV (n=5) and normalization of differences with nimodipine (1 \(\mu\)mol/L) and Ca\(^{2+}\) removal. B, Exemplary traces illustrating that myogenic tone at 60 mm Hg is attenuated in arteries treated with \(\alpha_2\delta\)-1shV vs \(\alpha_2\delta\)-1scrm. Nimodipine (1 \(\mu\)mol/L) fully dilates both \(\alpha_2\delta\)-1scrm- and \(\alpha_2\delta\)-1shV–treated arteries. C, Mean myogenic tone in endothelium-intact arteries treated with \(\alpha_2\delta\)-1shV or \(\alpha_2\delta\)-1scrm at 20 mm Hg (\(\alpha_2\delta\)-1shV, n=7; \(\alpha_2\delta\)-1scrm, n=10); 60 mm Hg (\(\alpha_2\delta\)-1shV, n=13; \(\alpha_2\delta\)-1scrm, n=16), and 100 mm Hg (\(\alpha_2\delta\)-1shV, n=7; \(\alpha_2\delta\)-1scrm, n=10) in physiological saline solution and in response to 60 mmol/L K\(^+\) at 60 mm Hg (\(\alpha_2\delta\)-1shV, n=10; \(\alpha_2\delta\)-1scrm, n=13) or nimodipine at 60 mm Hg (1 \(\mu\)mol/L; \(\alpha_2\delta\)-1shV, n=9; \(\alpha_2\delta\)-1scrm, n=8). At 60 mm Hg, tone in endothelium-intact noncultured arteries (see Figure 3) and arteries treated with \(\alpha_2\delta\)-1scrm were similar (P<0.05). D, Pregabalin–induced vasodilation is attenuated in endothelium-intact arteries treated with \(\alpha_2\delta\)-1shV (n=6) vs \(\alpha_2\delta\)-1scrm (n=5). *P<0.05 vs \(\alpha_2\delta\)-1scrm in 6 K\(^+\); #P<0.05 vs \(\alpha_2\delta\)-1shV in 6 K\(^+\); and §P<0.05 vs \(\alpha_2\delta\)-1scrm in 60 K\(^+\).

**Figure 6.** Pregabalin reduces plasma membrane expression of \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) subunits in cerebral arteries. A, Representative blots illustrating the effects of a 24-hour exposure to pregabalin (+P) (100 \(\mu\)mol/L) on total, cytosolic, and surface protein levels of \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) subunits. B, Bar graph indicating the percentage of total, cytosolic, and surface protein for Ca\(_{1.2}\) \(\alpha_1\) and \(\alpha_2\delta\)-1 in untreated controls (white bars; n=3 to 4) and following 24-hour exposure to pregabalin (100 \(\mu\)mol/L) (black bars; n=3 to 4). *P<0.05 vs control.

**Pregabalin Inhibits Plasma Membrane Expression of \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) Subunits, Leading to Vasodilation**

Chronic application of gabapentinoids inhibits surface expression of recombinant \(\alpha_2\delta\)-1, \(\alpha_2\delta\)-2, and Ca\(_{1.2}\) \(\alpha_1\) subunits and reduces membrane trafficking of \(\alpha_2\delta\)-1 in dorsal root ganglion neurons.\(^{28,29}\) Therefore, the regulation of Ca\(_{1.2}\) \(\alpha_1\) subunit membrane expression by chronic exposure to pregabalin was studied in cerebral arteries. Pregabalin (24 hours) did not alter total \(\alpha_2\delta\)-1 or Ca\(_{1.2}\) \(\alpha_1\) subunit protein (Figure 6A and 6B). However, pregabalin (24 hours) decreased plasma membrane-localized \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) proteins to \(\approx 55\%\) and 75% of untreated (24 hours) controls, respectively (Figure 6A and 6B). In accordance, pregabalin elevated cytosolic \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) subunit protein (Figure 6A and 6B). GABA (24 hours) did not alter \(\alpha_2\delta\)-1 or Ca\(_{1.2}\) \(\alpha_1\) subunit surface expression (Figure 6B). These data indicate that prolonged exposure to pregabalin reduces plasma membrane localization of \(\alpha_2\delta\)-1 and Ca\(_{1.2}\) \(\alpha_1\) subunits in cerebral artery smooth muscle cells.

The regulation of arterial diameter by chronic exposure to pregabalin was studied. Arteries were exposed to pregabalin for 24 hours and then pressurized. Pregabalin (24 hours) reduced myogenic tone at 60 mm Hg to \(\approx 40\%\) of untreated controls (Figure 7A and 7B). Pregabalin also reduced 60 mmol/L K\(^+\)–induced vasoconstriction to \(\approx 63\%\) of control (Figure 7B). Removal of pregabalin (2-hour wash) from the bath solution caused an immediate partial recovery of myogenic tone, consistent with washout of the acute pregabalin-induced vasodilation (see Figures 3A, 3B, 7A, and 7B). Two hours following pregabalin washout, myogenic tone and...
data show that cerebral artery smooth muscle cells express 1 αδ-1 subunits.

60 mmol/L K⁺–induced vasoconstriction partially recovered but was still attenuated, consistent with prolonged Cav1.2 inhibition (Figure 7A and 7B). Twelve hours after pregabalin washout, myogenic tone and 60 mmol/L K⁺–induced vasoconstriction fully recovered (Figure 7B).

These data indicate that αδ-1 subunits are essential regulators of cerebral artery contractility by promoting Cav1.2 α1 subunit plasma membrane insertion. These results also demonstrate that αδ-1 can be targeted either to cause acute vasodilation by modulating membrane-resident subunits or prolonged vasodilation by altering plasma membrane expression of Cav1.2 α1 subunits.

**Discussion**

We have examined the molecular identity and physiological functions of αδ-1 subunits in arterial smooth muscle cells. Our data show that cerebral artery smooth muscle cells express 1 αδ isoform, αδ-1. αδ-1 ligands inhibited Cav1.2 currents in isolated arterial smooth muscle cells. αδ-1 knockdown reduced plasma membrane-localized αδ-1 and Cav1.2 α1 subunits and [Ca²⁺]. Prolonged (24 hours) pregabalin exposure reduced plasma membrane insertion of αδ-1 and Cav1.2 α1 subunits. αδ-1 knockdown, and acute and prolonged pregabalin, all caused vasodilation. Taken together, these data indicate that αδ-1 stimulates plasma membrane insertion of smooth muscle cell Cav1.2 α1 subunits and that αδ-1 is a viable therapeutic target for causing both acute and prolonged vasodilation.

Cav1.2 channels regulate a variety of physiological functions in arterial smooth muscle cells, including contraction and gene expression. However, the macromolecular composition of vascular Cav1.2 channels was unclear. The present investigation was designed to determine physiological functions of arterial smooth muscle cell αδ-1 subunits. The few previous studies that have investigated physiological functions of αδ-1 subunits in other native cell types generated different findings. One explanation for these diverse findings is that physiological functions of αδ-1 subunits may depend on native Cav channel isoforms and splice variants. Skeletal muscle cells express Cav1.1 α1 subunits, whereas cardiac and smooth muscle cells both express Cav1.2 α1 subunits. Cardiac and smooth muscle cells express different Cav1.2 α1 subunit splice variants. For instance, resistance-size cerebral artery smooth muscle cell Cav1.2 α1 subunits primarily contain exon 1c, whereas most cardiac myocyte Cav1.2 α1 subunit mRNA contains exon 1b. Exon 1 splicing modified membrane insertion and voltage-dependent regulation of recombinant Cav1.2 α1 subunits by αδ-1. One possibility is that exon splicing underlies different physiological functions of αδ-1 in smooth versus cardiac muscle. Another possibility is that cell-specific auxiliary subunits, including different β isoforms, may modify physiological functions of αδ-1 subunits. Regardless of the molecular mechanisms involved, our data indicate that αδ-1 subunits modulate arterial contractility by regulating plasma membrane insertion of Cav1.2 α1 subunits in smooth muscle cells. This is in stark contrast to the apparent lack of effect of αδ-1 on membrane targeting of Cav1.2 subunits in skeletal and cardiac muscle cells.

Gabapentinoid drugs, including gabapentin and pregabalin, a higher-affinity analog, are antiallodynic and antihyperalgesic and are useful in the treatment of neuropathic pain that can result from nerve damage. Gabapentinoid drugs were originally developed as GABA receptor ligands but were subsequently found not to bind to GABA receptors. Gabapentinoid drugs to αδ-1 and αδ-2 subunits were discovered to underlie their therapeutic effects. Acute effects of gabapentin, the most widely studied gabapentinoid drug, and pregabalin on Cav currents produced variable results. Gabapentin inhibits a variety of Cav currents, including L-, N-, and P/Q-type. Gabapentin has also been reported to have no acute effect on Cav currents. These different observations may occur because of Cav channel isoform, cell culture conditions, auxiliary subunits present in the cells studied, or the affinity for αδ subunits of the gabapentinoid ligand that was used. Gabapentinoid regulation of arterial smooth muscle cell Cav currents and arterial contractility had not previously been examined. Our data indicate that acute pregabalin application inhibits cerebral artery smooth muscle cell Cav currents and dilates pressurized cerebral arteries. We show that pregabalin similarly dilated endothelium-intact and -denuded arteries, αδ-1 knockdown reduced pregabalin-induced vasodilation, and GABA had no effect, consistent with a previous report. These data indicate that pregabalin causes vasodila-
tion by binding to smooth muscle cell α₃δ-1 subunits. Acute application of pregabalin inhibits Caᵥ currents in cultured dorsal root ganglion neurons without causing a shift in current voltage dependence,⁴⁰ which is similar to the results shown here. Voltage-independent Caᵥ current inhibition is consistent with pregabalin acting as a pore blocker, an effect that may be facilitated by prior binding to an α₃δ-1 extracellular domain. Supporting this conclusion, we show that α₃δ-1 antibody also inhibited arterial myocyte Caᵥ,1.2 currents without shifting the I-V relationship. Taken together, our data indicate that α₃δ-1 ligands inhibit arterial smooth muscle cell Caᵥ,1.2 currents, leading to vasodilation.

Chronic exposure to gabapentin disrupted membrane trafficking of both recombinant and dorsal root ganglion neuron α₃δ and Caᵥ,2 α₁ subunits, reduced α₃δ-1 trafficking in the spinal cord, and alleviated allodynia.²⁵ α₃δ subunits modify Caᵥ channel trafficking through the von Willebrand factor A domain in the α₁ subunit.⁴¹ It has been proposed that gabapentinoid drugs may displace an endogenous ligand that is necessary for α₃δ subunit trafficking.²⁸,²⁹ To measure membrane expression of Caᵥ,1.2 α₁ and α₃δ-1 subunits, we used membrane biotinylation. Our successful implementation of this technique provides a basis from which future studies can measure the proportion and localization of a wide variety of native vascular proteins. Biotinylation indicated that >95% of both Caᵥ,1.2 α₁ and α₃δ-1 subunits are present in the cerebral artery smooth muscle cell plasma membrane. These data indicate that there is essentially no intracellular reserve of α₃δ-1 and Caᵥ,1.2 α₁ subunits that could be triggered to traffic to the plasma membrane, for example, in response to stimuli. We show that α₃δ-1 knockdown and 24-hour pregabalin treatment both reduced plasma membrane expression of α₃δ-1 and Caᵥ,1.2 α₁ subunits in arterial smooth muscle cells, leading to vasodilation. In contrast, GABA had no effect on Caᵥ,1 subunit trafficking, indicating that pregabalin prevents α₃δ-1 and Caᵥ,1.2 α₁ membrane expression by binding to α₃δ-1 subunits in smooth muscle cells. α₃δ-1 knockdown did not alter β₃ subunit expression but elevated Caᵥ,1.2 α₁ subunit protein. One explanation for this effect is that the decrease in plasma membrane Caᵥ,1.2 channels caused by α₃δ-1 knockdown, and the subsequent reduction in voltage-dependent Ca²⁺ influx, leads to a compensatory mechanism that stimulates Caᵥ,1.2 α₁ expression. Inhibition of α₃δ-1 and Caᵥ,1.2 α₁ membrane expression by knockdown and pregabalin treatment blocked pressure-induced vasoconstriction by reducing voltage-dependent Ca²⁺ activity. This was measured as a reduction in nimodipine-sensitive Ca²⁺ influx and tone. Pregabalin washout after a 24-hour exposure caused an immediate vasoconstriction that was consistent with removal of acute pregabalin-induced Caᵥ,1.2 inhibition. Reversal of the chronic functional effect of pregabalin on α₃δ-1 membrane expression took between 2 and 12 hours, consistent with protein trafficking being slower than washout of a pharmacological channel inhibitor. Because α₃δ-1 knockdown did not alter β-subunit expression, these data indicate that α₃δ-1 can function in a β-subunit-independent manner. Thus, α₃δ-1 expression and functionality is essential for plasma membrane insertion of Caᵥ,1.2 α₁ subunits in arterial smooth muscle cells and the regulation of arterial contractility.

In summary, we identify α₃δ-1 as a vasoregulatory protein and show that this subunit promotes Caᵥ,1.2 α₁ subunit membrane insertion in cerebral artery smooth muscle cells. Our data also indicate that molecular and pharmacological targeting of α₃δ-1 may be used as novel therapeutic mechanism to regulate arterial contractility.

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Disclosures

None.

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Smooth Muscle Cell α2δ-1 Subunits Are Essential for Vasoregulation by CaV1.2 Channels
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Supplemental Documentation

Materials and Methods

Reverse Transcription and Polymerase Chain Reaction
Total RNA was extracted from rat brain or intact cerebral arteries using Trizol (Invitrogen). Total RNA was extracted from ~100 selected smooth muscle cells using the Absolutely RNA Nanoprep kit (Stratagene). First strand cDNA was synthesized using oligo d(T) and reverse transcriptase (SuperscriptTM III, Invitrogen) from 2-3 μg of total RNA. To identify the presence of α2δ isoforms, nested PCR was performed on rat brain, cerebral arteries, and cerebral artery smooth muscle cell first strand cDNA. Standard PCR reactions of an initial 2 min denaturation at 94 ºC followed by 40 thermo cycles of denaturation (94 ºC for 30 s), annealing (55 ºC for 30 s) and extension (72 ºC for 1 min) were used for all PCR reactions. First round primer sequences were as follows: α2δ-1 For (GCACCAAGGGAATACTGCAATGACC), Rev (CCACCATCATCTAGAATGAC), α2δ-2 For (GATGACTATGTGGAATGCTGCTTC), Rev (CCAGGGCCATCTGTGTCAGGTTG), α2δ-3 For (AATCCATTGCTGCAATGACTC), Rev (AAAGTACTGCCATATGAGGAGAC), and α2δ-4 For (TGTTAGAGTGGAGTCCTGG) and Rev (GAGACATCCACAGCGATGA). Second round primers were as follows: α2δ-1 ForNest (TGAATTTATGGATGAAAACTCC), RevNest (TAAAATTTTCTATCCAGGAGTT), α2δ-2 ForNest (GCTTTGAGTATGCTTTGACCA), RevNest (AGCCCATCAGCACCAT ACTACA), α2δ-3 ForNest (ATTGAAGAATCGACGGTCTCC), RevNest (CATGTACATGGCGAGATTGCT), and α2δ-4 ForNest (TGTGACACAGATCCAGT) and RevNest (CCAGTGATAGCAGGACAGA).

Immunofluorescence
Freshly dissociated cerebral artery smooth muscle cells were allowed to settle onto poly-L-lysine coated coverslips. Smooth muscle cells were exposed to either polyclonal α2δ-1 antibody (1:500, Aviva Systems Biology), α2δ-1 antibody together with antigenic peptide, or with no α2δ-1 antibody. Cells were washed and incubated with Cy3-conjugated secondary antibody (Jackson ImmunoResearch), and imaged using a Zeiss LSM5 Pascal laser-scanning confocal microscope. Cy3 was excited with 543 nm light and emission collected at 560 nm.

Reverse permeabilization
To insert either α2δ-1shV or α2δ-1sclr expression vectors (10 μg/ul) into smooth muscle cells, reverse permeabilization was performed, as previously described. Briefly, cerebral arteries were first placed into a Ca2+-free (EGTA, 10 mM), high K+ (120 mM) saline solution. Arteries were then incubated in the presence of α2δ-1shV or α2δ-1sclr vectors in a Ca2+ free, Mg2+ (2 mM)-containing solution supplemented with ATP (5 mM). Extracellular [Mg2+] was then increased to 10 mM, after which the solution was changed to one containing Na+ (140 mM) and K+ (5 mM). Extracellular Ca2+ was then increased incrementally to 1.8 mM. Arteries were subsequently placed into DMEM-F12 supplemented with penicillin and streptomycin (1 %) for 4 days (37ºC, 5% CO2) prior to use.

Protein analysis and Biochemistry
For each experiment, cerebral arteries were obtained from 1-2 rats per sample and homogenized in 1x Laemmll buffer containing 2% β-Mercaptoethanol to obtain whole arterial lysate. Cellular debris was removed by centrifugation. Protein concentrations were determined using the method of Henkel et al. Proteins were separated using SDS-PAGE and analyzed by Western blotting. Antibodies used were anti
αδ-1 (Aviva Systems Biology), α1 (Alomone), β1 (Santa Cruz), and actin (Santa Cruz). Bands were visualized on a Kodak Image F-Pro system using a West Pico Chemiluminescence kit (Pierce). For quantification, protein band intensities were normalized to actin and then to control samples.

**Biotinylation**

Arteries were incubated for 1 h in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Pierce) in phosphate-buffered saline (PBS, Invitrogen). Unbound biotin was removed by quenching using 100 mM glycine and washing with PBS. For fluorescence measurements, biotinylated arteries were incubated with Texas red-conjugated streptavidin for 2 h at 22°C. Control arteries were not incubated with biotin reagents prior to addition of Texas red-streptavidin. Texas red was excited at 561 nm and emission collected at 575-632 nm using a Zeiss 5-live laser-scanning confocal microscope.

For protein determination, biotinylated arteries were homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. The supernatant comprised the total protein lysate. Total protein was then determined to allow normalization for Avidin (Monomeric Avidin, Pierce) pull-down of biotinylated surface proteins. Following pull down, the supernatant comprised the non-biotinylated (cytosolic) protein fraction, while surface proteins remained bound to the Avidin beads. Proteins were eluted from beads by boiling in 1x Laemmli-buffer containing 2-mercaptoethanol (2%). Total, surface, and cytosolic proteins were analyzed using Western blotting. Band intensity was determined using Quantity One software (BioRad). Surface and cytosolic proteins were then calculated as a percentage of total protein.

**Patch Clamp Electrophysiology**

Whole cell patch clamp recordings were obtained from acutely isolated smooth muscle cells at room temperature using an Axopatch 200B amplifier (Axon Instruments) and pCLAMP 8.2 or 9.2. Borosilicate glass electrodes of resistence 1-3 MΩ were filled with pipette solution containing (in mmol/l): CsMeSO4 135, CsCl 5, EGTA 5, MgATP 4, Na2GTP 0.25, HEPES 10, and glucose 10 (pH 7.2, adjusted using CsOH). Extracellular bath solution contained (in mmol/l): BaCl2 20, NMDG 120, MgCl2 1, HEPES 10, and glucose 10 (pH 7.4, adjusted using Aspartate). All solutions were ~300 mOsm, as measured using a Vapor Pressure Osmometer. Cell capacitance was measured by applying a 5 mV test pulse and correcting the capacitance transients using series resistance compensation. To measure the time course of pregabalin and anti-αδ-1 antibody on Cav currents, cells were clamped at -40 mV and whole cell currents were activated every 30 s by 300ms step depolarizations from -80 mV to +20 mV. For measurement of current voltage (I-V) relationships, cells were clamped at -40 mV and whole cell currents were evoked every 5 s by 300 ms step depolarizations from -80 mV to between -60 to +60 mV in 10 mV increments. To measure steady-state inactivation, 1 s conditioning pulses from -80 to +60 mV were applied in 10 mV increments every 15 s prior to a 200 ms test pulse to +20 mV. Whole cell currents were filtered at 1 kHz and digitized at 5 kHz. Leak and capacitative transients were subtracted using P/-4 protocols. Steady state inactivation curves and tail currents were fit with the Boltzmann function:

\[
\frac{I}{I_{\text{max}}} = \frac{R_{\text{in}} + (R_{\text{max}} - R_{\text{in}})/(1 + \exp((V - V_{1/2})/k))}
\]

Where \(I/I_{\text{max}}\) is the normalized peak current, \(V\) the conditioning pre-pulse voltage, \(V_{1/2}\) the voltage for half maximal inactivation or activation, \(k\) the slope factor, \(R_{\text{in}}\) the proportion of non-inactivating current and \(R_{\text{max}}\) the maximal current. The rate of current inactivation was calculated as the time to half maximal inactivation.
Intracellular Ca$^{2+}$ concentration measurement

Endothelium-denuded cerebral artery segments were incubated with fura-2AM (10 μM), a fluorescent Ca$^{2+}$ indicator, and 0.05% pluronic F-127 for 1 hour, followed by a 15 minute wash. Arteries were cannulated at each end in an experimental chamber and alternately excited at 340 or 380 nm using a PC driven Hyperswitch (Ionoptix, MA, USA). Background corrected ratios were collected at every 0.2 s at 510 nm using a photomultiplier tube. [Ca$^{2+}$]$_i$ concentrations were calculated using the following equation: 

$$[\text{Ca}^{2+}] = \frac{K_d (R - R_{\text{min}})}{(R_{\text{max}} - R)} \frac{(S_{f2})}{(S_{b2})}$$

where R is the 340/380 nm ratio, $R_{\text{min}}$ and $R_{\text{max}}$ are the minimum and maximum ratios determined in Ca$^{2+}$ free and saturating Ca$^{2+}$ solutions, respectively, $S_{f2}/S_{b2}$ is the Ca$^{2+}$ free/Ca$^{2+}$ replete ratio of emissions at 380 nm excitation, and $K_d$ is the dissociation constant for fura-2. $R_{\text{min}}$, $R_{\text{max}}$, $S_{f2}$ and $S_{b2}$ were determined at the end of each experiment by increasing the Ca$^{2+}$ permeability of smooth muscle cells with ionomycin (10 μM), and perfusing cells with a high Ca$^{2+}$ (10 mM) or Ca$^{2+}$-free (10 mM EGTA) solution. The in situ apparent dissociation constant ($K_d$) for fura-2 used in this study was 224 nM.

Pressurized artery myography

Where appropriate, the endothelium was denuded by introducing an air bubble into the artery lumen for 1 min followed by a wash with PSS, as previously described. Endothelium-denudation was confirmed by the lack of vasodilation to carbachol (10 μM), an endothelium-dependent vasodilator. Cerebral artery segments were cannulated at each end in a perfusion chamber (Living Systems Instrumentation). The chamber was continuously perfused with PSS and maintained at 37 ºC. Intravascular pressure was altered using an attached reservoir and monitored using a pressure transducer. Intravascular solution was static, i.e. no-flow. Wall diameter was measured at 1 Hz using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix). Pharmacological agents were applied via chamber perfusion. Passive Diameter was determined by applying Ca$^{2+}$-free PSS supplemented with 5 mM EGTA.
References


Supplemental Figure Legends

**Supplemental Figure 1.** Cerebral artery smooth muscle cells express only αδ-1. A. Isoform-specific primers amplified transcripts for αδ-1, -2, -3, and -4 in rat cerebral artery lysate (CA), but only amplified transcript corresponding to αδ-1 in isolated myocytes (CASM). Image is representative of 3-5 experiments. August 2014: Supplemental Figure 1 retracted by the authors. See Correction.

**Supplemental Figure 2.** Pregabalin blocks voltage-dependent Ba^{2+} currents, but does not alter inactivation, in cerebral artery smooth muscle cells. A. Exemplary traces of voltage-dependent Ba^{2+} currents elicited from a holding potential of -80 mV by 300 ms voltage steps to +20 mV in control and pregabalin (100 μM). B. Normalized (I/I_{max}) representative traces illustrating currents activated by a 1 s conditioning voltage pulse from -80 to +30 mV, followed by a 200 ms step to +20 mV.
August 2014: Supplemental Figure 1 retracted by the authors. See Correction.

Supplemental figure 1
Supplemental figure 2