Transcriptional Upregulation of α₂δ-1 Elevates Arterial Smooth Muscle Cell Voltage-Dependent Ca²⁺ Channel Surface Expression and Cerebrovascular Constriction in Genetic Hypertension


Abstract—A hallmark of hypertension is an increase in arterial myocyte voltage-dependent Ca²⁺ (Ca₁.2) currents that induces pathological vasoconstriction. Ca₁.2 channels are heteromeric complexes composed of a pore-forming Ca₁.2α₁ with auxiliary α₂δ and β subunits. Molecular mechanisms that elevate Ca₁.2 currents during hypertension and the potential contribution of Ca₁.2 auxiliary subunits are unclear. Here, we investigated the pathological significance of α₂δ subunits in vasoconstriction associated with hypertension. Age-dependent development of hypertension in spontaneously hypertensive rats was associated with an unequal elevation in α₂δ-1 and Ca₁.2α₁ mRNA and protein in cerebral artery myocytes, with α₂δ-1 increasing more than Ca₁.2α₁. Other α₂δ isoforms did not emerge in hypertension. Myocytes and arteries of hypertensive spontaneously hypertensive rats displayed higher surface-localized α₂δ-1 and Ca₁.2α₁ proteins, surface α₂δ-1:Ca₁.2α₁ ratio, Ca₁.2 current density and noninactivating current, and pressure- and depolarization-induced vasoconstriction than those of Wistar-Kyoto controls. Pregabalin, an α₂δ-1 ligand, did not alter α₂δ-1 or Ca₁.2α₁ total protein but normalized α₂δ-1 and Ca₁.2α₁ surface expression, surface α₂δ-1:Ca₁.2α₁, Ca₁.2 current density and inactivation, and vasoconstriction in myocytes and arteries of hypertensive rats to control levels. Genetic hypertension is associated with an elevation in α₂δ-1 expression that promotes surface trafficking of Ca₁.2 channels in cerebral artery myocytes. This leads to an increase in Ca₁.2 current-density and a reduction in current inactivation that induces vasoconstriction. Data also suggest that α₂δ-1 targeting is a novel strategy that may be used to reverse pathological Ca₁.2 channel trafficking to induce cerebrovascular dilation in hypertension. (Hypertension. 2012;60:00-00.)

Key Words: calcium channels | genetic hypertension | vasodilation | vasoconstriction

Hypertension is associated with an elevation in arterial contractility that increases systemic blood pressure and restricts organ blood flow, leading to end-organ damage. Hypertension is also a major predictor for a variety of cerebral diseases, including stroke, Alzheimer disease, and dementia. One characteristic pathological alteration that occurs in hypertension is an elevation in vascular smooth muscle cell (myocyte) voltage-dependent Ca²⁺ influx. Voltage-dependent L-type Ca²⁺ (Ca₁.2) channels are the primary Ca²⁺ entry pathway in arterial myocytes and are essential for contractility regulation by a wide variety of stimuli, including intravascular pressure, membrane potential, and vasoconstrictors. A hypertension-associated elevation in Ca₁.2 currents leads to an increase in intracellular Ca²⁺ concentration and vasoconstriction. However, molecular mechanisms that elevate arterial myocyte Ca₁.2 currents in hypertension, leading to vasoconstriction, are unclear.

Ca₁.2 channels are heteromeric complexes composed of a pore-forming α with auxiliary α₂δ and β subunits. Four α₂δ (1–4) subunit isoforms have been identified that are each encoded by different genes. α₂δ subunits undergo posttranslational cleavage into a highly glycosylated extracellular α₁ and a smaller δ subunit, which are subsequently coupled by a disulfide bond to form a single functional protein. α₂δ subunits are membrane bound by the bilayer-spanning δ subunit. Recently, α₂δ-1 was identified as being critical for functional trafficking of Ca₁.2α₁ subunits to the plasma membrane (surface) in arterial myocytes. To date, no studies have investigated pathological or disease-associated molecular changes in Ca₁.2 auxiliary subunits, including α₂δ subunits, in myocytes of resistance-sized arteries. In addition, it is unclear whether the subunit composition of arterial myocyte surface Ca₁.2 channels is altered in disease.
Given that arterial myocyte Ca$_{2.1.2}$ currents are elevated during hypertension, leading to vasoconstriction, we determined the subunit composition of Ca$_{2.1.2}$ channels and investigated the involvement of α$_2$δ subunits in this pathological alteration. Elucidating molecular mechanisms governing α$_2$δ subunit regulation of Ca$_{2.1.2}$ channels in hypertension could lead to the development of novel approaches to treat cardiovascular diseases.

Here, we used a genetic model of hypertension, the spontaneously hypertensive rat (SHR), to investigate the pathological significance of arterial myocyte α$_2$δ subunits in hypertension. We showed that, during hypertension, an elevation in α$_2$δ-1 expression increases plasma membrane Ca$_{2.1.2}$ currents in arterial myocytes, leading to vasoconstriction. We also identified α$_2$δ-1 as a novel therapeutic target to induce cerebrovascular dilation in hypertension.

**Methods**

**Cell Isolation and Tissue Preparation**

All of the animal protocols used were reviewed and approved by the animal care and use committee at the University of Tennessee Health Science Center. Male 6- or 12-week–old SHR and Wistar-Kyoto (WKY) rats were euthanized by IP injection of sodium pentobarbital (150 mg/kg of body weight, Vortech Pharmaceuticals, Dearborn, MI). Middle cerebral, posterior cerebral, and cerebellar arteries (≈100–200 µm diameter) were studied. Myocytes were enzymatically dissociated from dissected cerebral arteries, as described previously.

**Blood Pressure Measurements**

Diastolic and systolic blood pressures were measured in conscious rats using a tail-cuff sphygmomanometer (Kent Scientific, Torrington, CT).

**RT-PCR**

RT-PCR was performed on myocytes individually collected under a microscope using an enlarged patch-clamp pipette to prevent contamination from other arterial wall cell types, as described previously.

**Quantitative Real-Time PCR**

Total RNA was isolated from cerebral arteries using TRizol (Invitrogen, Grand Island, NY). cDNA was transcribed using Affinity Script Multiplex temperature reverse transcriptase (Stratagene, Clara, CA). Gene specific primers and probes were designed using the Universal Probe Library. Sequences of primers and probes used for quantitative PCR are given in Table S1 (available in the online-only Data Supplement). In contrast, at 12 weeks of age, diastolic, systolic, and mean arterial blood pressures were similar (Figure S1, available in the online-only Data Supplement). Rps5 mRNA levels were similar in WKY and SHR arteries and, thus, Rps5 was used as the reference gene for these experiments (Table S2).

**Protein Analysis and Biochemistry**

Proteins were separated on SDS-PAGE gels and analyzed by Western blotting. Blots were cut at the 75-kDa marker to allow simultaneous probing of the upper section for α$_2$δ-1 and lower section for actin. The upper portion of the blot was then reprobed for Ca$_{1.2}$α$_1$. Protein band intensities were determined using Quantity One (BioRad, Hercules, CA) software. For quantification, protein band intensities were first normalized to actin and then to appropriate control samples.

**Artery Surface Biotinylation**

To determine the distribution of α$_2$δ-1 and Ca$_{1.2}$α$_1$ subunit proteins between surface and intracellular compartments, artery surface biotinylation was used, as described previously.

**Patch-Clamp Electrophysiology**

Whole cell Ca$_{1.2}$ currents were recorded in isolated myocytes using the whole cell patch clamp configuration, as described previously.

**Pressurized Artery Myography**

Endothelium-denuded artery diameter was measured over a range of intravascular pressures (20–100 mm Hg) in the presence and absence of nimodipine (1 µmol/L) using edge-detection myography, as described previously. Diameter responses to elevating extracellular K+ from 6 to between 20 and 60 mmol/L at 10 mm Hg in the presence of pinacidil (10 µmol/L), a K$_{ATP}$ channel opener, were also recorded. Arteries treated with pregabalin for 24 hours were also maintained in pregabalin throughout these experiments to inhibit Ca$_{1.2}$ subunit membrane reinsertion.

**Statistical Analysis**

Summary data are presented as mean±SEM. Significance was determined using paired or unpaired t tests with Welch correction or ANOVA followed by Student-Newman-Keuls for multiple groups. $P<0.05$ was considered significant. Power analysis was carried out where $P$ value was >0.05 to verify that sample size was sufficient to give a value of >0.8.

An expanded Methods section is available in the online-only Data Supplement.

**Results**

**Arterial Myocyte α$_2$δ-1 and Ca$_{1.2}$α$_1$ Subunit Expression**

The pathological involvement of arterial myocyte Ca$_{1.2}$ subunits was studied using a rat genetic model of hypertension. At 6 weeks of age, WKY and SHR diastolic, systolic, and mean arterial blood pressures were similar (Figure S1, available in the online-only Data Supplement). In contrast, at 12 weeks of age, diastolic, systolic, and mean arterial pressures were ≥63, 65, and 72 mm Hg higher in SHRs than WKY rats, respectively (Figure S1).

Four different α$_2$δ isoforms have been described, with α$_2$δ-1 the only isoform expressed in normotensive Sprague-Dawley (SD) rat cerebral artery myocytes. We tested the hypothesis that hypertension is associated with a shift in α$_2$δ isoform expression in myocytes of resistance-size arteries. RT-PCR detected only α$_2$δ-1 in pure cerebral artery myocytes from 12-week–old WKY rats and hypertensive SHRs (Figure 1A). In contrast, the same primers amplified transcripts for all of the α$_2$δ isoforms in WKY and SHR whole brain (Figure 1A).

Quantitative PCR was performed to compare α$_2$δ-1 and Ca$_{1.2}$α$_1$ message levels in 6- and 12-week–old WKY and SHR cerebral arteries. Eight different reference genes were screened to identify those with similar mRNA levels in cerebral arteries of WKY rats and SHRs (Table S1, available in the online-only Data Supplement). Rps5 mRNA levels were similar in WKY and SHR arteries and, thus, Rps5 was used as the reference gene for these experiments (Table S2). Quantitative PCR indicated that mean α$_2$δ-1 and Ca$_{1.2}$α$_1$ mRNA levels were similar in 6-week–old WKY and SHR arteries (Figure 1B). In contrast, α$_2$δ-1 and Ca$_{1.2}$ mRNAs were ≈2.1- and 1.5-fold higher, respectively, in 12-week–old SHR compared with WKY arteries (Figure 1B).
Age-dependent development of hypertension was also associated with a larger increase in α,δ-1 than CaV1.2α, mRNA (Figure 1B). These data indicate that hypertension is associated with an elevation in α,δ-1 and CaV1.2α, subunit mRNA and protein, but not with the appearance of other α,δ isoforms in arterial myocytes.

Next, we investigated whether age-dependent development of genetic hypertension is associated with upregulation of α,δ-1 and CaV1.2α, proteins in cerebral arteries. α,δ-1 and CaV1.2α, protein levels were similar in 6-week-old WKY and SHR arteries (Figure 1C and 1D). Aging between 6 and 12 weeks did not alter α,δ-1 and CaV1.2α, protein in WKY rat arteries, but increased these proteins in SHR arteries (Figure 1C and 1D). At 12 weeks of age, α,δ-1 and CaV1.2α, proteins were more than 2.5- and 1.7-fold higher in SHR compared with age-matched WKY arteries (Figure 1C and 1D). In agreement with message levels, age-dependent development of hypertension also increased α,δ-1 more than CaV1.2α, protein (Figures 1C, 1D, and S2).

In summary, these data indicate that genetic hypertension is associated with transcriptional upregulation of both α,δ-1 and CaV1.2α, in cerebral artery myocytes. α,δ-1 and CaV1.2α, proteins are elevated more than their respective mRNAs (Figures 1B through 1D and S2), suggesting that hypertension-associated changes in posttranslational events also contribute to increased CaV1.2 channel subunit expression during hypertension. Furthermore, during hypertension there is a larger increase in mRNA and protein for α,δ-1 than for CaV1.2α,.

Hypertension Is Associated With an Elevation in Surface α,δ-1 and CaV1.2α, Proteins In Arteries

α,δ-1 induces membrane trafficking of CaV1.2α, subunits in SD rat arterial myocytes. Therefore, we tested the hypothesis that an increase in α,δ-1 contributes to elevated surface CaV1.2 expression in hypertension. Surface (plasma membrane) and intracellular α,δ-1 and CaV1.2α, proteins were measured in age-matched WKY and hypertensive SHR cerebral arteries using biotinylation. Surface-localized α,δ-1 and CaV1.2α, proteins were 2.6- and 2-fold higher, respectively, in SHR compared with WKY rat arteries (Figure 2A and 2B). A larger percentage of total α,δ-1 and CaV1.2α, was located at the plasma membrane in SHR compared with WKY arteries (Figure 2A and 2C). In WKY arteries, more of the total amount of α,δ-1 (≈85%) than CaV1.2 (≈77%) was located at the surface. In contrast, in SHR arteries, the percentages of total α,δ-1 (≈93%) and CaV1.2 (≈92%) located at the surface were similar (Figure 2A and 2C). These data indicate that, during hypertension, an elevation in α,δ-1 and CaV1.2α, total protein translates to an increase in surface expression of these subunits in arterial myocytes. Furthermore, hypertension is associated with an alteration in the distribution of α,δ-1 and CaV1.2α, proteins between intracellular and surface compartments.

Pregabalin Reduces Surface Trafficking of CaV1.2 Channel Subunits More Effectively in Hypertensive Than Normotensive Rat Arteries

Pregabalin, an α,δ-1/2 ligand, reduces surface trafficking of CaV1.2, 2.1, and 2.2 channels in neurons and arterial myocytes. Next, we studied pregabalin regulation of α,δ-1 and CaV1.2α, subunit surface expression and subunit cellular distribution in WKY and SHR cerebral arteries. For these experiments, arteries were incubated for 24 hours with or without pregabalin. Pregabalin (24 hours) did not alter total protein of α,δ-1 (WKY, 115±9; SHR, 118±20) or CaV1.2α (WKY, 116±10; SHR, 109±14;
Figure 3A; WKY, n=4–5; SHR, n=5; *P>0.05 for each). In contrast, pregabalin reduced surface αδ-1 and Ca_{1.2}δ subunits and increased intracellular levels of these proteins in both WKY and SHR arteries (Figure 3A through 3C and Figure S3). Pregabalin reduced plasma membrane αδ-1 and Ca_{1.2}δ subunits, αδ-1/Ca_{1.2}δ ≈ 3.1 and 1.9-fold more, respectively, in hypertensive SHR compared with WKY control arteries (Figure 3C). To evaluate pregabalin regulation of αδ-1 and Ca_{1.2} cellular distribution, surface:intracellular protein ratios were calculated. Consistent with data shown in Figure 2C, a larger proportion of αδ-1 and Ca_{1.2} subunits were present at the plasma membrane in SHR compared with WKY arteries (Figure 3D). Pregabalin induced a larger reduction in surface intracellular αδ-1 and Ca_{1.2} in SHR compared with WKY arteries (Figure 3D).

Hypertension was associated with a larger increase in surface αδ-1 than Ca_{1.2}α, protein in arteries (Figure 2A and 2B). We calculated the band intensity ratio of surface αδ-1: Ca_{1.2}α and regulation by pregabalin. Although this methodology cannot determine subunit stoichiometry, total protein loaded in each lane is identical, allowing for comparison of this ratio in SHR and WKY arteries from the same blot. The mean surface αδ-1/Ca_{1.2}α band intensity ratio was ≈ 1.38 in SHR arteries and ≈ 1.06 in WKY arteries, or ≈ 1.3-fold higher in SHR (Figure 3E). Pregabalin reduced the surface αδ-1/Ca_{1.2}α band intensity ratio to ≈ 0.91 in SHR arteries but did not change the ratio in WKY rat arteries (Figure 3E).

Collectively, these data indicate that pregabalin blocks surface expression of αδ-1 and Ca_{1.2}δ subunits more...
effectively in hypertensive than in normotensive rat arteries.

During hypertension, surface $\alpha_2\delta$-1 protein is elevated more than $\alpha_1.2\delta$ α protein, leading to an increase in the ratio of plasma membrane $\alpha_2\delta$:$\alpha_1.2\delta$ α subunits. Pregabalin reverses this elevation in surface $\alpha_2\delta$:$\alpha_1.2\delta$ α subunits. These data also indicate that $\alpha_2\delta$-1 is essential for upregulation of surface $\alpha_1.2$ channels in arterial myocytes during genetic hypertension.

$\alpha_2\delta$-1 Targeting Reverses Hypertension-Associated Modifications in CaV1.2 Current Density and Inactivation in Arterial Myocytes

To investigate the functional impact of elevated $\alpha_2\delta$-1 expression and effects of $\alpha_2\delta$-1 targeting, CaV1.2 currents were measured in age-matched WKY and hypertensive SHR cerebral artery myocytes. Mean peak CaV1.2 current density (Ba2+ as charge carrier) was $\sim$5.3 pA/pF in hypertensive SHR cells compared with $\sim$2.4 pA/pF in WKY cells or $\sim$2.2-fold larger (Figure 4A and 4B and Table). Pregabalin (24 hours) reduced peak CaV1.2 current density in SHR cells to $\sim$2.2 pA/pF, or by $\sim$59%, and to $\sim$1.6 pA/pF in WKY cells, or by $\sim$32% (Figure 4A and 4B and Table). Pregabalin reduced peak CaV1.2 current density in SHR myocytes to the current density of untreated WKY cells (Figure 4A and 4B and Table). The relationship between cell capacitance and peak CaV1.2 current was investigated (Figure 4C). When data were fit with a linear function, the slope was $-5.41$ for SHR cells and $-2.40$ for WKY cells, or 2.3-fold higher (Figure 4C). Pregabalin reduced slopes by $\sim$58% and 25% in SHR and WKY cells, respectively (Figure 4C). Slopes were similar for untreated WKY cells and pregabalin-treated SHR cells (Figure 4C; $P>0.05$). Mean cell capacitance for WKY (16.3 $\pm$ 0.8 pF) and SHR cells (14.8 $\pm$ 0.8 pF) were similar and were not altered by pregabalin (WKY, 18.8 $\pm$ 1 pF; SHR, 15.8 $\pm$ 0.8 pF; $P>0.05$ when comparing all), indicating that current density and slope increased because of changes in CaV1.2 channels (Figure 4B and 4C).

The voltage-dependence of half-maximal CaV1.2 current activation and slope were similar in untreated control and pregabalin-treated WKY and SHR arterial myocytes (Figure 4D and Table). The voltage dependence of half-maximal inactivation and slope were also similar in untreated control and pregabalin-treated WKY and SHR cells (Figure 4E and Table). In contrast, untreated SHR cells displayed a non-activating CaV1.2 current that was $\sim$2-fold larger than in WKY cells (Figure 4A and 4E). Pregabalin (24 hours) reduced the...
noninactivating current in SHR cells such that it was similar to WKY cells (Figure 4A and 4E). \( \alpha_{\delta} \) current inactivation rates (\( \tau \)) were similar in control and pregabalin-treated WKY and SHR cells (Figure S4).

In addition to acting as an inhibitor of \( \alpha_{\delta} \)-1-induced \( \alpha_{\delta} \) currents, pregabalin is a weak \( \alpha_{\delta} \) channel pore blocker that does not directly alter \( \alpha_{\delta} \) current voltage dependence in normotensive SD rat arterial myocytes. To determine whether the reduction in \( \alpha_{\delta} \) current amplitude in pregabalin-treated WKY and SHR myocytes was because of \( \alpha_{\delta} \) pore block, we measured \( \alpha_{\delta} \) current regulation in untreated cells by acute bath application of pregabalin. Acute pregabalin reduced \( \alpha_{\delta} \) currents in WKY cells by \( \approx 12\% \) (Figure 4F). In contrast, pregabalin reduced \( \alpha_{\delta} \) currents in SHR myocytes by \( \approx 23\% \), or \( \approx 1.9 \)-fold more than in WKY cells (Figure 4F). Acute pregabalin-induced \( \alpha_{\delta} \) current inhibition was significantly smaller than that induced by 24-hour pregabalin treatment in both WKY (\( \approx 32\% \) inhibition) and SHR (\( \approx 59\% \) inhibition) cells (Figure 4B, 4C, and 4F). When combined with the biochemical data illustrated in Figure 3, these data indicate that acute and chronic pregabalin inhibit \( \alpha_{\delta} \) currents through distinct mechanisms in arterial myocytes.

Collectively, data indicate that genetic hypertension is associated with an elevation in \( \alpha_{\delta} \)-1 expression that stimulates surface expression of \( \alpha_{\delta} \) subunits, leading to a \( \alpha_{\delta} \) current elevation and an increase in noninactivating current. \( \alpha_{\delta} \)-1 targeting reduces the hypertension-associated \( \alpha_{\delta} \)-1-induced elevation in \( \alpha_{\delta} \) surface expression, leading to a reduction in \( \alpha_{\delta} \) current density. \( \alpha_{\delta} \)-1 targeting also restores \( \alpha_{\delta} \) current inactivation.

### Table. Properties of Arterial Myocyte \( \alpha_{\delta} \)-1 Currents

<table>
<thead>
<tr>
<th>Variable</th>
<th>WKY</th>
<th>WKY + Pregabalin</th>
<th>SHR</th>
<th>SHR + Pregabalin</th>
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<tr>
<td>IV relationship</td>
<td></td>
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<tr>
<td>Peak current density (pA/pF)</td>
<td>2.40±0.15 (17)</td>
<td>1.65±0.13 (13)*</td>
<td>5.33±0.38 (16)*</td>
<td>2.23±0.14 (16)</td>
</tr>
<tr>
<td>Peak voltage (mV)</td>
<td>10.25±0.74 (17)</td>
<td>12.31±1.45 (13)</td>
<td>11.09±1.03 (16)</td>
<td>11.26±0.89 (18)</td>
</tr>
<tr>
<td>Voltage-dependent activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{1/2 \text{act}} ) (mV)</td>
<td>9.24±1.88 (12)</td>
<td>10.70±3.72 (9)</td>
<td>11.62±1.80 (12)</td>
<td>11.18±3.29 (5)</td>
</tr>
<tr>
<td>Slope</td>
<td>13.09±1.55 (12)</td>
<td>14.56±1.66 (9)</td>
<td>12.74±2.84 (12)</td>
<td>10.51±1.09 (5)</td>
</tr>
<tr>
<td>Voltage-dependent inactivation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{1/2 \text{inact}} ) (mV)</td>
<td>−14.23±1.57 (8)</td>
<td>−17.20±1.28 (13)</td>
<td>−11.33±1.90 (16)</td>
<td>−14.71±1.15 (11)</td>
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<tr>
<td>Slope</td>
<td>6.44±0.76 (8)</td>
<td>8.58±1.16 (13)</td>
<td>7.86±0.70 (16)</td>
<td>8.09±0.69 (11)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate experimental No. WKY indicates Wistar-Kyoto rat; SHR, spontaneously hypertensive rat; \( V_{1/2 \text{act}} \) half-maximal \( \alpha_{\delta} \) current activation.

*P<0.05 vs WKY.

#P<0.05 vs SHR+pregabalin.

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**Figure 5.** Pregabalin reverses elevated pressure-induced vasoconstriction in hypertension. A. Representative traces illustrating steadystate myogenic tone in response to increasing intravascular pressure in a Wistar-Kyoto (WKY) and spontaneously hypertensive rat (SHR) artery. **Horizontal black bars** indicate an increase in bath K+ from 6 to 60 mmol/L. B. Pregabalin (24 hours, 100 µmol/L) reduced pressure (20–100 mmHg)-induced myogenic tone (**filled symbols**) more so in arteries from hypertensive rats than in controls. Myogenic tone was abolished by nimodipine (1 µmol/L, **empty symbols**). Mean data (n: WKY, 6–10; WKY + pregabalin, 6–9; SHR, 6–10; SHR+pregabalin, 6–7). *P<0.05 vs untreated WKY; #P<0.05 for SHR+pregabalin vs untreated SHR.
Pregabalin reduced myogenic tone in WKY and SHR arteries, decreased tone more in SHR than in WKY arteries (eg, % reduction in myogenic tone at 60 mm Hg: SHR, ≈41%; WKY, ≈28%), and reduced tone in SHR arteries to levels in untreated WKY arteries (Figure 6). Nimodipine (1 µmol/L), a voltage-dependent Ca\(^{2+}\) channel blocker, fully dilated control and pregabalin-treated WKY and SHR arteries at all pressures (20–100 mm Hg) studied, indicating that myogenic tone occurred because of Ca\(_{\alpha1.2}\) channel activation (Figure 5B). Passive arterial diameters were similar for WKY (249±11 µm) and hypertensive SHR (242±9 µm) cerebral arteries (values given at 60 mm Hg; n=10 for each; P>0.05).

Elevating extracellular K\(^{+}\) induces depolarization, activation of voltage-gated Ca\(^{2+}\) channels, Ca\(^{2+}\) influx, and vasoconstriction.\(^{3}\) As an alternative approach to investigate the functional impact of α\(_{\delta-1}\)-targeting, we studied K\(^{+}\)-induced vasoconstriction in WKY and SHR arteries. Increasing extracellular K\(^{+}\) from 6 to 20, 40, or 60 mmol/L induced graded vasoconstriction that was larger in SHR than in WKY cerebral arteries (Figure 6). Pregabalin reduced K\(^{+}\)-induced vasoconstriction more in arteries from hypertensive rats than controls. Mean data (n: Wistar-Kyoto rat WKY, 6; WKY + pregabalin, 6; spontaneously hypertensive rat SHR, 6; SHR + pregabalin, 6). *P<0.05 vs untreated WKY; #P<0.05 for SHR + pregabalin vs untreated SHR.

Discussion

To date, no studies have investigated involvement of Ca\(_{\alpha1.2}\) channel auxiliary subunits in the pathological elevation of arterial myocyte Ca\(_{\alpha1.2}\) currents and vasoconstriction in hypertension. Here, we demonstrated for the first time that genetic hypertension is associated with transcriptional and posttranslational upregulation of α\(_{\delta-1}\) subunits in myocytes of resistance-size arteries. The additional α\(_{\delta-1}\) subunits increase surface trafficking of Ca\(_{\alpha1.2}\) subunits, which are also elevated in hypertension. The consequent increase in surface α\(_{\delta-1}\) and Ca\(_{\alpha1.2}\) proteins generates Ca\(_{\alpha1.2}\) current density and generates a nonactivating current, leading to vasoconstriction. We also demonstrated that α\(_{\alpha1.2}\)-targeting normalizes myocyte α\(_{\delta-1}\) and Ca\(_{\alpha1.2}\) surface expression, re-establishes Ca\(_{\alpha1.2}\) current density and inactivation, and reduces hypertensive rat artery contractility to levels in controls. These data indicate that α\(_{\delta-1}\) elevates Ca\(_{\alpha1.2}\) currents and Ca\(_{\alpha1.2}\)-dependent vasoconstriction during hypertension and demonstrate that α\(_{\delta-1}\)-targeting is a viable therapeutic strategy to reverse these pathological alterations and induce cerebrovascular dilation.

Our data indicate that the development of genetic hypertension is associated with a transcriptional and posttranslational increase in α\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\), in arterial myocytes. In contrast, other α\(_{\delta}\) isoforms did not emerge during hypertension, an alteration that could have contributed to pathological Ca\(_{\alpha1.2}\) current modifications. Previous studies have described that Ca\(_{\alpha1.2}\), mRNA and protein are higher in mesenteric arteries and aorta of hypertensive SHRs than WKY rat controls.\(^{21,22}\) In contrast, angiotensin II– and hypoxia-induced hypertension did not alter Ca\(_{\alpha1.2}\) mRNA but elevated Ca\(_{\alpha1.2}\) protein in cultured mesenteric arteries and neonatal piglet pulmonary arteries.\(^{23,24}\) These findings lead to the proposal that hypertension may not be associated with an increase in Ca\(_{\alpha1.2}\) message but posttranslational upregulation of Ca\(_{\alpha1.2}\) protein.\(^{21,24}\) Here, we used both age-dependent development of hypertension in SHRs and comparison with WKY rat controls to investigate relative changes in α\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\) mRNA and protein. Our data indicate that the increase in α\(_{\alpha1.2}\) (≈2.1-fold) and Ca\(_{\alpha1.2}\) (≈1.5-fold) mRNA cannot fully account for the elevation in α\(_{\alpha1.2}\) (≈2.5-fold) and Ca\(_{\alpha1.2}\) (≈1.7-fold) proteins during hypertension. These data indicate that both transcriptional and posttranslational mechanisms elevate α\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\) proteins in cerebral artery myocytes during hypertension.

Using a novel application of biotinylation, we recently determined the surface to intracellular distribution of arterial α\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\) proteins in normotensive rats.\(^{16}\) Essentially all (>95%) α\(_{\alpha1.2}\) and Ca\(_{\alpha1.2}\) proteins locate to the surface in cerebral artery myocytes of normotensive SD rats.\(^{16}\) Here, a smaller percentage of total α\(_{\alpha1.2}\) (≈85%) and
Gaba-pentin reduced surface expression of both αδ-1 and CaV1.2α protein and higher relative surface expression elevates plasma membrane levels of these proteins. Our data also indicate that there is a fractional shift in surface αδ-1:CaV1.2α during hypertension, a change that occurs because of a larger elevation in surface αδ-1 than in CaV1.2α. These results provide evidence that an elevation in αδ-1:CaV1.2α subunit ratio can modify native CaV1.2 current properties and that there may not be rigid αδ-1:CaV1.2α subunit stoichiometry in arterial myocytes. Also possible is that, in normotension, a proportion of arterial myocyte CaV1.2 channel complexes may not contain αδ-1 subunits. During hypertension, the higher elevation in surface αδ-1 than CaV1.2α may increase the proportion of channels that contain αδ-1. Future studies should be designed to further investigate native CaV1.2 channel stoichiometry in arterial myocytes and changes that occur in cardiovascular disease. Collectively, these results indicate that αδ-1 increases surface expression and functionality of CaV1.2α subunits in arterial myocytes during hypertension.

Pregabalin is a gabapentinoid drug used to treat neuropathic pain, fibromyalgia, and epileptic seizures.14,20 Of all 4 αδ isoforms, only αδ-1 and -2 contain complete metal ion adhesion site and RRR motifs, which are required for gabapentinoid drug binding.14,20 Gabapentin reduced αδ subunit recycling from Rab11–positive recycling endosomes.17 Pregabalin also reduced surface expression of both αδ-1 and CaV1.2α proteins in cerebral artery myocytes of normotensive SD rats.16 Here, pregabalin did not alter total αδ-1 or CaV1.2α protein. Rather, pregabalin reduced surface αδ-1 and CaV1.2α more in hypertensive arteries than in control rat arteries, essentially normalizing surface levels of these proteins compared with those in WKY rats. Pregabalin also reduced the αδ-1:CaV1.2α subunit ratio in hypertensive rat arteries compared with that in WKY controls. Given that pregabalin normalized elevated CaV1.2α current density and the proportion of nonactivating current to those in WKY cells, our data indicate that upregulated αδ-1 functionality contributes to the increase in CaV1.2 currents in arterial myocytes in hypertension.

Voltage-dependent Ca2+ currents are elevated in myocytes from vasculature, including renal, cerebral, and mesenteric arteries, when studying a variety of different hypertension models, such as SHR, angiotensin II–induced, aortic banding, stroke-prone SHR, hypoxia-induced pulmonary hypertension, and Osborne-Mendel rats on a high fat diet.2,3,7,9,21,23,24,28–30 CaV1.2 current density measured here is consistent with that reported previously in cerebral artery myocytes when using WKY and SHR models.2,30 Our data indicate that CaV1.2 current density was ≈2.2-fold larger in hypertensive than in control rat arterial myocytes. In contrast, half-maximal CaV1.2 current activation and voltage dependence of half-maximal inactivation were similar in WKY and SHR cells, consistent with previous reports.2,9,28,30 A nonactivating CaV1.2 current in myocytes of hypertensive rats was double that in controls, a modification that would significantly increase Ca2+ influx at steady-state membrane potentials, thereby stimulating vasoconstriction. Pregabalin (24 hours) reduced elevated CaV1.2 current density and nonactivating current to levels in controls, suggesting that these pathological modifications occurred because of an increase in αδ-1 surface expression. Our data are consistent with pregabalin acting as both a weak CaV1.2 channel pore blocker and an effective chronic inhibitor of αδ-1 surface expression in hypertensive rat arterial myocytes. In our previous study, acute pregabalin reduced CaV1.2 currents by ≈33% in SD rat cerebral artery myocytes.16 Here, the same acute pregabalin concentration reduced CaV1.2 currents by ≈12% in WKY rat myocytes. Our previous study used 7-week–old SD rats, whereas here acute pregabalin effects were measured in 12-week–old WKY rat myocytes. Our data indicate that CaV1.2 channel properties in 12-week–old WKY myocytes are not identical to those in 7-week–old SD rat myocytes, including the percentage of CaV1.2α protein that is located at the surface. Data here indicate that acute pregabalin is a more effective inhibitor of CaV1.2 currents in SHR compared with WKY myocytes. This may be because of the higher number of surface αδ-1 subunits and the higher αδ-1:CaV1.2α ratio in SHR myocytes. Acute gabapentin also inhibited voltage-dependent Ca2+ currents in pyramidal neocortical cells but did not alter currents generated by recombiant CaV2.1 channels or endogenous Ca2+ channels in dorsal root ganglia neurons.31,32 In a model of neuropathic pain in which αδ-1 is upregulated, chronic pregabalin inhibited αδ-1 trafficking to presynaptic terminals, thereby inhibiting Ca2+ channel function.33 Our data indicate that chronic pregabalin inhibits αδ-1–induced trafficking of CaV1.2α channel subunits, thereby reducing CaV1.2α currents in arterial myocytes during hypertension.

Intravascular pressure and depolarization both stimulated a larger vasoconstriction in arteries of hypertensive rats than in controls. Consistent with our findings, pressure-induced Ca2+ influx and associated vasoconstriction were larger in arteries from animal models with both genetic and induced hypertension.2,7,21,23,24 We show that nimodipine abolished myogenic tone at all pressures, indicating that CaV1.2 channel activity was essential to generate tone in hypertensive and control rat arteries. Chronic pregabalin (24 hours) was a more effective vasodilator of hypertensive than control rat arteries, effectively reversing the pathological vasoconstriction. Pregabalin is also a weak CaV1.2 channel pore blocker, which induces a small vasodilation.16 Thus, pregabalin-induced CaV1.2 pore block may also have contributed to vasodilation in both WKY and SHR arteries. Although unlikely, pregabalin could have caused vasodilation through additional mechanisms, including through inducing membrane hyperpolarization. Our data are inconsistent with this possibility, because pregabalin similarly reduced surface CaV1.2 subunits, CaV1.2 currents, myogenic tone, and K+-induced constriction, and inhibitions of
pressure- and depolarization-induced vasoconstriction were equivalent. Thus, data demonstrate that pregabalin dilates hypertensive rat arteries primarily by reducing surface expression of Ca\textsubscript{\textalpha}1,2 subunits in myocytes.

Hypertension is associated with increased risk for cerebral diseases, including stroke, Alzheimer disease, and dementia. Cerebral blood flow is reduced in hypertensive humans and 12-week-old SHRs when compared with normotensive controls.\textsuperscript{33,34} Voltage-dependent Ca\textsuperscript{2+} channel blockers have been used for >2 decades to treat hypertension.\textsuperscript{35} However, Ca\textsuperscript{2+} channel blockers inhibit Ca\textsubscript{\textalpha}1,2 channels in multiple cell types in vivo and induce multiple adverse effects, including sweating, edema, and nausea.\textsuperscript{35,36} Therefore, the development of alternative approaches to target Ca\textsubscript{\textalpha}1,2 channels in arterial myocytes could provide significant benefits over current inhibitors. Here we used pregabalin as an in vitro tool to test the concept that \(\textalpha\textdelta\)-targeting induces vasodilation in cerebral arteries of hypertensive animals. Data here provide a foundation for future studies aimed at developing novel approaches to target \(\textalpha\textdelta\)-1 in arterial myocytes. All of the data in our study were obtained by studying cerebral arteries that regulate brain regional blood flow but do not control systemic blood pressure. Clinical pregabalin does not appear to modify systemic blood pressure in normotensive humans at doses used to treat neuropathic pain, fibromyalgia, and epileptic seizures.\textsuperscript{26} There are several explanations for this observation. First, there are a large number of distinct mechanisms that control cerebral and systemic artery contractility. To date, no studies have examined the molecular identity or physiological functions of \(\textalpha\textdelta\) subunits in systemic artery myocytes that regulate diastolic and systolic blood pressures. \(\textalpha\textdelta\)-1 may not be the principal \(\textalpha\textdelta\) isof orm or \(\textalpha\textdelta\) subunits may not regulate Ca\textsubscript{\textalpha}1,2 channel activity in systemic artery myocytes. Pregabalin is an \(\textalpha\textdelta\)-1/2 ligand. If \(\textalpha\textdelta\)-1 or \(\textalpha\textdelta\)-2 is not expressed or does not regulate Ca\textsubscript{\textalpha}1,2 channels in systemic artery myocytes, pregabalin should not induce systemic vasodilation or alter blood pressure. Second, clinical doses of pregabalin that are used to treat neuropathic pain, fibromyalgia, and epileptic seizures may be insufficient to induce vasodilation in vivo. Gabapentin, a lower affinity pregabalin analog, enters cells through system-L neutral amino acid transporters.\textsuperscript{32} Arterial myocytes may not uptake pregabalin as effectively as neurons. In vivo, intracellular myocyte pregabalin concentrations may be less than those obtained in vitro that alter Ca\textsubscript{\textalpha}1,2 function. Third, many in vivo mechanisms, including those mediated by baroreceptors or the renin-angiotensin system, may compensate for pregabalin-induced systemic vasodilation, leading to net change in blood pressure. Fourth, our data indicate that pregabalin is more effective at inhibiting Ca\textsubscript{\textalpha}1,2\textalpha\textgamma subunit trafficking in cerebral artery myocytes of hypertensive compared with normotensive rats. In vivo, pregabalin may be a more effective vasodilator in hypertensive subjects and have a smaller effect in normotensive subjects in which clinical systemic blood pressure measurements have been obtained. Our study provides the first evidence that arterial myocyte \(\textalpha\textdelta\)-1 functionality is upregulated in hypertension and that \(\textalpha\textdelta\)-1 targeting is a novel approach for reducing pathological vasoconstriction in hypertension. Data also indicate that \(\textalpha\textdelta\)-1 targeting can modify cerebral artery contractility, setting the stage for future studies to use a variety of other \(\textalpha\textdelta\)-1 targeting strategies, including RNA interference and genetic models, to investigate physiological and pathological involvement of \(\textalpha\textdelta\) subunits in arteries of other vascular beds and in vivo.

In summary, we identify for the first time that a hypertension-associated increase in \(\textalpha\textdelta\)-1 elevates Ca\textsubscript{1,2\textalpha} surface expression in arterial myocytes leading to pressure- and depolarization-induced vasoconstriction. Our data also indicate that \(\textalpha\textdelta\)-1 targeting is a novel approach to reverse elevated Ca\textsubscript{1,2} channel surface expression in arterial myocytes and vasoconstriction in hypertension.

Perspectives

A hallmark of hypertension is an increase in voltage-dependent Ca\textsubscript{1,2} currents in arterial myocytes that induces vasoconstriction.\textsuperscript{1–3} Molecular mechanisms that elevate arterial myocyte Ca\textsubscript{1,2} currents in hypertension and the significance of auxiliary subunits in this pathological alteration are unclear. We show that the development of genetic hypertension is associated with a transcriptional and posttranslational upregulation of \(\textalpha\textdelta\)-1 subunits in cerebral artery myocytes. This increase in \(\textalpha\textdelta\)-1 subunits elevates Ca\textsubscript{1,2} channel surface expression, Ca\textsubscript{1,2} current density, and vasoconstriction. \(\textalpha\textdelta\)-1 targeting using pregabalin, an \(\textalpha\textdelta\)-1 ligand, reduced \(\textalpha\textdelta\)-1 and Ca\textsubscript{1,2} surface expression and Ca\textsubscript{1,2} current density in myocytes. Pregabalin also dilated cerebral arteries of hypertensive rats. Our study provides the first evidence that \(\textalpha\textdelta\)-1 subunits are upregulated in cerebral artery myocytes during hypertension and contribute to the pathological elevation in myocyte Ca\textsubscript{1,2} currents and vasoconstriction. We also identified \(\textalpha\textdelta\)-1 as a potential novel therapeutic target for inducing cerebrovascular dilation in hypertension.

Acknowledgment

We thank Dr Marie Dennis Leo for critical reading of the article.

Sources of Funding

This work was supported by National Institutes of Health grants to J.H.J.

Disclosures

None.

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Novelty and Significance

**What Is New?**

- We demonstrate for the first time that genetic hypertension is associated with a transcriptional and posttranslational upregulation of α,δ-1 subunits in myocytes of resistance-sized cerebral arteries that increase Ca2,1.2 subunit surface trafficking, thereby elevating Ca2,1.2 current density and arterial contractility.

- Pharmacological targeting of α,δ-1 inhibits the pathological increase in Ca2,1.2 current density and cerebral artery contractility during hypertension. This study identifies α,δ-1 as a novel therapeutic target for inducing cerebrovasoconstriction in hypertension.

**What Is Relevant?**

- Upregulation of α,δ-1 subunits is essential for the elevation in Ca2,1.2 current density and cerebrovascular tone in genetic hypertension.

**Summary**

Upregulation of α,δ-1 subunits during genetic hypertension increases Ca2,1.2 channel surface expression and Ca2,1.2 current density, leading to vasoconstriction. α,δ-1 targeting reverses this pathological increase in Ca2,1.2 channel surface expression, Ca2,1.2 current density, and contractility in cerebral arteries.
Transcriptional Upregulation of \(\alpha_2\beta_1\) Elevates Arterial Smooth Muscle Cell Voltage-Dependent Ca\(^{2+}\) Channel Surface Expression and Cerebrovascular Constriction in Genetic Hypertension

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_Hypertension_. published online September 4, 2012;
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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http://hyper.ahajournals.org/content/early/2012/09/04/HYPERTENSIONAHA.112.199661

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TRANSCRIPTIONAL UPREGULATION OF α₂δ-1 ELEVATES ARTERIAL SMOOTH MUSCLE CELL CAᵥ1.2 CHANNEL SURFACE EXPRESSION AND CEREBROVASCULAR CONSTRICTION IN GENETIC HYPERTENSION

by

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Short Title: α₂δ-induced vasoconstriction in hypertension

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Supplemental Methods

Cell isolation and tissue preparation
All animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Male 6 or 12 week old SHR and Wistar Kyoto (WKY) rats were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/Kg body weight). The brain was removed and placed in oxygenated (21 % O2/5 % CO2) physiological saline solution (PSS) containing (in mmol/L): KCl 6, NaCl 112, NaHCO3 24, MgSO4 1.2, KH2PO4 1.2, CaCl2 1.8, and glucose 10. Middle cerebral, posterior cerebral and cerebellar arteries (~100-200 µm diameter) were dissected from the brain.

Blood pressure measurements
Diastolic, systolic, and mean arterial blood pressures were measured in 6 and 12 week old WKY and SHR rats using a CODA 2 tail cuff system (Kent Scientific, Torrington, Conn). Rats were acclimatized to the chamber and tail cuff on a heated platform (37°C) for 3 days prior to acquisition of blood pressure data. On the day of data acquisition, rats were again acclimatized for 30 min before blood pressure measurements were acquired. For each rat, 10-15 acclimatization cycles were run and data taken as the mean of 5-9 measurements.

RT-PCR
Total RNA was isolated from brain and cerebral arteries using Trizol (Invitrogen) and from isolated smooth muscle cells using the Absolutely RNA nanoprep kit (Strategene). RT-PCR was performed using α2δ isoform-specific primers, as previously described.1 Standard PCR reactions consisting of a 2 min heat shock (94°C) and 40 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 1 min), were used for all reactions.

Quantitative real time PCR
Quantitative Taqman PCR reactions were performed using an LC480 light cycler (Roche Applied Science). Reaction conditions were an initial denaturation step at 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 10 s. Negative control without cDNA was run for each reaction. Standard curves using four 10-fold dilutions of cDNA were run for all probe and primer pairs to determine PCR efficiency. α2δ-1 or CaV1.2α1 mRNA expression was calculated from the difference between fluorescence (Ct) values (ΔCt) of α2δ-1 or CaV1.2α1 and Rps5. ΔΔCt was calculated from the difference between the ΔCt values for SHR and WKY. α2δ-1 or CaV1.2α1 mRNA levels in SHR compared to WKY were calculated using the formula 100 x 2(-ΔΔCt).2 All PCR reactions including standard curves were performed in triplicate. Gene specific primers and probes were designed using the Universal Probe Library (UPL). The overall efficiency of each primer pair and probe for PCR reactions was calculated. Primer sequences, probes, gene accession numbers, amplicon length, and real-time PCR reaction efficiencies are provided in Table S1.

The following reference genes were screened: beta (β) actin, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), TATA box binding protein (TBP), ribosomal RNA 18s (18S rRNA), ribosomal subunit protein 16 (Rps16), platelet/endothelial cell adhesion molecule 1 (Pecam1), cyclophilin B and Rps5. Rps5 was identified as the most suitable reference gene for this study after comparing both the fold change in mRNA levels in SHR compared to WKY and the coefficient of variation (expressed as %) in Ct values of WKY and SHR samples (Table S2). For each reference gene, coefficient of variation of the Ct values in all (SHR and WKY) samples was calculated using the following equation:

\[ \text{Coefficient of variation} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100 \]
**Protein analysis and biochemistry**

Arteries were homogenized in 1x Laemmli buffer supplemented with 2 % β-mercaptoethanol. Cellular debris was removed by centrifugation. The method of Henkel et al. was used to determine protein concentration. Proteins were separated on 7.5 % SDS-PAGE gels and analyzed by Western blotting. Blots were cut at the 75 kDa marker to allow simultaneous probing of higher molecular mass (>75 kDa) proteins for αδ-1 and lower mass proteins (<75 kDa) for actin. The higher molecular mass blot was stripped and re-probed for Cav1.2α1. Antibodies used were anti-αδ-1 (Aviva Systems Biology), anti-Cav1.2α1 (Neuromab) and anti-actin (Millipore). Bands on Western blots were visualized using HRP conjugated secondary antibodies and a West Pico Chemiluminescence kit (Pierce), using a Kodak Image F-Pro system. Protein band intensities were determined using Quantity One (BioRad) software. For quantification, protein band intensities were first normalized to actin and then to appropriate control samples.

**Artery surface biotinylation**

Intact, cleaned arteries were incubated for 1 h in a mixture of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Pierce, 1 mg/ml each) in phosphate-buffered saline (PBS, Invitrogen). Unbound biotin was removed by quenching with PBS supplemented with 100 mmol/L glycine and washing with PBS. For determination of protein concentration, arteries were homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. Protein concentration was determined using the method of Henkel et al. Equal amounts of biotinylated arterial lysate for each sample were then used for Avidin (Monomeric Avidin, Pierce) pulldown of biotinylated proteins. Following pulldown, the supernatant comprised the intracellular proteins and biotinylated surface proteins remained bound to Avidin. Following removal of the supernatant (intracellular fraction), biotinylated surface proteins were eluted from Avidin beads by boiling in 1x Laemmli buffer supplemented with 2 % β-mercaptoethanol. The entire supernatant comprising the intracellular and the entire eluate comprising the surface protein fraction from the pulldown, were separated on SDS-PAGE gels and analyzed by Western blotting. Band intensities were determined using Quantity One software (BioRad). Total protein was calculated as the sum of surface and intracellular band intensities. To determine the proportion of a protein located at the plasma membrane, surface band intensity was divided by total. Alternatively, the surface:intracellular ratio of a protein was calculated by dividing surface band intensity by intracellular band intensity. Pregabalin-induced changes in protein cellular distribution were calculated by normalizing to control protein distribution. The ratio of surface αδ-1 to Cav1.2α1 was calculated by dividing surface αδ-1 by surface Cav1.2α1 band intensity from the same blots.

**Patch-clamp electrophysiology**

Cav1.2 currents were recorded in isolated smooth muscle cells using the whole cell patch-clamp configuration at room temperature using an Axopatch 200B amplifier (Axon Instruments). Borosilicate glass electrodes (4-5 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 10, NMDG 130, MgCl2 1, HEPES 10 and glucose 10 (pH 7.4 adjusted using L-Aspartic acid). Cell capacitance was measured by applying a 5 mV test pulse and correcting transients with series resistance compensation. Pregabalin (100 µmol/L) was present in the bath solution during recordings for chronically-treated cells, and applied acutely to the bath otherwise.

To measure whole-cell Cav1.2 currents and steady-state inactivation, 1 s conditioning pulses to between -80 and +60 mV were applied in 10 mV increments prior to a 200 ms test pulse to 0 mV. Current-voltage (IV) relationships were generated from the peak current obtained during the 1 s conditioning pulses. The rate of current inactivation was calculated from the current decay during each 1 s conditioning pulse. Steady state inactivation was determined from the current generated during the 200
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ms test pulse to 0 mV. To measure steady state activation, tail currents were elicited by repolarization to -80 mV from 20 ms test pulses from -60 to +60 mV in 10 mV increments. To measure the time course and magnitude of acute pregabalin (100 µmol/L) inhibition, CaV1.2 currents were elicited by repetitive 300 ms steps to 10 mV from -80 mV every 30 s. Whole cell currents were filtered at 1 or 5 kHz and digitized at 5 or 20 kHz for the inactivation and activation protocols, respectively. P/-4 protocols were used to subtract leak and capacitive transients.

Scatter plot data points were fit with a linear function: y = mx, where m is the slope, r is the strength of the linear relationship between the x and y variables, and p is the probability of the correlation co-efficient, with P<0.05 significant. Steady state inactivation and activation curves were fit with a single power Boltzmann function: I/I_{MAX} = R_{in} + (R_{MAX} - R_{in})/(1 + exp((V - V_{1/2})/k)), where I/I_{MAX} is the normalized peak current, V is the conditioning pre-pulse voltage, V_{1/2} is the voltage for half-inactivation or half-activation, k is the slope factor, R_{in} is the proportion of non-inactivating current and R_{MAX} is the maximal current amplitude. Inactivation kinetics data were fit with a single exponential function: I_t = (A x e^{(-t/τ)}) + I_0, where I_t is the inward current at time t, A the amplitude and I_0 the residual current.

**Pressurized artery myography**

The endothelium was denuded by introduction of an air bubble into the artery lumen for ~1 min followed by PSS. Cerebral artery segments were cannulated at each end in a perfusion chamber (Living Systems Instrumentation) that was maintained at 37°C and continuously perfused with PSS. Intravascular pressure was monitored using a pressure transducer and altered using an attached reservoir. Wall diameter was measured using a charge-coupled device camera and the edge detection function of IonWizard (Ionoptix), at 1 Hz. Arteries treated with pregabalin for 24 h were maintained in pregabalin throughout these experiments to inhibit CaV1.2 subunit membrane re-insertion. Myogenic tone (%) was calculated as: 100 x (1 – D_{active}/D_{passive}), where D_{active} is active arterial diameter and D_{passive} is the passive arterial diameter determined by applying Ca^{2+}-free PSS supplemented with 5 mM EGTA.

**Chemicals**

Pregabalin was a gift from Pfizer Inc (Groton, Conn) or was purchased from Sigma Aldrich (Milwaukee, WI).
References


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</table>

**Table S1**
Gene specific oligonucleotide sequences for primers, probes, amplicon length, and reaction efficiencies of quantitative PCR experiments.
<table>
<thead>
<tr>
<th>Reference Gene</th>
<th>Fold difference in mRNA levels (SHR/WKY)</th>
<th>Coefficient of variation in Ct values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>8.51 ± 0.37 (3)</td>
<td>6.02</td>
</tr>
<tr>
<td>β-actin</td>
<td>3.77 ± 0.27 (3)</td>
<td>3.35</td>
</tr>
<tr>
<td>TBP</td>
<td>4.70 ± 3.2 (3)</td>
<td>4.15</td>
</tr>
<tr>
<td>HGPRT</td>
<td>2.96 ± 0.26 (3)</td>
<td>2.61</td>
</tr>
<tr>
<td>Pecam1</td>
<td>2.27 ± 0.19 (3)</td>
<td>2.24</td>
</tr>
<tr>
<td>Rps16</td>
<td>4.39 ± 0.07 (3)</td>
<td>4.41</td>
</tr>
<tr>
<td>Rps5</td>
<td>1.14 ± 0.10 (3)</td>
<td>0.37</td>
</tr>
<tr>
<td>Cyclophilin B</td>
<td>1.88 ± 0.22 (3)</td>
<td>1.88</td>
</tr>
</tbody>
</table>

**Table S2**
Comparison of mRNA levels for eight potential reference genes in 12 week old WKY and SHR cerebral arteries. Experimental number is given in parentheses.
Figure S1. Diastolic, systolic, and mean arterial blood pressures are elevated more so in 12 week hypertensive SHR than age-matched WKY rats. A-C, Bar graphs showing mean diastolic pressures (A), mean systolic pressures (B), and mean arterial pressures (C) obtained from 6 week WKY, 6 week SHR, 12 week WKY and 12 week SHR rats by tail cuff. N numbers were as follows: 6 week WKY (n=10), 6 week SHR (n=6), 12 week WKY (n=10), 12 week SHR (n=11). * indicates P<0.05 when compared to 6 week WKY, # indicates P<0.05 when compared to 6 week SHR and § indicates P<0.05 when compared to 12 week WKY.
Figure S2. $\alpha_\delta$-1 and $\text{Ca}_{v1.2}\alpha_1$ subunit protein are elevated during development of hypertension. Mean data illustrating that $\alpha_\delta$-1 and $\text{Ca}_{v1.2}\alpha_1$ subunit protein increase with age in SHR but not in WKY cerebral arteries. * indicates $P<0.05$ when compared with WKY. # indicates $P<0.05$ when compared with $\text{Ca}_{v1.2}\alpha_1$ in SHR (n=8 for each protein at each age).
Figure S3. A longer time exposure of the intracellular lanes of the blot in Figure 3B illustrating that pregabalin increased intracellular $\alpha\delta$-1 and $\text{Ca}_{y}1.2\alpha_{1}$ in WKY and SHR cerebral arteries.
**Figure S4.** Pregabalin (100 μmol/L) does not alter the rate of Ca_{1.2} current inactivation in WKY or SHR cells. Graph showing that the rate of current inactivation (τ) was similar in untreated WKY (n=17), pregabalin-treated WKY (n=13), untreated SHR (n=16) and pregabalin-treated SHR (n=18) cells.