Ca\(_{\alpha 1.2}\) splice variant with exon 9* is critical for regulation of cerebral artery diameter

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Nystoriak MA, Murakami K, Penar PL, Wellman GC. Ca\(_{\alpha 1.2}\) splice variant with exon 9* is critical for regulation of cerebral artery diameter. Am J Physiol Heart Circ Physiol 297: H1820–H1828, 2009.—L-type voltage-dependent Ca\(^{2+}\) channels (VDCCs) are essential for numerous processes in the cardiovascular and nervous systems. Alternative splicing modulates proteomic composition of Ca\(_{\alpha 1.2}\) to generate functional variation between channel isoforms. Here, we describe expression and function of Ca\(_{\alpha 1.2}\) channels containing alternatively spliced exon 9* in cerebral artery myocytes. RT-PCR showed expression of Ca\(_{\alpha 1.2}\) splice variants both containing (α\(_1\)C\(_{9/9*/10}\)) and lacking (α\(_1\)C\(_{9/10}\)) exon 9* in intact rabbit and human cerebral arteries. With the use of laser capture microdissection and RT-PCR, expression of mRNA for both α\(_1\)C\(_{9/9*/10}\) and α\(_1\)C\(_{9/10}\) was demonstrated in isolated cerebral artery myocytes. Quantitative real-time PCR revealed significantly greater α\(_1\)C\(_{9/9*/10}\) expression relative to α\(_1\)C\(_{9/10}\) in intact rabbit cerebral arteries compared with cardiac tissue and cerebral cortex. To demonstrate a functional role for α\(_1\)C\(_{9/9*/10}\), smooth muscle of intact cerebral arteries was treated with antisense oligonucleotides targeting α\(_1\)C\(_{9/9*/10}\) (α\(_1\)C\(_{9/9*/10-AS}\)) or exon 9 (α\(_1\)C\(_{9-AS}\)), expressed in all Ca\(_{\alpha 1.2}\) splice variants, by reversible permeabilization and organ cultured for 1–4 days. Treatment with α\(_1\)C\(_{9/9*/10-AS}\) reduced maximal constriction induced by elevated extracellular K\(^+\) ([K\(^+\)]\(_o\)) by ~75% compared with α\(_1\)C\(_{9/9*/10}\)-sense-treated arteries. Maximal constriction in response to the Ca\(^{2+}\) ionophore ionomycin and [K\(^+\)]\(_o\) EC\(_{50}\) values were not altered by antisense treatment. Decreases in maximal [K\(^+\)]\(_o\)-induced constriction were similar between α\(_1\)C\(_{9/9*/10-AS}\) and α\(_1\)C\(_{9-AS}\) groups (22.7 ± 9% and 25.6 ± 4% constriction, respectively). We conclude that although cerebral artery myocytes express both α\(_1\)C\(_{9/9*/10}\) and α\(_1\)C\(_{9/10}\) VDCC splice variants, α\(_1\)C\(_{9/9*/10}\) is functionally dominant in the control of cerebral artery diameter.

vascular smooth muscle; calcium channels; cerebral blood flow

L-TYPE VOLTAGE-DEPENDENT Ca\(^{2+}\) channels (VDCCs) play a crucial role in the physiological processes of numerous cell types. In the resistance circulation, arterial constriction is dependent upon membrane potential depolarization and Ca\(^{2+}\) entry via Ca\(_{\alpha 1.2}\) channels in vascular smooth muscle (15, 21). An increase in global cytosolic Ca\(^{2+}\) leads to Ca\(^{2+}\)-calmodulin-dependent activation of myosin light chain kinase, myosin light chain phosphorylation, increased actin-myosin interaction, smooth muscle contraction, and decreased vessel diameter. This mechanism is essential for proper regulation of organ perfusion and systemic blood pressure.

VDCCs are multimeric protein complexes composed of an α\(_1\)-pore-forming subunit associated with β- and α\(_{\delta}\)-auxiliary subunits (5, 8, 24). L-type VDCC currents are distinguished by high activation potentials, slow inactivation of barium currents, and selective inhibition by dihydropyridines (DHPs), phenylalkylamines, and benzoalderazines (5). The Ca\(_{\alpha 1.2}\) gene CACNA1C consists of 55 exons, 19 of which are subject to extensive alternative splicing with 40 splice variations found at 12 loci (34). cDNA library screening studies have allowed the identification of the cardiac and smooth muscle Ca\(_{\alpha 1.2}\) isoforms, differing in composition at four alternative splice sites (2, 22, 28, 31). The purported smooth muscle splice combination consists of exons 1/8/ +9*/32, whereas the cardiac form consists of exons 1a/8a/ +9*/31. Smooth muscle L-type channels are reported to activate at more hyperpolarized (~15 mV) membrane potentials (14, 30) and display greater DHP sensitivity than analogous channels in the heart (35). A previous study suggests that the presence of exon 8 rather than 8a to form transmembrane segment 6 of domain I in smooth muscle channels contributes to differences in DHP inhibition (36). Other work has shown that the inclusion of the 25 amino acid insertion exon 9* in the intracellular linker region between homologous domains I and II affects channel gating properties resulting in a hyperpolarizing shift in activation potential and current-voltage relationship (26). The electrophysiological alteration imposed by the addition of exon 9* to the channel protein structure suggests that expression of exon 9* may be a critically important mechanism for the fine-tuning of channel function such that smooth muscle VDCCs activate at physiologically relevant membrane potentials. Although such a role for Ca\(_{\alpha 1.2}\) channels expressing exon 9* would be suitable for proper vascular function, the physiological significance of this splice variant in the regulation of blood vessel diameter has not been directly investigated.

Here, the objective was to determine the role of the exon 9* Ca\(_{\alpha 1.2}\) splice variant in constriction of resistance size cerebral arteries. Consistent with previous findings by others (3, 13, 26), we provide evidence for exon 9* expression in cerebral arteries and further show a significantly higher ratio of exon 9* mRNA relative to total Ca\(_{\alpha 1.2}\) mRNA in cerebral arteries compared with cerebral cortex and cardiac tissue. RT-PCR performed on cDNA obtained from myocytes isolated by laser-capture microdissection found expression of both splice variants in cerebral artery smooth muscle. Antisense oligodeoxynucleotides were used to selectively suppress α\(_1\)C\(_{9/9*/10}\) in cerebral artery smooth muscle to examine the functional role for this splice variant in cerebral artery constriction. Our findings indicate that despite heterogeneous mRNA expression of both α\(_1\)C\(_{9/9*/10}\) and α\(_1\)C\(_{9/10}\) isoforms by cerebral artery myocytes, α\(_1\)C\(_{9/9*/10}\) channels play a dominant role in constriction of these vessels.
EXON 9* Ca,1.2 regulates cerebral artery constriction

H1821

METHODS

Animals. New Zealand White rabbits (males, 3.0–3.5 kg) were used in this study. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication 85-23, Revised 1996] and followed protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. Animals were euthanized under deep pentobarbital anesthesia (150 mg/kg iv) by exsanguination and decapitation. Posterior cerebral and cerebellar arteries were dissected in ice-cold physiological saline solution (PSS) of the following composition (in mM): 118.5 NaCl, 4.7 KCl, 24 NaHCO3, 1.18 KH2PO4, 2.25 CaCl2, 1.2 MgCl2, 0.023 EDTA, and 11 glucose, aerated with 5% CO2–20% O2–75% N2 (bath pH, 7.4). Cerebral artery myocytes (40–60 cells/sample) were collected from enzymatically dissociated freshly isolated posterior cerebral arteries (23, 37) using a PALM Laser Capture Microdissection system (Zeiss, Bernried, Germany). Human cerebral arteries, removed as a necessary part of a Department of Health and Human Services covering this activity (Assurance identification number: FWA723; IRB identification number 0485).

RT-PCR. Total RNA was extracted using RNA STAT-60 total RNA/mRNA isolation reagent (Tel-test, Friendswood, TX) (4). Total RNA was reverse transcribed to cDNA using SuperScript First-Strand synthesis system (Invitrogen, Carlsbad, CA). Semi-quantitative PCR was performed using primers detecting the region spanning exons 7–11 of Ca,1.2 (Genbank accession No. X55763; Fig. 1A) of the following sequences: forward 5'-TGCTTTGCGATGACTGGC-3' and reverse 5'-GATTTGCGATGGAGATCCGG-3'. Amplification was performed with Taq PCR core kit (Qiagen) using the following protocol: 94°C for 3 min; 35 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 1 min; and final extension at 72°C for 10 min. 

Table 1. Diameter values for [K+]o concentration-response experiments

<table>
<thead>
<tr>
<th>Day</th>
<th>Diameter, μm</th>
<th>RP</th>
<th>αCa9p*10-Sense</th>
<th>αCa9p*10 Antisense</th>
</tr>
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<tr>
<td>Day 1</td>
<td>6 mM [K+]o</td>
<td>170±27.8</td>
<td>186±18.7</td>
<td>182±21.8</td>
</tr>
<tr>
<td></td>
<td>80 mM [K+]o</td>
<td>62±16.1</td>
<td>66±9.2</td>
<td>63±7.6</td>
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<tr>
<td></td>
<td>Ionomycin</td>
<td>37±13.1</td>
<td>37±7.5</td>
<td>29±2.6</td>
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<tr>
<td></td>
<td>Ionomycin induced</td>
<td>81±4.6</td>
<td>80±3.1</td>
<td>85±2.7</td>
</tr>
<tr>
<td></td>
<td>Constriction, % of maximum diameter</td>
<td>35±3.5</td>
<td>34±1.2</td>
<td>34±2.3</td>
</tr>
<tr>
<td></td>
<td>EC50</td>
<td>12.7±0.1</td>
<td>89±8.9</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>80 mM [K+]o</td>
<td>85±11.7</td>
<td>87±23.0</td>
<td>87±12.5</td>
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<tr>
<td></td>
<td>Ionomycin</td>
<td>53±9.7</td>
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<td>83±2.7</td>
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<td>31±1.3</td>
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<td>EC50</td>
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<td>194±13.0</td>
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<tr>
<td>Day 3</td>
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<td>81±10.3</td>
<td>89±14.3</td>
<td>149±13.0*</td>
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<tr>
<td></td>
<td>Constriction, % of maximum diameter</td>
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<td>30±2.1</td>
<td>32±3.0</td>
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<tr>
<td></td>
<td>EC50</td>
<td>16.4±0.7</td>
<td>201±50.7</td>
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<tr>
<td>Day 4</td>
<td>80 mM [K+]o</td>
<td>80±5.4</td>
<td>83±2.8</td>
<td>194±13.0</td>
</tr>
<tr>
<td></td>
<td>Ionomycin</td>
<td>37±12.7</td>
<td>51±15.5</td>
<td>50±15.3</td>
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<tr>
<td></td>
<td>Ionomycin induced</td>
<td>85±4.9</td>
<td>75±2.6</td>
<td>73±3.0</td>
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<tr>
<td></td>
<td>Constriction, % of maximum diameter</td>
<td>33±2.3</td>
<td>31±2.3</td>
<td>30±2.8</td>
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</table>

Values are means ± SE. Diameter values are shown for αCa9p*10 antisense, αCa9p*10 sense, and reversible permeabilization control (RP) arteries in physiological saline solution of 6 mM extracellular K⁺ ([K+]o), 80 mM [K+]o, and 120 mM [K+]o, containing 10 μM ionomycin (ionomycin group) for all time points tested. Ionomycin constriction, expressed as percent decrease from maximum diameter, and EC50 values, calculated from [K+]o, concentration-response curves, are also shown. *P < 0.05 vs. αCa9p*10 sense and RP groups.
The introduction of oligonucleotides into smooth muscle of intact cerebral arteries was achieved by a reversible permeabilization procedure (9, 25). Arterial segments were first incubated at 4°C for 30 min. Following reversible permeabilization, arteries not achieving >70% constriction in response to ionomycin were not used for analysis. Half-maximal effective concentration (IC50) was calculated for each agonist.

**Fig. 1.** Cerebral arteries express α1C9/10. A: PCR primer design for the detection of α1C9/10. Amplification of transcripts containing exon 9* results in 535 nucleotide (nt) product, whereas amplification of transcripts excluding exon 9* results in 460 nt product. B: representative gel demonstrating 2 bands corresponding to both α1C9/10 and α1C9/10 present in whole cerebral arteries (n = 7); α1C9/10 band is most prominent in brain (n = 5) and heart tissue (n = 8). C: resulting RT-PCR gel using cDNA obtained from whole cerebral arteries from human (n = 2) demonstrates presence of 2 bands corresponding to α1C9/10 and α1C9/10. Fwd, forward; Rvs, reverse.

**Fig. 2.** Quantitative real-time PCR (qPCR) shows high expression of α1C9/10 in cerebral arteries. A: PCR primer sets for the specific detection of α1C9/10 and α1C9/10. α1C9/10-specific (left) forward primer recognizes sequence specific to exon 9*; α1C9/10 (right) forward primer recognizes sequence of boundary between exons 9 and 10. Sequence analysis of PCR products confirmed specificity of primers for target sequences. B: summary qPCR data for cerebral arteries, brain (cortex), and heart (left ventricle). The ratio of α1C9/10 mRNA to α1C9/10 mRNA was significantly greater in cerebral arteries relative to brain and heart (0.289 ± 0.008; n = 7 compared with 0.006 ± 0.001; n = 4 and 0.050 ± 0.006; n = 8, respectively). **P < 0.01 vs. brain and heart.
(EC50) was determined from each [K+]o concentration-response experiment.

Statistical analysis. Values are presented as means ± SE. One-way ANOVA followed by Tukey multiple comparison test was used in the comparison of multiple groups. Student’s t-test was used in the comparison of two groups. Statistical significance was considered at the level of P < 0.05 (*) or P < 0.01 (**).

RESULTS

Cerebral arteries demonstrate enhanced expression of \( \alpha_1 \)C99/9/10 compared with brain and heart. Previous work has shown selective expression of \( \alpha_1 \)C99/9/10 splice variants by smooth muscle-containing tissues. However, mRNA expression of both \( \alpha_1 \)C splice variants including (\( \alpha_1 \)C99/10) and excluding (\( \alpha_1 \)C9/10) exon 9* has been reported in aorta (3, 13, 26). Therefore, our first objective was to investigate whether small diameter (100–250 \( \mu \)m) cerebral arteries express mRNA for both \( \alpha_1 \)C99/9/10 and \( \alpha_1 \)C9/10. To detect expression of \( \alpha_1 \)C99/10, we designed PCR primers to generate products consisting of exons 7–11 of Cav1.2 (see Fig. 1A) such that a shift in product size would result if the 75 nucleotide (nt) insertion for exon 9* was expressed. RT-PCR analysis resulted in two distinct bands (Fig. 1B; \( n = 7 \)). Sequence analysis confirmed the lower band of 460 nt represents \( \alpha_1 \)C9/10, whereas the upper band of 535 nt represents \( \alpha_1 \)C99/10. Human cerebral arteries were also analyzed and found to express both \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 similar to arteries from rabbit (Fig. 1C). In contrast, cardiac tissue (left ventricle; \( n = 8 \)) and brain tissue (cerebral cortex; \( n = 5 \)) were found to express one dominant product corresponding to \( \alpha_1 \)C9/10 (Fig. 1B).

Quantitative real-time PCR was used to evaluate relative expression levels of \( \alpha_1 \)C99/10 in these tissues. We hypothesized the fraction of mRNA for Ca,1.2 expressing exon 9* is greater in cerebral arteries than cardiac and brain tissue. \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 expression was measured separately by using either a PCR primer specific for exon 9* sequence (\( \alpha_1 \)C99/10 specific) or a primer specific for the boundary of exons 9 and 10 (\( \alpha_1 \)C9/10 specific; Fig. 2A). \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 expression was normalized to 18S ribosomal RNA levels. This approach allows quantification of the \( \alpha_1 \)C99/10-to-\( \alpha_1 \)C9/10 mRNA ratio, which is representative of \( \alpha_1 \)C99/10 relative to overall \( \alpha_1 \)C9/1.2 expression considering that \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 are mutually exclusive splice variants. We found that cerebral arteries express significantly higher relative levels of \( \alpha_1 \)C99/10 compared with brain and heart tissues (Fig. 2B; –52-fold and –6-fold difference, respectively). Together, these results demonstrate mRNA for both \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 splice variants in cerebral arteries and enhanced relative expression of \( \alpha_1 \)C99/10 in cerebral arteries compared with brain and heart.

Isolated cerebral artery myocytes express both \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 splice variants. We next chose to clarify whether cerebral artery myocytes express mRNA for both splice variants or whether \( \alpha_1 \)C9/10 detection was due to the presence of non-smooth muscle cell types within the vascular wall, such as fibroblasts or perivascular neurons, which are reported to express Ca,1.2 (10, 13, 17, 32). Live freshly isolated cerebral artery myocytes (~40–60 cells) were collected by laser capture microdissection for RT-PCR analysis (Fig. 3B). These elongated spindle-shaped cells showed strong immunofluorescent staining for SM-MHC or smooth muscle-\( \alpha \)-actin, markers commonly used to identify smooth muscle (Fig. 3A) (1, 11). RT-PCR was used to examine expression of specific cell

![Fig. 3. Cerebral artery myocytes express both \( \alpha_1 \)C99/10 and \( \alpha_1 \)C9/10 splice variants. A: immunostaining of isolated cerebral artery myocytes. Red: smooth muscle (SM) myosin heavy chain (SM-MHC); green: SM-\( \alpha \)-actin (color changed from red to green to distinguish from SM-MHC); blue: 4,6-diamidino-2-phenylindole nuclear stain. Scale bars represent 10 \( \mu \)m. A lack of staining was observed for SM-MHC or smooth muscle-\( \alpha \)-actin by cells incubated without primary antibody (No 1° Ab; right). B: microdissection of isolated cerebral artery myocytes. Live myocytes plated on PALM Duplex dish (Ziess) were identified by cell morphology and the surrounding dish membrane was cut. Myocytes were then catapulted onto PALM Adhesivecap collection tubes (Ziess) for mRNA extraction. Scale bars represent 25 \( \mu \)m. C: representative gel showing the presence of cell markers: SM-MHC (smooth muscle), endothelin-1 (ET-1: endothelin), fibroblast specific protein-1 (FSP-1; fibroblast), and growth associated protein-43 (GAP43; neuronal). All markers are amplified using cDNA from whole cerebral arteries. cDNA from cerebral artery myocytes samples collected by laser capture microdissection demonstrate amplification of SM-MHC, whereas other markers were not detected. D: results of nested PCR performed on cDNA from isolated cerebral artery myocytes. First round of amplification (35 cycles) was performed using primers for exons 7–11 of Ca,1.2 (see Fig. 1A). Second round of amplification (35 cycles) was done using nested primers (see METHODS) and 1:100 dilution of first-round PCR products. Final products represent expression of \( \alpha_1 \)C99/10 (top band) and \( \alpha_1 \)C9/10 (lower band; \( n = 5 \)).]
markers: SM-MHC (smooth muscle), ET-1 (endothelium) (38), FSP-1 (fibroblast) (33), and GAP43 (neuron) (39) in mRNA isolated from both intact arteries and isolated myocytes. The mRNA markers: SM-MHC (smooth muscle), ET-1 (endothelium) (38), FSP-1 (fibroblast) (33), and GAP43 (neuron) (39) in mRNA isolated from both intact arteries and isolated myocytes. The above cell markers all amplified using intact cerebral artery mRNA (Fig. 3C), consistent with the presence of multiple cell types in intact vessels. However, only the smooth muscle marker SM-MHC was expressed in freshly isolated myocytes, confirming purity of our samples. Nested PCR for exons 7–11 of Ca,1.2 demonstrated two bands corresponding to α1C9/10 and α1C9/10 band intensity following treatment with α1C-AS and organ culture for 4 days (right; n = 4) is shown. Total RNA used was similar as shown by endogenous control 18S ribosomal RNA. B: quantification of changes in mRNA levels using qPCR in antisense-treated arteries compared with sense-treated arteries from same animal. *P < 0.05 (α1C9/10-AS/S, n = 4; α1C-AS/S, n = 4).

α1C9/10 plays critical role in depolarization-induced constriction of cerebral arteries. High expression levels of α1C9/10 in cerebral artery myocytes suggest this splice variant may have an important role in vascular function. We therefore tested the hypothesis that α1C9/10 splice variants are an important regulator of cerebral artery diameter. Two antisense oligonucleotide sequences were designed to examine the functional role of α1C9/10. First, α1C9/10-AS targeting a sequence specific for exon 9* was used for selective suppression of the α1C9/10 variant. Second, α1C-AS targeting a sequence specific for exon 9, which is constitutively expressed in all L-type VDCC splice variants, was used for nonselective suppression of all Ca,1.2 isoforms. Four days following treatment, standard RT-PCR and quantitative real-time PCR demonstrated a significant reduction (43 ± 6.9%) in α1C9/10 expression following treatment with α1C-AS compared with α1C9/10-sense-treated arteries (Fig. 4, A and B). However, suppression of α1C9/10 was not observed following treatment with α1C9/10-AS. In contrast with the effect of α1C9/10-AS, arteries treated with α1C-AS exhibited a significant reduction in expression for both α1C9/10 and α1C9/10 splice variants (Fig. 4, A and B).

To examine the effect of suppressing α1C9/10 on arterial constriction, luminal diameter measurements were performed while increasing external K⁺ ([K⁺]o) in the bath solution over a range of concentrations from 6 to 120 mM following a period of 1 to 4 days in organ culture. The relationship between extracellular [K⁺]o and membrane potential at [K⁺]o > 16 mM closely follows that which is predicted by the Nernst equation (16, 21).

**Freshly isolated**

<table>
<thead>
<tr>
<th>Extracellular K⁺ (mM)</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
</tr>
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<tbody>
<tr>
<td>10 µM</td>
<td>268 µm</td>
<td>150 µm</td>
<td>125 µm</td>
<td>100 µm</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>62 µm</td>
<td>51 µm</td>
<td>45 µm</td>
<td>40 µm</td>
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<tr>
<td>Extracellular K⁺ (mM)</td>
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<tr>
<td>Ionomycin</td>
<td>51 µm</td>
<td>45 µm</td>
<td>40 µm</td>
<td>35 µm</td>
</tr>
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</table>

Fig. 5. Representative arterial diameter traces demonstrating important functional role for α1C9/10 in arterial constriction. All arteries were cannulated, pressurized to 20 mmHg, and perfused with physiological saline solution (PSS) for 30 min before stepwise increases in extracellular K⁺ ([K⁺]o). Control freshly isolated and sense-treated (day 4) arteries responded to increases in [K⁺]o by graded constriction. α1C9/10-AS-treated (day 4) arteries exhibited a marked reduction in [K⁺]o-induced constriction. Ionomycin (10 µM) was applied at the end of each experiment.
Stepwise increases in \([K^+]_o\) caused graded arterial constriction of control freshly isolated vessels (Figs. 5 and 6). \(\alpha_1C_{99+/10}\)-AS, \(\alpha_1C_{99+/10}\)-sense, and RP only groups all responded similarly to freshly isolated vessels following 1 day in culture (Fig. 6). However, \(K^+\)-induced constrictions, expressed as a percentage of maximum constriction caused by ionomycin (see METHODS), were significantly reduced in \(\alpha_1C_{99+/10}\)-AS arteries compared with control \(\alpha_1C_{99+/10}\)-sense-treated and RP arteries after 2 days. For example, \(\alpha_1C_{99+/10}\)-AS-treated vessels exhibited 52 ± 5.9% constriction in response to 60 mM \(K^+\) on day 2 compared with 80 ± 2.1% and 75 ± 2.9% constriction in \(\alpha_1C_{99+/10}\)-sense-treated and RP vessels, respectively. Further reductions were observed in \(\alpha_1C_{99+/10}\)-AS-treated arteries on day 3 (e.g., 26 ± 4.1% in \([K^+]_o = 60 \text{mM}\) and day 4 (e.g., 23 ± 4.9% in \([K^+]_o = 60 \text{mM}\)) of organ culture (Fig. 6). \(\alpha_1C_{99+/10}\)-sense-treated and RP arteries constricted to a similar degree as freshly isolated vessels at all time points tested (Figs. 5 and 6). Mean diameters of arteries exposed to these treatments are shown in Table 1. No significant differences in \(EC_{50}\) values for \(K^+\) were observed between groups (Table 1). Diameter values and maximal constriction to ionomycin were similar between groups at all time points (Table 1). Diameter values and maximal constriction to \(60 \text{mM} [K^+]_o\) that was similar to that observed in arteries treated with \(\alpha_1C_{99+/10}\)-AS (Fig. 7; \(\alpha_1C_{99+/10}\)-AS: 23.5 ± 3.6%, \(n = 6\); and \(\alpha_1C\)-AS: 25.6 ± 4.3%, \(n = 5\), suggesting that suppression of all \(Ca^2+\) splice variants had no greater effect on constriction to 60 mM \([K^+]_o\), than selectively suppressing \(\alpha_1C_{99+/10}\). These data suggest that the isoform containing exon 9* is the functionally dominant \(Ca_{1.2}\) splice variant in cerebral artery constriction.

**DISCUSSION**

Here we demonstrate that cerebral artery myocytes express \(Ca_{1.2}\) splice variants both containing (\(\alpha_1C_{99+/10}\)) and lacking (\(\alpha_1C_{99/10}\)) exon 9* and that \(\alpha_1C_{99+/10}\) plays a dominant role in cerebral artery constriction. The following observations are consistent with this novel finding: 1) cerebral arteries have a significantly higher ratio of \(\alpha_1C_{99+/10}\) to \(\alpha_1C_{99/10}\) mRNA compared with cerebral cortex or cardiac tissue; 2) RT-PCR performed on cDNA from isolated cerebral artery myocytes confirmed expression of both \(\alpha_1C_{99+/10}\) and \(\alpha_1C_{99/10}\) in smooth muscle; 3) selective suppression of the \(\alpha_1C_{99+/10}\) splice variant...
EXON 9* Cav1.2 regulates cerebral artery constriction

Fig. 7. α1C9/9*/10 plays a dominant role in cerebral artery constriction. A: representative diameter traces showing response to 60 mM [K\(^+\)]\(_o\), followed by application of 10 μM ionomycin. B: summary of 60 mM [K\(^+\)]\(_o\) for AS-treated arteries. All constrictions were normalized to minimum diameter obtained in ionomycin and maximum diameter obtained in Ca\(^{2+}\)-free PSS with diltiazem (100 μM) and forskolin (1 μM). After 4 days in organ culture following oligonucleotide treatment, arteries treated with α1C-AS exhibited similar response to 60 mM [K\(^+\)]\(_o\), as arteries treated with α1C9/9*/10-AS, α1C9/10-AS and α1C-Sense groups were significantly decreased compared with corresponding sense-treated groups. No significant difference (NS) was observed between α1C9/9*/10-AS and α1C-AS groups (\(P < 0.05\); α1C9/9*/10-Sense, n = 4; α1C9/10-AS, n = 6; α1C-Sense, n = 5; α1C-AS, n = 5).

caused a marked reduction in K\(^+\) induced arterial constriction; and 4) suppression of all Ca\(_{1.2}\) splice variants caused no further reduction in K\(^+\) induced arterial constriction. In addition, we found a similar expression profile of α1C9/9*/10 and α1C9/10 splice variants in cerebral arteries from humans.

This work expands our understanding of the molecular composition of smooth muscle VDCCs responsible for the control of arterial diameter and blood flow. Unlike cardiac myocytes, vascular smooth muscle operates in a state of tonic contraction responding to modest changes in membrane potential caused, for example, by changes in intravascular pressure, neurotransmitter release, circulating catecholamines, and endothelial influences. Thus L-type VDCCs expressed by vascular smooth muscle respond to small voltage changes at relatively negative membrane potentials compared with more depolarized membrane potentials reached during action potentials in cardiac myocytes or neurons. Recent studies using cell expression systems have found expression of the 25 amino acid insertion exon 9* causes a 9-mV hyperpolarizing shift in L-type VDCC V\(_{0.5}\),act (6, 26). This hyperpolarizing shift in the activation of this splice variant would be well-suited to the range of vascular smooth membrane potential in vivo (−45 to −35 mV) (29). We now report that exon 9*-containing L-type VDCCs are the dominant Ca\(_{1.2}\) splice variant contributing to cerebral artery constriction. To our knowledge, this is the first study directly demonstrating a physiological role for a single Ca\(_{1.2}\) splice variant.

We have observed that whole arterial tissue containing smooth muscle, endothelial, fibroblast and perivascular neural cells expresses both α1C9/9*/10 and α1C9/10 splice variants. Although endothelial cells are known to lack expression of VDCCs (13), Ca\(_{1.2}\) expression by fibroblasts and neurons is well documented (10, 17, 32). To further explore the composition of Ca\(_{1.2}\) splice variants in vascular smooth muscle, we performed PCR on mRNA obtained from freshly isolated vascular myocytes using laser capture microdissection. This approach demonstrated mRNA for both α1C9/9*/10 and α1C9/10 isofoms in a pure population of native vascular smooth muscle. When compared with vascular tissue, we found a relatively low level of α1C9/9*/10 mRNA in heart and cerebral cortex tissue, providing additional support that exon 9* is selectively expressed by smooth muscle. It is possible that apparent differences in the stoichiometry of α1C9/9*/10 and α1C9/10 splice variants may reflect discrepancies in total levels of Ca\(_{1.2}\) expressed between tissues. However, an earlier study by Graf et al. (13) demonstrated that the ratio of +9* to −9* Ca\(_{1.2}\) is not well correlated with expression levels of total Ca\(_{1.2}\). For example, it was shown that human cardiac ventricle and aorta express similar levels of total Ca\(_{1.2}\), although the +9* to −9* ratio is greater in aorta. Increased α1C9/9*/10 mRNA ratio observed in vascular smooth muscle is, therefore, unlikely to be due to differential expression of total Ca\(_{1.2}\) levels compared with brain and heart.

The use of antisense oligonucleotides to specifically suppress 9*-containing Ca\(_{1.2}\) channels revealed a substantial decrease in K\(^+\) induced constriction, supporting a functional role for this splice variant in vascular physiology. In fact, antisense oligonucleotides targeting a region common to all Ca\(_{1.2}\) splice variants (exon 9) caused no greater decrease in contractility than suppression of only α1C9/9*/10. These data suggest exon 9*-containing Ca\(_{1.2}\) channels play a dominant role in regulating smooth muscle contraction. It should be noted that this conclusion is based on the assumptions that both antisense oligonucleotides used in this study suppress translation of their target with similar efficiency and that functional effects are not due to nonspecific suppression of total Ca\(_{1.2}\) levels. Consistent with these assumptions, we observed a maximal response using both antisense oligonucleotides at day 4 with no greater suppression occurring after 5 days of treatment (data not shown). Furthermore, the EC\(_{50}\) for [K\(^+\)]\(_o\) was not altered by α1C9/9*/10-AS, consistent with a functional population of Ca\(_{1.2}\) channels with a uniform V\(_{0.5}\),act. With the consideration of the loss of endothelial function following prolonged organ culture of arteries (20), it is possible that compensatory changes in Ca\(_{1.2}\) splice variant expression may occur. However, we have found using RT-PCR that the ratio of
\(\alpha_1C_{99}\text{-}10\) to \(\alpha_1C_{910}\) does not change following up to 4 days in culture (data not shown). Future studies are needed to address whether \(\alpha_1C_{99}\text{-}10\) also plays a role in vasoconstriction to endogenous compounds and physiological increases in intravascular pressure.

The unique location of exon 9* within the structure of the \(\alpha_1\)-subunit may also play a role in post-translational regulation of VDCCs in vascular smooth muscle. Interestingly, the site of \(\alpha_1B\)-subunit interaction, known as the \(\alpha_1\) interaction domain, is found 18 amino acids upstream of exon 9* within the I-II intracellular linker region of the \(\alpha_1\)-C-protein. It has previously been shown that functional interaction between the \(\alpha_1\)- and \(\beta\)-subunit is required for correct targeting of the channel to the plasma membrane (12). It remains possible that \(\alpha_1\)-subunits expressing exon 9* may differentially bind specific Ca\(_2+\) isoforms compared with \(\alpha_1\)-subunits lacking the insertion, leading to favored trafficking of \(\alpha_1C_{99}\text{-}10\) to the membrane and a dominant functional role for this splice variant. Further research is needed to elucidate whether the presence of exon 9* alters the binding of \(\beta\) subunit subtypes or splice isoforms. It should be noted that smooth muscle-selective alternatively spliced exons other than exon 9* could be preferentially expressed in vascular smooth muscle VDCCs. For example, it has recently been shown that inclusion of exon 1c in full-length cloned \(\alpha_1\text{-}C_{12}\) channels can lead to a hyperpolarizing shift in \(V_{0.5,\text{act}}\) in HEK293 cells, similar to inclusion of exon 9*(7).

In summary, this study suggests that the exon 9*-containing \(\alpha_1\text{-}C_{12}\) splice variant controls cerebral artery myocyte Ca\(_2+\) influx, arterial diameter, and cerebral blood flow. We propose that \(\alpha_1C_{99}\text{-}10\) may represent a novel target for therapeutics against vascular pathologies associated with increased Ca\(_2+\) influx in vascular smooth muscle leading to enhanced arterial constriction. Future genetic or pharmacological strategies targeting smooth muscle-selective \(\alpha_1\text{-}C_{12}\) splice variants could provide a valuable means of modulating vascular tone while avoiding widespread effects on VDCC isoforms in cardiac or nervous systems.

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