Calcium Efflux Activity of Plasma Membrane Ca\textsuperscript{2+} ATPase-4 (PMCA4) Mediates Cell Cycle Progression in Vascular Smooth Muscle Cells\textsuperscript{*}

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We explored the role played by plasma membrane calcium ATPase-4 (PMCA4) and its alternative splice variants in the cell cycle of vascular smooth muscle cells (VSMC). A novel variant (PMCA4e) was discovered. Quantitative real-time-PCR-quantified PMCA4 splice variant proportions differed in specific organs. The PMCA4a:4b ratio in uninjured carotid arteries (\textasciitilde 1:1) was significantly reduced by wire denudation injury (to \textasciitilde 1:3) by modulation of alternative splicing, as confirmed by novel antibodies against PMCA4a/e and PMCA4b. Laser capture microdissection localized this shift to the media and adventitia. Primary carotid VSMC from PMCA4 knock-out (P4KO) mice showed impaired \textsuperscript{32}P(thymidine incorporation and G1 phase arrest as compared with wild type (P4WT). Electroporation of expression constructs encoding PMCA4a, PMCA4b, and PMCA4ab unable to bind PDZ- proteins but not by inactive PMCA4.

Significance: PMCA4 Ca\textsuperscript{2+} efflux regulates cell cycle.

Progression through the cell cycle involves Ca\textsuperscript{2+} transporters including Ca\textsuperscript{2+} channels and Ca\textsuperscript{2+} efflux pumps for review, see Ref. 1, and sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase-2 (SERCA2) splice variants are differentially expressed at various stages of the cell cycle (for review, see Ref. 2). However, whether and how plasma membrane Ca\textsuperscript{2+} ATPases (PMCA)\textsuperscript{2} a family of low capacity, high affinity plasma membrane-associated Ca\textsuperscript{2+} efflux pumps encoded by four genes (PMCA1\textendash 4) (for review, see Refs. 3 and 4) also participated in this process was not known.

Alternative splicing at the amino (N) and carboxyl (C) terminal sites of PMCA4 pre-mRNA can produce \textit{x} or \textit{z} splice variants at site N and \textit{a}, \textit{b}, or \textit{d} splice variants at site C, each leading to the expression of a different protein (4\textendash 6). PMCA4 is expressed later in development than PMCA1 but is expressed more or less ubiquitously in adult mammalian organs (4). PMCA gene transcription is controlled by c-Myb, leading to changes and increases in the free intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]).

The initial observations in VSMC showed that inhibiting c-Myb expression or function prevented cell cycle progression and lowered [Ca\textsuperscript{2+}], (10). We found these changes to be mediated by increased PMCA1 and PMCA4 expression. Indeed,

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\textsuperscript{1}This article contains supplemental Tables S1\textendash S8 and Figs. S1\textendash S9.

\textsuperscript{2}The abbreviations used are: PMCA, plasma membrane Ca\textsuperscript{2+} ATPases; qRT, quantitative real-time; VSMC, vascular smooth muscle cells; EGFP, enhanced GFP; IHC, immunohistochemistry; NFAT, nuclear factor of activated T-cells; CREB, cAMP-response element-binding protein; PI, pro-pidium iodide; N5, not significant; Rgs16, regulator of G-protein signaling-16; Dcn, Decorin.

\textsuperscript{3}The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{TM}/EBI Data Bank with accession number(s) JF683384, EU493095, and EU493094.

\textsuperscript{4}PMCA4 microarray data have been placed in the PubMed microarray database GEO under accession number GSE38320.

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PMCA4 Efflux Activity Regulates Cell Cycle in VSMC

transient overexpression of PMCA1a in VSMC elevated the Ca\(^{2+}\) efflux rate, decreased the [Ca\(^{2+}\)]\(_i\), and reduced the rate of cell proliferation by over 2.5-fold (10). Whereas PMCA4b over-expression in mice increased blood pressure and myogenic tone (11), mice with functional knock-out of PMCA4 showed impaired phasic contractions and apoptosis in portal vein smooth muscle in vitro provided the mice were also heterozygous for the PMCA1 gene deletion (12). Together, these findings suggested that PMCA4 plays a role in VSMC biology.

We now present evidence that the relative proportions of PMCA4a and PMCA4b splice variants are regulated after wire denudation injury of carotid arteries in vivo. Quiescent VSMC in vivo have roughly equal proportions of PMCA4a and PMCA4b splice variants until arterial injury leads to a significant decrease in the ratio of PMCA4a to PMCA4b. Mice lacking functional PMCA4 (P4KO) show reduced remodeling after injury in vivo, although primary VSMC isolated from such mice manifest reduced cell proliferation and a G\(_1\) phase arrest that can be rescued by either the PMCA4a or PMCA4b splice variant alone. A PMCA4b mutant lacking the carboxyl-terminal PDZ binding domain also rescued the G\(_1\) phase arrest of P4KO VSMC, whereas a PMCA4b mutant with only 10% of the Ca\(^{2+}\) efflux activity of the wild-type pump could not. Microarray data show that functional PMCA4 ablation caused strong increases in Decorin (a G\(_1\) arrest marker) and regulator of G-protein signaling-16 (Rgs16; mitogen-activated protein kinase (MAPK) inhibitor, nuclear factor of activated T-cells (NFAT) target) expression, although rescue with either PMCA4a or PMCA4b restored normal Decorin and Rgs16 expression levels. Having said this, PMCA4a and PMCA4b rescues did differ in their global outcomes, with PMCA4a causing decreased expression of the transcription factor AP-2\(\beta\) (an inhibitor of c-Myc, Ap-2a, and cell cycle), whereas PMCA4b caused decreased expression of p15 (Cdkn2b (cyclin-dependent kinase inhibitor 2b)), an inhibitor of Cyclin D1/Cdk4. Together, these findings lead us to conclude that the Ca\(^{2+}\) efflux activity of PMCA4 mediates G\(_1\) phase cell cycle progression in VSMC and that PMCA4a and PMCA4b appear to work through different effector proteins to do so.

MATERIALS AND METHODS

**Mice and Cell Culture**—C57BL6/J mice were purchased (Charles River Laboratories, Wilmington, MA). Frozen PMCA4 knock-out hearts (lacking exons 2 and 3) were a gift from Ludwig Neyses (Manchester, UK) (13, 14). Because deletion of exons 2–3 disrupts the reading frame of the resulting PMCA4 locus, these mice do not produce any PMCA4 protein (supplemental Fig. S2, panel B). PMCA4 functional knock-out mice distinct from the Neyses laboratory mice and lacking exon-11 (encoding codons 440–520, including aspartate 466, which is the catalytic phosphorylation site; GenBank\(^\text{TM}\) accession number NM_001167949.2) were a kind gift from Gary Shull (12). These exon-11-deficient mice express a mutant PMCA4 mRNA as a consequence of exon-10 splicing to exon-12 (12), which preserves the reading frame and enables these mice to produce functionless mutant PMCA4a and PMCA4b proteins (devoid of a catalytic site). These mice are referred to as P4KO mice, with their wild-type littermates denoted as P4WT. Protocols for mouse tissue harvest and carotid artery wire denudation injury have been described (15) and were approved by the Animal Care Committee of University Health Network. Primary VSMC cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Wisent; Montreal, Quebec, Canada), 50 ng/ml rat recombinant PDGF-BB (Sigma), and 1% penicillin-streptomycin in humidified air containing 5% CO\(_2\) at 37 °C.

**Cloning of PMCA4 cDNA Variants**—PMCA4a, PMCA4b, and PMCA4e cDNA were cloned from total RNA extracted from mouse bladder, a mouse VSMC cell line (MOVAS; ATCC number CRL-2797), and mouse brain of C57 BL/6/j mice, respectively (NucleoSpin RNA II kit; Takara-Clontech, Mountain View, CA) and PCR-cloned using mouse PMCA4-specific primers (see supplement Table S1) targeting either the 5’ or 3’ half of PMCA4 mRNA into pGemT-Easy vector (Promega). Clones containing the 5’ and 3’ halves of PMCA4 were digested with BamHI, gel-eluted, and ligated to generate full-length cDNA clones for each splice variant and sequenced. These cDNA sequences have been deposited in GenBank\(^\text{TM}\) under accession numbers JF683384 (PMCA4a), EU493095 (PMCA4b), and EU493094 (PMCA4e).

**Antibody Generation and Validation**—Peptides (supplemental Fig. 1B) specific for PMCA4b and PMCA4a/e carboxyl termini were designed (Aves Labs; Tigard, OR) and used to inject chickens. Polyclonal chicken antibodies with expected specificity for PMCA4 splice variants were collected and affinity-purified on columns containing the immobilized peptide antigen.

**Western Blotting**—Protein extracts made from primary VSMC and various mouse organs including uninjured and injured carotid arteries were resolved on mini-PAGE gels (Novex; Invitrogen), blotted, and hybridized to various antibodies and visualized either by HRP-conjugated secondary antibodies (ECL kit; PerkinElmer Life Sciences) or by near infrared dye conjugated secondary antibodies using the Odyssey laser scanner (LiCor, Lincoln, NE).

**qRT-PCR**—Total RNA was extracted from various organs of C57BL6/J mice with the Picopure RNA extraction kit (Invitrogen). After DNase treatment and the addition of 1.0 pg of EGFP RNA to each sample (reverse transcription efficiency control), cDNA was synthesized, and qRT-PCR was carried out with primers specific for EGFP (cDNA synthesis efficiency), β2-microglobulin (housekeeping gene), PMCA4a, PMCA4b, and PMCA4e splice variants (see supplemental Table S1) using SYBR GREEN master mix in an ABI HT-7900 (Invitrogen) thermal cycler. qRT-PCR primers for PMCA4 splice variants were first used to gel elute amplified products from each primer pair, clone them into pGemT-Easy vector, and sequence them to confirm the identity of the amplicon for each splice variant. Next, the PMCA4a, PMCA4b, and PMCA4e primer pairs were tested under various amplification conditions to measure amplification efficiency. Amplification conditions were chosen where the individual Ct values for each splice variant differed by <5% from the mean Ct value for all three variants. This ensured that amplification efficiency would not vary significantly among the PCR reactions for the three PMCA4 splice variants. The amplification conditions employed consisted of a hot start.
of 94 °C for 10 min followed by 50 cycles of 94 °C for 15 s (denaturation), 69 °C for 30 s (annealing), and 72 °C for 30 s (extension). Gel eluted amplicons for EGFP, microglobulin, and the three PMCA4 splice variants were diluted to generate copy numbers, (annealing), and 72 °C for 30 s (extension). Gel eluted amplicons for EGFP, microglobulin, and the three PMCA4 splice variants were diluted to generate copy numbers containing 1 × 10⁶, 1 × 10⁵, 1 × 10⁴, 1 × 10³, 100, and 10 copies/μl. The absolute copy number for each particular mRNA was calculated via the absolute quantification method (Protocol 4304965; Applied Biosystems) that employs Ct values of copy number standards to calculate absolute copy numbers of each template in each sample. The absolute copy numbers of PMCA4 splice variants were then normalized to the copies of EGFP (cDNA synthesis efficiency control) and microglobulin (housekeeping gene control) cDNA in each sample. Finally, normalized copy numbers of each PMCA4 splice variant in each sample were summed, and copy numbers of each splice variant were divided by this sum to obtain the relative proportion of each splice variant in each sample.

**Immunohistochemistry (IHC), Immunofluorescent Staining, and Morphometry**—Mouse carotid artery cross-sections (paraformaldehyde-fixed, paraffin-embedded) were used for immunostaining with various antibodies and fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch and Invitrogen) and visualized by confocal imaging on the Olympus Fluoview microscope using Fluoview software (Advanced Optical Microscopy Facility, University Health Network). Regions of interest were drawn on the images, and fluorescence intensity was quantified in the regions of interest using Fluoview software. Quantification was done on at least two different regions of interest of both uninjured and injured carotid artery sections embedded in one paraffin section (three uninjured and three injured arteries per section), immunostained together on one slide, and imaged via z-stacks using the same laser intensity for all sections. For carotid artery morphometry, sections from P4KO and P4WT mice (day 9 post injury) were immunostained with anti-PMCA4a/e antibody, and the area of the entire media and entire remodeled adventitia was measured at 40× resolution on the Olympus Fluoview microscope using Fluoview software.

**Laser Capture Microdissection**—Mouse carotid arteries (uninjured and injured) or mouse bladder fresh-frozen in OCT (Optical Cutting Temperature Compound, Sakura Finetek, Torrance, CA) were sectioned (10 μm thick) and mounted on laser capture microdissection microscope slides. Sections were lightly stained with Histogene (Invitrogen), fixed with 70% ethanol, and taken through a dehydration series ending in xylene and dried. An laser capture microdissection microscope and laser set up (Advanced Optical Microscopy Facility, University Health Network) was used to microdissect and capture medial and adventitial layer regions of carotids (or bladder smooth muscle layer) from the stained and fixed sections. RNA was extracted with the PicoPure kit, and qRT-PCR was carried out as described above.

**Tritiated Thymidine Uptake Assays**—Primary VSMC were seeded into 4 replicate wells of 6-well plates, allowed to grow overnight, and then serum-starved (0.1% serum; DMEM) for 24 h. Serum-synchronized cells were then stimulated with 10% serum and 50 ng/ml PDGF-BB for 24 h. Cells were labeled with BrdU for the last 1 h before harvest. Cells were harvested, fixed in 70% ethanol, and processed for BrdU-PI flow cytometry. Cells were harvested, fixed in 70% ethanol, and processed for anti-BrdU FITC-conjugated antibody staining as well as propidium iodide staining and used for flow cytometry.

**Microarray Analysis**—Primary VSMC from the carotid arteries of P4KO and P4WT mice were grown in quadruplicate T-75 flasks, allowed to grow overnight, and then serum-starved (0.1% serum; DMEM) for 24 h. Synchronized cells were then stimulated with 10% serum and 50 ng/ml PDGF-BB for 24 h. Cells were labeled with BrdU for the last 1 h before harvest, and then flow-sorted to obtain the G0/G1, S, and G2/M subpopulations (Primary VSMC were seeded into 4 replicate wells of 6-well plates, allowed to grow overnight, and then serum-starved (0.1% serum; DMEM) for 24 h. Synchronized cells were then stimulated with 10% serum and 50 ng/ml PDGF-BB for 24 h. Cells were labeled with BrdU for the last 1 h before harvest, and then flow-sorted to obtain the G0/G1, S, and G2/M subpopulations. The pre-processing includes three steps: background correction (performed in GenomeStudio software), quantile normalization (16), and log2 transformation of normalized data. The last two steps were performed in lumi R package (17). The Center for Applied Genomics (SickKids Hospital, Toronto, ON) for microarray analysis. Table 1 and supplemental Tables S2–S8 show genes increased or decreased in expression or which are known to be implicated in G1 phase arrest or are part of the NFAT pathway and were seen to be strongly modulated in P4KO cells as compared with P4WT cells. The preprocessing includes three steps: background correction (performed in GenomeStudio software),quantile normalization (16), and log2 transformation of normalized data. The last two steps were performed in lumi R package (17). The Center for Applied Genomics used LIMMA (linear models for microarray data) (18) to identify differentially expressed gene signatures under different conditions. Briefly speaking, it starts by fitting a linear model for each gene in the data, then an empirical Bayes method is used to moderate the standard errors for estimating the moderated t-statistics for each gene, which shrinks the standard errors toward a common value. This test is similar to an analysis of variance method for each gene except that the residual standard deviations are moderated across genes to ensure more stable inference for each gene. The moderated standard deviations are a compromise

### TABLE 1
**Summary of microarray results**

| Comparison                  | Gene modulated (-fold change; p value) |
|-----------------------------|----------------------------------------|
| Early G_{4} P4KO vs. P4WT  | Dcn (p = 8.95E-09)                      |
|                            | Rgs16 (+ 39.4; p = 6.38E-09)            |
|                            | Ezr2 (-3.6; p = 4.02E-05)              |
| Late G_{1}/S P4KO vs. P4WT | Dcn (+9.0; p = 3.25E-20)               |
|                            | Rgs16 (+15.1; p = 2.00E-18)            |
| PMCA4a-rescued vs. P4KO     | Tfp2b (-3.4; p = 2.5E-10)              |
|                            | Dcn (-1.2; p = NS)                     |
| PMCA4b-rescued vs. P4KO     | Rgs16 (+1.1; p = NS)                   |
|                            | Cdkn2b (p15) (-5.0; p = 8.51E-24)      |
|                            | Dcn (-2.6; p = 1.09E-13)               |
|                            | Rgs16 (-18.5; p = 3.07E-19)            |
between the individual gene-wise standard deviations and an overall pooled standard deviation. We evaluated the false discovery rate using the Benjamini multiple testing procedure (19). Our PMCA4 microarray data have been placed in the PubMed microarray database GEO under accession number GSE38320.

**Cloning Mouse PMCA4 Splice Variants**—cDNA constructs encoding PMCA4a, PMCA4b, and the novel splice variant termed PMCA4e were cloned from total RNA extracted from mouse bladder, the murine VSMC cell line MOVAS, and mouse brain, respectively. Sequence analysis showed that the PMCA4e cDNA clone contains a novel exon-20 sequence that is 10 nucleotide longer than the evolutionarily conserved 181-nucleotide sequence of other mammalian PMCA4a variants. These additional 10 nucleotide result in a premature stop codon being created in the mature, spliced PMCA4e mRNA. The predicted amino acid sequences at the C terminus of the PMCA4e pre-mRNA that generates PMCA4a, -4b, and -4e splice variants. To separate the consequences of transcriptional regulation (via the endogenous PMCA4 promoter) from those of alternative splicing modulation, the relative proportions of each PMCA4 splice variant were calculated from absolute copy number data obtained by quantitative RT-PCR. We reasoned that promoter up- or down-regulation would increase the absolute copy numbers of all alternatively spliced transcripts (so that analysis using absolute copy numbers would tend to reflect the effects of promoter regulation), whereas changes in the relative proportions of each PMCA4 splice variant would accurately reflect regulation of alternative splicing. Others engaged in studies of alternative splicing have also used this strategy (20).

In this manner we determined the relative proportions of PMCA4 splice variants in various mouse organs (Fig. 1A). We found that mouse organs could be grouped into three categories: organs predominantly expressing PMCA4e (brain), organs predominantly expressing PMCA4a (bladder), and organs predominantly expressing PMCA4b (all other organs tested), excluding liver, which showed negligible PMCA4 expression. We then used our PMCA4b and PMCA4a/e antibodies to probe PMCA4 splice variant proteins (Fig. 1B) and found that the organs surveyed also displayed distinct PMCA4a/e and PMCA4b expression profiles.

**PMCA4 Splice Variant-specific Antibodies**—The PDZ binding domain lies within the last seven amino acids of the carboxyl terminus of mouse PMCA4b (viz. PSLETPV), which is absent from the mouse PMCA4a tail. Two peptides were designed (Aves Labs); one coding for the PMCA4b carboxyl tail and the other coding for an exon-20-specific epitope expected in both PMCA4a and PMCA4e variants (supplemental Fig. S1B), which corresponds to the carboxyl end of the calmodulin binding motif.

Hearts from homozygous Nesyse laboratory-generated PMCA4 knock-out adult mice (PMCA4-/-) and littermate wild-type controls (PMCA4-/-) as well as C57BL6/J mice were used in genomic PCR (14). This confirmed the genetic deletion of PMCA4 (exons 2–3) in knock-out hearts, with the 816-bp PMCA4 (exon3–4)-specific band identified only in hearts from PMCA4-/- and C57BL6/J mice (supplemental Fig. S2A). Next, Western blots revealed that the PMCA4b antibody recognized a ~130-kDa protein from brain, stomach, and heart of C57BL6/J mice but did not bind to protein extracts made from PMCA4-/- heart (supplemental Fig. S2B). Similarly, the PMCA4a/e antibody detected a ~130-kDa protein in extracts from C57BL6/J brain and stomach but not heart from C57BL6/J mice, presumably because of very low or absent PMCA4a/e expression in the normal mouse heart.

**PMCA4 Splice Variants Show Differential mRNA and Protein Expression Levels in Specific Mouse Organs**—Having previously demonstrated a functional consequence of PMCA1a and PMCA4b overexpression (10, 11), we were interested in exploring the regulation of alternative splicing at splice site C in the PMCA4 pre-mRNA that generates PMCA4a, -4b, and -4e splice variants. To separate the consequences of transcriptional regulation (via the endogenous PMCA4 promoter) from those of alternative splicing modulation, the relative proportions of each PMCA4 splice variant were calculated from absolute copy number data obtained by quantitative RT-PCR. We reasoned that promoter up- or down-regulation would increase the absolute copy numbers of all alternatively spliced transcripts (so that analysis using absolute copy numbers would tend to reflect the effects of promoter regulation), whereas changes in the relative proportions of each PMCA4 splice variant would accurately reflect regulation of alternative splicing. Others engaged in studies of alternative splicing have also used this strategy (20).
showed that injured media and remodeled adventitia have a higher proportion of the PMCA4b transcript as compared with uninjured media, whereas a sample from bladder smooth muscle expressed a higher proportion of PMCA4a variant (supplemental Fig. S3).

IHC confocal imaging showed the expression of a smooth muscle marker (sm22α/H9251) in uninjured and injured carotid artery cross-sections, whereas trichrome staining showed increased collagen deposition in remodeled adventitia (Fig. 3A). IHC staining with a proliferation marker (proliferating cell nuclear antigen) was only visible in injured arteries. Consistent with qRT-PCR and Western blot, IHC showed a decrease in PMCA4a/e and an increase in PMCA4b staining in injured carotids as compared with uninjured arteries that was further corroborated by quantifying immunofluorescent intensities with PMCA4 splice variant-specific antibodies in injured and uninjured medial layers (Fig. 3B).

PMCA4 functional knock-out mice kindly provided by Gary Shull (Cincinnati, OH) lack exon-11, which encodes the central catalytic loop (81 amino acids; residues 440–520; see “Materials and Methods”) (12), resulting in a protein product devoid of any Ca2+ efflux activity (12). In the current manuscript, these mice are referred to as P4KO mice, with their wild-type littermates termed P4WT. Carotid artery cross-sections from P4WT and P4KO mice at day 9 post-injury were used for morphometry (Fig. 3, C and D). There was a 2-fold decrease in the adventitia: media ratio of injured P4KO arteries compared with P4WT arteries. This decreased remodeling in P4KO mice suggested two interrelated possibilities: (i) absence of functional PMCA4 impairs VSMC proliferation in vivo, and (ii) increased proportion of PMCA4b observed in injured arteries may play a physiologically relevant role in VSMC proliferation.

**PMCA4 Knock-out Causes G1 Phase Arrest, Which Can Only Be Rescued by PMCA4 with Intact Ca2+ Efflux Activity**—To probe the correlation between relative proportions of PMCA4 splice variants and VSMC proliferation, we isolated primary carotid VSMC from P4WT and P4KO mice. [3H]Thymidine uptake assays using cell cycle-synchronized VSMC cultures showed a 70% reduction in G1 to S phase cell cycle progression of P4KO VSMC compared with P4WT cells (Fig. 4A). Similarly, BrdU-PI flow cytometry showed a 30% increase in G1-stage P4KO cells as compared with P4WT (Fig. 4B), suggesting a G1 stage arrest.

Importantly, electroporation of either mouse PMCA4a or PMCA4b expression constructs rescued the cell cycle phenotype of P4KO cells (Fig. 4B), i.e. PMCA4a- or PMCA4b-rescued P4KO VSMC manifest a 40–50% increase in S phase cells and a 50% reduction in G1 phase cells. Western blots confirmed (supplemental Fig. S7) that as a result of these electroporations, PMCA4a was overexpressed 1.5-fold in P4KO +4a cells, whereas PMCA4b was overexpressed 1.8-fold in P4KO +4b
cells as compared with P4KO cells. Although expression of a PMCA4b mutant lacking the PDZ binding domain was still able to rescue the G1 arrest of P4KO cells, expression of a PMCA4b-Asp mutant (with only 10% of the Ca2+/H11001 efflux activity of wild-type PMCA4) failed to rescue the G1 phase arrest of P4KO cells (Fig. 4B).

In a separate series of experiments assessing cell cycle progression at multiple time points after serum stimulation of G0-synchronized P4WT, P4KO, and PMCA4a- or PMCA4b-rescued cell populations, we confirmed that P4KO cells suffer from a G1 phase arrest that is completely alleviated by either PMCA4a or PMCA4b cDNA expression (Fig. 5). Together these data show that whereas specific PDZ binding domain-mediated PMCA4b/nNOS (21) or PMCA4b/CASK interactions (22) are not necessary for PMCA4b to regulate cell cycle, the presence of Ca2+ efflux activity is required for PMCA4 to mediate G1-to-S phase progression in VSMC.

**Microarray Analysis and Validation of Identified Targets**—Early G1 stage P4KO and P4WT cells, expression of a PMCA4b-Asp mutant (with only 10% of the Ca2+/H11001 efflux activity of wild-type PMCA4) failed to rescue the G1 phase arrest of P4KO cells (Fig. 4B).

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**Microarray Analysis and Validation of Identified Targets**—Early G1 stage P4KO and P4WT cells were collected via flow sorting (by gating for G0/G1 phase Hoechst-stained cells) and used for microarray analysis. This unbiased exploration revealed that Decorin (+69.1-fold; \( p = 8.9E-09 \)) and Rgs16 (+39.4-fold; \( p = 6.3E-09 \); Fig. 6A and supplemental Tables 2 and 3) were markedly increased in P4KO cells as compared with P4WT cells (Fig. 6A and supplemental Tables 2 and 3). Decorin is known to reduce cyclin D1 expression via the epidermal growth factor receptor (EGFR) or NFAT pathways (24–26), with NFAT activation also known to inhibit Rgs16 expression (27) (see the proposed model, supplemental Fig. S8).
suggested that the high Rgs16 expression observed in P4KO cells (as compared with P4WT cells) may be due to reduced NFAT expression or activation in P4KO cells. Consistent with this, immunoblot analysis showed that Decorin and Rgs16 levels were increased, and Cyclin D1 levels were decreased in P4KO cells as compared with P4WT cells (Fig. 7).

Similar results were observed in vivo. First, Decorin-staining intensity trended 18% lower in medial layers of injured as compared with uninjured carotid arteries of P4WT mice (0.82 ± 0.05 versus 1.00 ± 0.06; n = 3, p = 0.1), suggesting injury-induced decreases in Decorin expression accompanied the reduced PMCA4a:PMCA4b efflux activity ratio. Second, this pattern was lost in P4KO mice, in which carotid artery denudation injury failed to reduce Decorin expression (1.10 ± 0.03 versus 1.00 ± 0.04; n = 3, p = NS), supporting a role for PMCA4 in regulating Decorin expression in vivo. Indeed, similar to the microarray-documented difference in Decorin expression observed in primary VSMC cultures from P4KO versus P4WT mice, levels of Decorin expression were significantly higher in injured carotid arteries of P4KO animals as compared with P4WT (1.10 ± 0.03 versus 0.82 ± 0.05; n = 3, p = 0.01) (data not shown).

Because Decorin and Rgs16 have both been implicated in the NFAT pathway, we next examined mRNA levels of NFAT facilitator proteins Rcan1, Rcan2, and the NFAT target E2F2 in our microarray results (supplemental Table S4). Rcan2 was reduced −6.6-fold (p = 3.8E−08) and E2F2 was decreased −3.6-fold (p = 4.0E−05) in P4KO as compared with P4WT cells, suggesting diminished NFAT action. To examine this more directly, cytoplasmic protein extracts were probed for NFATc3 levels and found to be significantly decreased in P4KO cells as compared with P4WT cells (Fig. 7).

PMCA4a rescue of P4KO cells followed by late G1/S microarray analysis (Fig. 6, B and C) suggested some reversal of the expression levels of Decorin (PMCA4a rescue versus P4KO = −1.27-fold; p = NS) and Rgs16 (PMCA4a rescue versus P4KO = +1.1; p = NS); however, these changes did not reach statistical significance (supplemental Table S5). Rather, PMCA4a-rescued P4KO cells showed a sharp decline in the expression of a known anti-proliferative transcription factor 

viz. AP-2β (PMCA4a rescue versus P4KO = 44.6; p = 2.5E−10 (supplemental Table S5)). AP-2β inhibits the activity of AP-2α (28) and also represses c-Myc transactivation (29). AP-2α is also known to bind to the ErbB2 promoter (EGFR family member) and promote proliferation via the EGFR pathway (30) activated by vessel injury in vivo (31). AP-2β is also implicated in VSMC proliferation in vivo by its role in the closure of the ductus arteriosus (32).

Additional microarray analysis using RNA harvested from late G1/S-stage P4KO, P4WT; and PMCA4b-rescued- versus vector-transfected-P4KO cells showed that Decorin was downregulated −2.4-fold (p = 1.59E−13) in PMCA4b-rescued versus
P4KO cells (down 15.3-fold in PMCA4b-rescued versus control P4KO cells; \( p = 5.5 \times 10^{-22} \)), whereas it remained up-regulated 9.3-fold (\( p = 3.24 \times 10^{-20} \)) in P4KO versus P4WT G1/S stage cells (Fig. 6D; and supplemental Tables S6–S8). Rgs16 was also decreased 18.5-fold (\( p = 3.07 \times 10^{-19} \)) in PMCA4b-rescued versus P4KO cells (3.9-fold in PMCA4b-rescued versus vector-control P4KO cells; \( p = 9.2 \times 10^{-14} \)), whereas it continued to be increased 15.1-fold (\( p = 2.0 \times 10^{-18} \)) in P4KO versus P4WT at the G1/S stage (Fig. 6D; and supplemental Tables S6–S8). Interestingly, Cdkn2b (cyclin-dependent kinase inhibitor 2b; also known as p15 or Ink4b) was markedly decreased in PMCA4b-rescued cells as compared with P4KO cells (PMCA4b versus P4KO = −50.4; \( p = 8.51 \times 10^{-24} \)). p15 is a cyclin-dependent kinase inhibitor that forms a complex with CDK4 and prevents activation of Cyclin D1 (33).

Immunoblot analysis using late G1/S-stage cytoplasmic protein extracts showed that Rgs16 was increased in P4KO (and control vector-rescued P4KO) cells versus P4WT cells but decreased in PMCA4b-rescued P4KO cells (Fig. 7). Western blots also showed that the decrease in NFATc3 expression observed in P4KO cells was reversed by rescue with PMCA4b (Fig. 7C). Together, these data clearly demonstrate that PMCA4 splice variants rescue common Decorin, Rgs16, and NFATc3 alterations observed in VSMC lacking any PMCA4 expression (P4KO) and also that expression of PMCA4a and PMCA4b result in the regulation of unique downstream mediators.

DISCUSSION

In the current study we present evidence that PMCA4 gene deletion impairs cell cycle progression and causes a G1 phase arrest in VSMC. Furthermore, quiescent VSMC in vivo have roughly equal proportions of PMCA4a and PMCA4b splice variants, whereas proliferating VSMC in vivo appear to have relatively higher levels of PMCA4b than PMCA4a expression. This suggested differential roles for these two PMCA4 splice variants in the VSMC cell cycle. However, when P4KO VSMC were electroporated with either PMCA4a or PMCA4b cDNA, cells are rescued from the G1 phase arrest, and altered expression levels of Decorin and Rgs16 are corrected. Also, our data show that whereas the PDZ binding domain unique to PMCA4b (i.e. absent in PMCA4a) is not needed for this effect, \( \text{Ca}^{2+} \) efflux activity is required to rescue the G1 phase arrest of P4KO cells. This observation suggest that the \( \text{Ca}^{2+} \) efflux activity of PMCA4 is critical to G1-to-S-phase progression in VSMC but leaves open the possibility that other domains within the PMCA4 protein may also regulate the VSMC cell cycle.
Prior to our current results, a possible mechanism through which PMCA4 alternatively spliced domains could regulate cell cycle included PMCA4b binding to the tumor suppressor RASSFl (34), which mediates G1 cell cycle arrest via p21 expression (35) or via p53 and p16 accumulation (36). Instead of directly testing the involvement of such a candidate, we used an unbiased, open-ended microarray strategy to discover putative PMCA4 targets. This analysis strongly implicated two proteins (Decorin, Rgs16) previously not known as PMCA4-regulated targets capable of modulating cell cycle. Both of these proteins are markedly increased upon PMCA4 gene deletion, which is reversed by expression of PMCA4a or PMCA4b (but not vector DNA) in P4KO cells. Interestingly, both these PMCA4 target proteins participate in the NFAT pathway. Decorin is an established natural onco-suppressive factor and a novel ligand for EGFR (37, 38), which is increased in VSMC in vivo after vascular injury (31) and triggers a signaling cascade involving Akt and terminating in cyclin D1 induction (39). Decorin can inhibit EGFR, has been implicated in G1 phase arrest (by inducing p21 expression), and is deregulated in many types of human cancers (23, 24, 40). Decorin is also known to inhibit the VSMC cell cycle by triggering Myc degradation (41) and can also inhibit FosL1 (42). Under normal conditions FosL1 and Jun dimerize to form AP-1, which can induce the NFAT family of transcription factors (43), leading to cyclin D1 expression (26). Important confirmation of our in vitro microarray and Western blot results regarding Decorin included the novel finding that the decrease in Decorin expression observed in injured carotid arteries of P4WT mice is lost in P4KO mice, which manifest significantly higher levels of Decorin expression after injury.

Rgs16, the other putative PMCA4 target identified here, is known to block cell cycle progression by inhibiting the MAPK and Akt pathways and is also modulated by the NFAT pathway. Rgs16 overexpression in MCF7 breast cancer cells inhibits EGFR-mediated proliferation and Akt phosphorylation, whereas shRNA-mediated extinction of Rgs16 augments cell growth (44). Transient as well as stable Rgs16 expression in CHO cells can attenuate IP3 production and p38 MAPK activation by platelet-activating factor receptor (45). In T cells, PKC activation induces TNFα, which increases Rgs16 expression. Importantly, the calcineurin/NFAT pathway can repress this effect on Rgs16 (27).

Our results suggest that PMCA4a (supplemental Fig. S8) as well as PMCA4b (supplemental Fig. S9) function to limit Decorin and Rgs16 expression (as well as increasing NFATc3 and cyclin D1 expression) and that PMCA4a may additionally function via inhibiting AP-2β expression (which de-represses c-Myc and induces EGFR expression), whereas PMCA4b may work via decreasing the CDK4 inhibitor p15, allowing cyclin D1 to activate CDK4.

It is indeed surprising that although PMCA4a and PMCA4b proteins show very different expression patterns in quiescent and injured carotid arteries in vivo, both these splice variants rescue the G1 arrest prevalent in proliferating primary VSMC derived from the carotid arteries of P4KO mice. Potential explanations include the following. (i) Our rescue of P4KO cells was with the PMCA4x/a splice variant, whereas the PMCA4a splice variant actually expressed in quiescent carotid artery media may be PMCA4z/a. RNA-Seq of injured and uninjured carotid arteries would be required to explore this possibility as would the cloning and rescue by a PMCA4z/a (and PMCA4z/b) splice variant in vitro. (ii) The P4KO mice we have employed express “non-functional” PMCA4a and PMCA4b. As such, endogenous non-functional PMCA4b proteins may form multimeric complexes with exogenous PMCA4a enabling the non-functional PMCA4b molecules to regain normal Ca2+ efflux activity within complexes functionally defined by the protein-protein interactions of PMCA4b. In this scenario, although P4KO cells were electroporated with PMCA4a, the cells were able to regenerate “functional” PMCA4b pumps associated with a normal Ca2+ efflux activity. (iii) Electroporation of PMCA4a into de-differentiated, proliferating P4KO VSMC in culture may not mimic differentiated, quiescent VSMC in vivo, where a balance between PMCA4a and PMCA4b expression levels might be the critical factor driving the differentiated, non-proliferative state. In this explanation, expressing PMCA4a alone in de-differentiated, proliferating VSMC may not be enough to regain the quiescent, non-proliferative state. To achieve the latter, a fine balance in the expression levels and possibly differential subcellular localization of the two splice variants of PMCA4a and PMCA4b might be what is required.

The eukaryotic cell cycle is known to be regulated by Ca2+ signals at various check points within the cycle (for review, see Ref. 1). Both voltage- and ligand-gated Ca2+ transporters are known to control cell cycle checkpoints by modulating intracellular Ca2+ signals or by hitherto unknown mechanisms involving macromolecular complexes with growth factor receptors, cell adhesion molecules, or cytoplasmic regulatory proteins (1). The latter may or may not depend on the Ca2+ transport function of these transporters but do involve the participation of their non-catalytic domains in signaling cascades that modulate cell cycle checkpoints. For example, the voltage-gated potassium channel subunit hERG1 (Kcnh2 or Kv11.1) regulates proliferation by a mechanism involving its recruitment into multiprotein membrane complexes including integrins and growth factor receptors (46). Other examples where Ca2+ channel domains not involved in the Ca2+ transport function regulate transcription via a protein signaling cascade include Cav1.2 (Cacna1c), the L-type Ca2+ channel 250-kDa α1C subunit that forms the Ca2+ transporting transmembrane pore. An endogenously cleaved 15-kDa carboxyl-terminal fragment of Cav1.2 (calcium channel-associated transcription regulator (or CCA1T)) translocates to the nucleus, binds to a nuclear protein, associates with endogenous promoters, and regulates the expression of a wide variety of endogenous genes including the bone morphogenetic protein inhibitor follistatin-like-1 (47, 48) protein and thus de-represses stem cell quiescence (45, 46). In a separate study involving the point mutation of the Cav1.2 calmodulin binding domain, it was shown that Ca2+/calmodulin binding to Cav1.2 enables specific activation of the Ras/MAPK pathway, CREB activation, and CREB-driven transcription (47). Importantly, it was discovered that the ability of Cav1.2 to generate a sustained [Ca2+]i rise was not needed for it to activate the Ras/MAPK/CREB pathway leading to gene transcription (47).
Further studies focused on elucidating the molecular mechanisms that allow PMCA4 to regulate the VSMC cell cycle are needed. The current study supplies the impetus for researchers examining PMCA s in other tissue systems to explore the roles of PMCA4a and PMCA4b in other cellular processes. The only other known example of physiological consequences of a PMCA4a/PMCA4b switch in vivo is the observed increase in relative PMCA4a splice variant expression (and decreased relative PMCA4b levels) during functional sperm maturation (49). In that model of differentiation, there was a steady shift from predominantly PMCA4b expression in the testis, caput, and corpus epididymis to predominantly PMCA4a expression in the cauda epididymis. It is interesting to note that differentiation (associated with lack of cell proliferation) appears to require an increase in the relative level of PMCA4a expression in this model of sperm maturation. This correlates with the observed decrease in relative PMCA4a expression in proliferating VSMC in vivo reported here and is reminiscent of the 2.5-fold reduction in rate of cell proliferation resulting from transient overexpression of PMCA1a splice variant in mouse VSMC (10) as well as the high relative proportion of PMCA4a variant in bladder smooth muscle cells (SMCs) (quiescent SMCs) and the roughly equal relative proportions of PMCA4a and PMCA4b in uninjured carotid artery SMCs (quiescent VSMC).

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