Calcineurin-independent regulation of plasma membrane Ca\textsuperscript{2+} ATPase-4 in the vascular smooth muscle cell cycle

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Calcineurin-independent regulation of plasma membrane Ca\textsuperscript{2+} ATPase-4 (PMCA4) expression in neurons, whereas c-Myb is known to repress PMCA1 expression in vascular smooth muscle cells (VSMC). Here, we describe a novel mouse VSMC line (MOVAS) in which \( ^{45}\text{Ca} \) efflux rates decreased 50\%, fura 2-AM-based intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]) increased twofold, and real-time RT-PCR and Western blot revealed a ~40\% decrease in PMCA4 expression levels from G\textsubscript{0} to G\textsubscript{1}/S in the cell cycle, where PMCA4 constituted ~20\% of total PMCA protein. Although calcineurin activity increased fivefold as MOVAS progressed from G\textsubscript{0} to G\textsubscript{1}/S, inhibition of this increase with either BAPTA or retroviral transduction with peptide inhibitors of calcineurin (CAIN), or its downstream target nuclear factor of activated T cells (NFAT) (VIVIT), had no effect on the repression of PMCA4 mRNA expression at G\textsubscript{1}/S. By contrast, Ca\textsuperscript{2+}-independent activity of the calmodulin-dependent protein kinase-II (CaMK-II) increased eightfold as MOVAS progressed from G\textsubscript{0} to G\textsubscript{1}/S, and treatment with an inhibitor of CaMK-II (KN-93) or transduction of a c-Myb-neutralizing antibody significantly alleviated the G\textsubscript{1}/S-associated repression of PMCA4. These data show that G\textsubscript{1}/S-specific PMCA4 repression in proliferating VSMC is brought about by c-Myb and CaMK-II and that calcineurin may regulate cell cycle-associated [Ca\textsuperscript{2+}], through alternate targets.

calcineurin; c-Myb; plasma membrane Ca\textsuperscript{2+}-ATPase-4; cell cycle

IN EUKARYOTIC CELLS, Ca\textsuperscript{2+} and Ca\textsuperscript{2+}/calmodulin-mediated signals act at multiple points in the cell cycle, including the G\textsubscript{0}/G\textsubscript{1} transition, the initiation of S-phase, and the initiation and completion of M phase (reviewed in Refs. 4, 31, 38, and 44). Of the four plasma membrane Ca\textsuperscript{2+}-ATPase genes (PMCA1–4) encoding pumps that extrude Ca\textsuperscript{2+} from cells (6, 35, 39), only PMCA1 and PMCA4 are ubiquitously expressed (43), and repression of both may be required for the reduced Ca\textsuperscript{2+} efflux rate and increased intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) needed for G\textsubscript{1}-to-S transitions in rat vascular smooth muscle cells (VSMC) (22, 23).

We previously showed that the cell cycle-associated repression of PMCA1 expression during G\textsubscript{0} to G\textsubscript{1}/S progression in rat VSMC is mediated by the c-Myb transcription factor (1). However, the mechanism(s) underlying the cell cycle-associated repression of PMCA4 had not been elucidated. Guerini et al. (18) demonstrated in mouse neurons that PMCA4 expression can be repressed by a calcineurin-dependent pathway. Given this result, we hypothesized that G\textsubscript{1}/S-associated repression of PMCA4 expression in VSMC may also be mediated by calcineurin. To explore these mechanisms in cell culture, we generated a clonal, immortalized mouse VSMC line (MOVAS). Immunostaining for smooth muscle-specific proteins such as SM22\alpha, calponin, smooth muscle-specific \( \alpha \)-actin and desmin, as well as SM22\alpha-promoter-driven enhanced green fluorescent protein (EGFP) expression, confirm the lineage and phenotype of these cells. \( ^{45}\text{Ca} \) efflux assays and fura 2-based ratiometric Ca\textsuperscript{2+} imaging reveal regulated Ca\textsuperscript{2+} efflux and [Ca\textsuperscript{2+}] at the G\textsubscript{1}/S transition point. Western blot and real-time RT-PCR reveal cell cycle-regulated repression of mouse PMCA1 and PMCA4. Drugs inhibiting calcineurin activity, such as the Ca\textsuperscript{2+}-chelating agent BAPTA and the calmodulin-dependent protein kinase-II (CaMK-II) inhibitor KN-93, and retroviruses encoding either a calcineurin-inhibitory peptide (CAIN), a peptide inhibitor (VIVIT) of the calcineurin-dependent nuclear factor of activated T cells [NFAT; a transcription factor inhibiting [Ca\textsuperscript{2+}]i], and PMCA4 expression during the MOVAS cell cycle.
MATERIALS AND METHODS

Establishment of a mouse vascular smooth muscle cell line. Aortic SMC were harvested from C57Bl6 mice (Charles River Laboratories, Wilmington, MA) as previously described (8) and grown for two passages in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS: CanSera, Rexdale, ON, Canada) and 1% penicillin-streptomycin (Canadian Life Technologies, Burlington, ON, Canada) in humidified air containing 5% CO2 at 37°C. These primary VSMC were transduced with retrovirus encoding the SV40 large-T antigen as well as the gene for neomycin resistance (5). Transduced cells were grown for 48 h, passed, and subjected to G418 selection at 400 μg/ml for 14 days. They subsequently underwent two rounds of clonal selection by limiting dilution. Three distinct clones were isolated and designated MOVAS-1, -2, and -3 (mouse vascular SMC). MOVAS-1 was selected for further study and has been maintained in medium with 200 μg/ml G418.

Cell migration. The migration rate of MOVAS cells was determined in a wound closure assay as previously described (27).

Immunofluorescence. Cells were stained with the following antibodies (Ab): FITC-conjugated anti-smooth muscle specific α-actin (1:200), anti-calponin (1:100), and anti-desmin (1:10) (all from Sigma, Mississauga, ON, Canada); anti-SM22α (1:100); rabbit polyclonal; Rolf Ryseck, Squibb, Princeton, NJ), Oregon Green-conjugated anti-Rabbit IgG (1:50; Molecular Probes, Eugene, OR), and FITC-conjugated antimouse IgG (1:50 dilution; Pierce, Rockford, IL). Slides were mounted and examined with a confocal microscope (RTS2000, Bio-Rad, Hercules, CA).

Calcium imaging. [Ca2+]i was measured using fura 2-AM as previously described (Photon Technology International, London, ON, Canada). Free [Ca2+]i was measured using fura 2-AM as previously described (22, 23). Free [Ca2+]i was measured by fluorescence ratio imaging using an Image-Master DeltaRAM system (Photon Technology International, London, ON, Canada) with an Olympus IX70 inverted microscope and an IC-200 intensified charge-coupled device camera. Actual [Ca2+]i (nM) were calculated from experimental ratios using established formulas derived from in situ calibrations with indo-1 and known [Ca2+]i after appropriate background subtraction (17).

Calcineurin- and c-Myb-responsive reporter assays. MOVAS cells were transduced with ANTI-MYB, CAIN (30), or VIVIT (2) for 24 h. Cells were then transfected with 2 μg of mimi-luc [a c-Myb-responsive luciferase reporter; courtesy of J. Lipsick, Palo Alto, CA (13)], 2 μg of mouse PMCA1-promoter-luciferase reporter (1), or 2 μg of a NFAT-IL-2 promoter-firefly luciferase reporter construct (NFAT-luc (37)) and 50 ng of a thymidine kinase promoter-rellina luciferase reporter (pRL-TK; Promega, Madison, WI) using Lipofectamine (Invitrogen, Burlington, ON). Cells were starved for 48 h posttransfection and harvested at 0 and 16 h post-transfection. Luciferase activity was measured with the DLR kit (Promega) with a luminometer (Berthold-Lumat; Wallac/Perkin-Elmer, Boston, MA). Raw relative light units (RLU) were corrected for transfection efficiency by subtracting the corresponding renilla luciferase-based RLU. The mean corrected RLU for nontransduced G0 cells was used to normalize corrected RLU for all other samples.

Retroviral constructs for CAIN, VIVIT, and anti-Myb. PA317 (ATCC no. CRL-9078, Manassas, VA) fibroblasts carrying a helper virus conferring retroviral packaging function [mamu-R5 (3)] were employed. Embracing vector MDR5 (courtesy of R. G. Hawley, Bethesda, MD), EGFP (BD Biosciences), CAIN [courtesy of J. Molkentin, Cincinnati, OH (10)] or VIVIT [courtesy A. Rao, Boston, MA (20)] encoding cDNAs were cloned into MINV while an anti-c-Myb antibody-encoding cDNA [courtesy of D. T. Curiel (28)] was cloned into a Dox-inducible retroviral expression vector (pTRE-Rev; BD Biosciences) to generate ANTI-MYB. These recombinant constructs were transfected into PA317 followed by drug selection to generate cell lines used for virion harvest.

CaMK-II activity assay. CaMK-II autophosphorylation activity assays were performed in vitro as previously described (46), with some modifications. Cytosolic (S-100) protein extracts were prepared from G0 and G1/S stage MOVAS cells treated or untreated with KN-93 (1 μM) for 16–24 h before harvest. For each sample, 100 μg of cytosolic extract were hybridized with 500 ng of anti-CaMK-II polyclonal antibody recognizing all CaMK-II isoforms (catalog no. SC9035; Santa Cruz Biotechnology, Santa Cruz, CA) and immunoprecipitated with protein G-Sepharose beads. The beads were exposed to 2.5 μCi [γ-32P]ATP (6000 Ci/mmol) and 0.1 μM calmodulin in kinase buffer (34) without exogenous substrate and with (Ca2+-dependent autophosphorylation) or without Ca2+ (Ca2+-independent autophosphorylation) at 30°C for 10 min, resolved on a 3–8% SDS-PAGE gradient gel, blotted onto nitrocellulose, and autoradiographed. NIH Image software was used to quantify intensities of the 53-kDa CaMK-II radiolabeled bands. Values were background subtracted and normalized to the untreated G0 value. In drug-treated samples, KN-93 (1 μM) was included during the protein extraction and the phosphorylation step of the activity assay. To measure Ca2+-independent CaMK-II activity, EGTA (2 mM) was added during the kinase step.

Pharmacological agents. For experiments employing induction of ANTI-MYB, Dox (0.5 μg/ml; Sigma) was added to the culture medium after the 24-h viral transduction step, and fresh aliquots of the drug were provided daily in fresh medium. BAPTA (5 μM) and the water-soluble form of KN-93 (1 μM) (Calbiochem, La Jolla, CA) were added to the culture medium for 16–24 h before assay.

Real-time RT-PCR. Total RNA was extracted (Nucleospin RNAII kit; BD Biosciences) from G0 and G1/S stage MOVAS cells. Known amounts of DNase-treated total RNA were used to amplify mouse PMCA1 and PMCA4 in separate real-time PCR assays (SYBR Green kit; Applied Biosystems, Foster City, CA). Reaction conditions of each primer set were opti-
mized to generate a single PCR product of expected size and melting temperature, and relative standard calibration curves were generated for each gene as per Protocol 4304965 (Applied Biosystems, Foster City, CA) and used to quantify the mRNA level of the PMCA4 and PMCA1 genes in MOVAS cells. Gene-specific real-time PCR primers employed were as follows: mouse PMCA1: 5'-TGA-GCA-GAA-GTA-CAC-CAT-GTC-AC-C-3' mouse PMCA4: 5'-CGA-GCC-GCA-CAC-ACT-GTG-TC-3'.

**RESULTS**

**VSMC-specific phenotype of MOVAS.** MOVAS cells display a spindle-like shape at low to medium confluence, migrate (mean migration rate = 29.7 μm/h), and show the presence of protein markers specific for SMC such as SMC-specific α-actin (Fig. 1), SM22α, and the actin filament-associated proteins desmin and calponin (data not shown). To confirm a transcriptional profile consistent with VSMC, EGFP expression driven by a VSMC-restricted SM22α promoter was monitored (pSE1–2; see MATERIALS AND METHODS). MOVAS cells showed abundant SM22α promoter-driven expression of EGFP at 72 h after transfection (Fig. 1), whereas CHO cells did not express EGFP when transfected with pSE1–2.

**S-phase entry of MOVAS.** Flow cytometry of BrdU-labeled MOVAS cells was conducted to assess our ability to growth arrest and synchronize entry into S phase. Serum deprivation for 48 h forced 63 ± 4% of cells into G0 phase, and only 16 ± 1% were in S phase under these conditions (n = 4 experiments; G0-0 h vs. S-0 h; P < 0.0001). The presence of some residual S-phase cells in the putative G0 samples is consistent with the known difficulty of perfectly synchronizing cells immortalized with large T antigen (32). However, upon serum stimulation, the proportion of cells in S phase increased steadily to reach a peak of 60 ± 4% after 16 h (n = 4; S-0 h vs. S-16 h; P < 0.0001), whereas the proportion of G0 cells decreased to 34 ± 4% (n = 4; G0-0 h vs. G0-16 h; P = 0.0038). Hence, most MOVAS cells enter S phase at ~16 h post-serum stimulation.

**Intracellular Ca2⁺ and Ca2⁺ efflux.** The Ca2⁺ efflux rate from MOVAS cells progressing through the cell cycle was assayed, and the contribution to this rate by PMCA activity was quantified. Inhibiting PMCA activity with La3⁺ yielded a 40% reduction in the total rate of Ca2⁺ efflux at G0 (Fig. 2A). The total Ca2⁺ efflux rate also dropped 50% as cell cycle-synchronized MOVAS cells moved from G0 to G1/S (P < 0.005), at which point further reductions with La3⁺ were not significant (Fig. 2A). These data demonstrate a decrease in La3⁺-sensitive 45Ca efflux activity during MOVAS cell cycle progression, a result consistent with previous observations in other cell lines (3, 22, 23).

Ratiometric fluorescence imaging was used to measure [Ca2⁺]i in MOVAS cells during G0-to-G1/S cell cycle progression. In nontransduced and untreated MOVAS, [Ca2⁺]i increased from 114 ± 5 nM at G0 to

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Fig. 1. Mouse vascular smooth muscle cell (VSMC) line (MOVAS) cells exhibit a smooth muscle cell-specific phenotype. A: MOVAS cells immunostained with smooth muscle-specific α-actin antibody and visualized by confocal fluorescence microscopy. B: spindle-shaped MOVAS cells imaged at a wavelength specific for the enhanced green fluorescent protein (EGFP, 507 nm) after transfection with a VSMC-specific SM22α promoter-driven EGFP reporter gene (C).
Inhibiting calcineurin activity with CAIN, VIVIT, and BAPTA. To monitor calcineurin-mediated transcriptional activity, we employed a NFAT promoter-luciferase reporter construct (NFAT-luc; see MATERIALS AND METHODS and Fig. 4A). Transient transfection assays showed that calcineurin activity rises approximately fivefold as MOVAS cells progress from G0 to the G1/S transition (Fig. 4B). Both CAIN and VIVIT encoding retroviral transduction suppressed this rise in calcineurin/NFAT activity, as did treatment with BAPTA (Fig. 4B). Of note, the CAIN- and VIVIT-mediated reductions in NFAT-dependent luciferase activity at G0, a time point where CAIN and VIVIT had no effect on MOVAS [Ca2+], suggest that these agents were acting as direct inhibitors of calcineurin/NFAT and not via indirect effects on [Ca2+]. By contrast, the reduction in calcineurin/NFAT-responsive reporter activity noted with BAPTA is likely dependent on its ability to chelate and lower [Ca2+].

Inhibiting calcineurin and CaMK-II activity with KN-93. The effect of the CaMK-II inhibitor KN-93 on calcineurin/NFAT-responsive reporter activity (Fig. 4B) may be due to both indirect effects on [Ca2+] and direct effects on reduced CaMK-II-dependent calcineurin activation. Indeed, KN-93 treatment reduced Ca2+-dependent CaMK-II activity by 33% at G0 (1.00 ± 192 ± 6 nM at G1/S (P < 0.001). Control transfections with empty virions or virions encoding EGFP reduced the G1/S [Ca2+] to 118 ± 4 nM. However, retroviral transduction with CAIN or VIVIT (Fig. 3) caused significant further reductions in [Ca2+]: [G1/S [Ca2+]] with CAIN = 75 ± 8 nM (P < 0.001) and with VIVIT = 95 ± 5 nM (P < 0.001) compared with control-transduced cells. Similarly, transduction with Dox-inducible ANTI-MYB reduced G1/S [Ca2+] level to 75 ± 3 nM (P < 0.001) upon addition of Dox (Fig. 3), whereas addition of Dox had no effect on [Ca2+] of nontransduced cells (G0 + Dox = 113 ± 11 nM, and G1/S + Dox = 195 ± 22 nM) or the [Ca2+] of control-transduced cells (data not shown). Thus inhibitors of calcineurin, NFAT, or c-Myb significantly reduced G1/S-associated [Ca2+]. Of note, treatment of nontransduced MOVAS with BAPTA or KN-93 also significantly reduced cytosolic Ca2+ levels at G1/S (Fig. 3). These data demonstrate a cell cycle-dependent increase in the [Ca2+] of MOVAS that can be blocked by peptide inhibitors of calcineurin and NFAT, a Ca2+-chelating agent, an inhibitor of CaMK-II, and the inducible expression of a neutralizing antibody to c-Myb.

CALCINEURIN-INDEPENDENT REPRESSION OF PMCA4

Fig. 2. Plasma membrane Ca2+ pump (PMCA)-dependent 45Ca efflux and PMCA protein levels in cell cycle-synchronized MOVAS. A: cell cycle-synchronized MOVAS cells were allowed to equilibrate with 45CaCl2 overnight in the presence or absence of La3+ (PMCA inhibitor), and Ca2+ efflux rates were measured at the G0 and G1/S stages as described in MATERIALS AND METHODS. A significant reduction in La3+-sensitive (i.e., PMCA-dependent) 45Ca efflux is observed during G0-to-G1/S cell cycle progression (*P < 0.01 vs. all other groups). La3+ treatment at G1/S does not further reduce the 45Ca efflux rate (G0/S vs. G1/S + La3+; P = not significant). B: microsomal protein was prepared from cell cycle-synchronized MOVAS as described in MATERIALS AND METHODS. Protein (50 μg) was resolved on a 5–15% SDS-PAGE gel, blotted onto nitrocellulose, and probed with nonisomeric specific anti-PMCA and anti-actin antibodies. Densitometric quantification revealed the top putative plasma membrane Ca2+-ATPase-1 (PMCA1) band (~132 kDa) to be 3.8-fold more abundant than the lower putative PMCA4 band (~126 kDa). However, both PMCA1 and PMCA4 bands showed a mean 40% reduction (n = 4, range 20–55%) in actin-normalized expression at G1/S vs. G0.

Fig. 3. Cell cycle-, calcineurin (CAIN)-, nuclear factor of activated T cells (NFAT)-, and c-Myb-dependent [Ca2+] in MOVAS. G1/S (16 h post-serum stimulation) synchronized cell populations were loaded with fura 2-AM, and the [Ca2+] was determined as outlined in MATERIALS AND METHODS. Data shown represent means ± SE (n = 10 cells for each experimental group). Effect of transduction with various viral constructs or treatment with KN-93 (1 μM) or BAPTA (5 μM) on [Ca2+] in MOVAS cells. *P < 0.001 vs. empty vector; #P < 0.001 vs. doxycyline (Dox)-inducible c-Myb neutralizing antibody (ANTI-MYB) without doxycyline (Dox); $P < 0.001 vs. untreated nontransduced MOVAS at G1/S (192 ± 6 nM); ±Dox = presence or absence of Dox (0.5 μg/ml). A significant decrease in [Ca2+] is noted at G1/S by CAIN, its downstream target NFAT VIVIT, BAPTA, KN93, and Dox-mediated induction of ANTI-MYB.
synchronized MOVAS (Fig. 6). Control reactions in which the reverse transcriptase had been omitted did not produce PCR products (data not shown), confirming that RNA samples were free of genomic DNA contamination. PMCA4 mRNA levels decreased by ~40% as cells progressed from G0 to G1/S (Fig. 6; G1/S value = 0.62 ± 0.01; P < 0.001 vs. nontransduced G0 cells). However, expression of CAIN (G1/S value = 0.66 ± 0.01) or VIVIT (G1/S value = 0.59 ± 0.01) had no significant effect on the G1/S-associated repression of PMCA4 expression (P = NS vs. empty vector). Although BAPTA had no significant effect on PMCA4 expression levels at G1/S, the CaMK-II inhibitor KN93 did appear to increase the expression of PMCA4 at this point in the cell cycle (0.87 ± 0.01; P < 0.001 vs. nontransduced cells). Together, these data suggest that calcineurin does not play a role in repressing cell cycle-dependent PMCA4 expression but that other targets of CaMK-II may play a role.

To exclude possible calcineurin-mediated effects on PMCA1, real-time RT-PCR was also performed for this pump. G0-normalized G1/S mRNA levels of PMCA1 were not significantly de-repressed with CAIN (0.82 ± 0.01; P = 0.17 vs. empty vector) or VIVIT (0.85 ± 0.03; P = 0.16 vs. empty vector), although the effect of these inhibitors was not as neutral on PMCA1 as it was on PMCA4.

The lack of a mouse-specific anti-PMCA4 antibody restricted our ability to specifically measure PMCA4 expression. PMCA4 mRNA levels were measured with quantitative real-time RT-PCR in cell cycle-synchronized MOVAS cells. CaMK-II autophosphorylation activity was assayed in cytosolic protein extracts from G0 and G1/S stage MOVAS treated or untreated with KN-93 (1 μM) for 16–24 h before harvest. Extracts (100 μg) were hybridized with anti-CaMK-II antibody, immunoprecipitated, incubated in kinase buffer with [γ-32P]ATP, resolved on a 3–8% SDS-PAGE gel, blotted onto nitrocellulose, and autoradiographed. Densitometric quantified intensities of 53-kDa CaMK-II radiolabeled bands were normalized to G0 values and are shown as means ± SE (n ≥ 4). EGTA (2 mM) was added to the kinase buffer to determine Ca2+-independent CaMK-II activity. Total and Ca2+-independent CaMK-II activities increased at G1/S (P < 0.05 vs. respective G0 values). KN-93 significantly inhibited both G0 and G1/S total CaMK-II activities (P < 0.01 vs. untreated G0 value).
protein in the MOVAS cell cycle. However, Western blots with the isofrom and species-nonspecific anti-PMCA antibody 5F10 detected a mean 40% decrease in the density of both putative PMCA1 and PMCA4 protein bands (as defined by molecular weight and relative abundance) at G1/S vs. G0 (Fig. 2B). These results are consistent with previous data in rat VSMC (23) and suggest that the transcriptional regulation of PMCA4 is translated into an effect at the protein level.

C-Myb activity and effects of ANTI-MYB on PMCA4 expression. Having previously demonstrated cell cycle-associated repression of PMCA mRNA expression in rat VSMC (23) and c-Myb-mediated repression of the mouse PMCA1 promoter (1), we sought to examine whether the observed repression of PMCA4 expression in MOVAS was also c-Myb-dependent. Using a c-Myb responsive reporter (mim-1-luc), we found that c-Myb-dependent reporter activity increased three- to sixfold as MOVAS moved from G0 to G1/S, which is consistent with data in other VSMC (22, 40, 41). Transduction with a mouse PMCA1 promoter-luciferase reporter (1) revealed a G1/S-specific repression of PMCA1 promoter activity (G0 = 1.0 ± 0.15 vs. G1/S = 0.49 ± 0.01; P < 0.05). Upon Dox-mediated induction of the anti-Myb antibody, the PMCA1 promoter was significantly de-repressed (G1/S without Dox = 0.49 ± 0.01 vs. G1/S + Dox = 0.94 ± 0.02; P < 0.001). We then explored the effect of ANTI-MYB on PMCA4 levels in proliferating MOVAS cells. Expression of the ANTI-MYB construct was observed to alleviate the G1/S-specific repression of PMCA4 (Fig. 6, G1/S: ANTI-MYB + Dox vs. ANTI-MYB without Dox = 0.85 ± 0.01 vs. 0.67 ± 0.01; P < 0.01).

DISCUSSION

CaMK-II and calcineurin are both involved in the Ca2+ and calmodulin-mediated signaling of growth regulation (31, 38, 44). In mammals, calcineurin mediates T cell and B cell proliferation (15, 24, 25, 36) and has been implicated in urotensin II-mediated proliferation of airway SMC (7), angiotensin II-induced cardiac fibroblast hyperplasia (12), and pituitary cell proliferation (11). Indeed, calcineurin has been shown to be essential for DNA synthesis in Swiss 3T3 fibroblasts (47), where inhibitors of calcineurin block the fibroblast growth factor-mediated expression of cyclins A and E (48).

Both c-Myb and calcineurin influence Ca2+-mediated signals of cell cycle (1, 22, 47, 48). A marked rise in [Ca2+]i is critically required for the G1/S transition of VSMC and is brought about by an increase in Ca2+ influx and a c-Myb-dependent reduction in Ca2+ efflux mediated via repression of PMCA1 (1, 22, 23, 40, 41). Indeed, ~60% of the c-Myb effect on VSMC proliferation is exerted via PMCA1 repression (22). However, PMCA4 also appeared to be regulated during the cell cycle progression of rat VSMC (23), and overexpression of PMCA4 had been shown to induce terminal differentiation of skeletal myoblasts (19). Given that PMCA4 expression levels in mouse neurons were repressed via calcineurin (18), we sought to examine whether this pathway participated in the cell cycle-regulated [Ca2+]i of mouse VSMC.

Primary VSMC cultures exhibit slow growth and cannot be used beyond a limited number of passages due to senescence and phenotypic changes occurring late in culture. Moreover, the use of primary cells is
labor intensive, expensive, and time consuming. Furthermore, an immortalized mouse VSMC line would allow complementary in vitro studies of mouse models of cardiovascular disease in which, for example, transgenic manipulations and/or their effects have been restricted to VSMC in vivo (26, 49). For these reasons, we developed and characterized a novel mouse VSMC cell line (MOVAS) and employed it in our study.

We showed that PMCA4 mRNA levels decreased twofold as MOVAS cells moved from G0 to the G1/S transition, and this correlated with a 50% reduction in Ca2+ efflux and a twofold increase in [Ca2+]i. We found a three- to sixfold rise in c-Myb activity at the G1/S transition of MOVAS cells and inhibiting c-Myb with the ANTI-MYB antibody de-repressed PMCA4 expression. Importantly, these data add to several lines of evidence supporting dynamic regulation of PMCA isoform expression in development, differentiation, proliferation, and in response to a variety of signaling pathways (reviewed in Ref. 43).

Our data show a fivefold rise in calcineurin activity as MOVAS progress from G0 to G1/S. Blocking the G1/S-specific increase in calcineurin activity with BAPTA, CAIN, or VIVIT reduced [Ca2+]i, but did not de-repress PMCA4. By contrast, inhibiting CaMK-II (with KN-93) or c-Myb (with ANTI-MYB) decreased [Ca2+]i, and also de-repressed PMCA4 at the G1/S transition. Although we cannot completely rule out a role of calcineurin in mediating some of the effect of KN-93, it appears more likely that the KN-93 mediated de-repression of PMCA4 occurs via a calcineurin-independent pathway. We speculate that calcineurin acts on regulators of cell cycle-associated [Ca2+]i, other than PMCA1 or PMCA4. For example, it may modulate the expression of the sarcoendoplasmic reticulum Ca2+-ATPase-2 (SERCA2) or the inositol 1,4,5-trisphosphate receptor type-1 (IP3R1). Indeed, calcineurin is known to increase transcription of IP3R1 in neurons (14, 16), and calcineurin has been shown to control calcium homeostasis in yeast by repressing SERCA activity (45). Further studies are needed to dissect the role of calcineurin in regulating cell cycle-associated [Ca2+]i in VSMC.

A particularly novel finding of our study is the potentially important role played by CaMK-II in cell cycle-dependent regulation of [Ca2+]i, and PMCA4 expression. Indeed, the eightfold increase in Ca2+-independent CaMK-II activity at G1/S, a measure of Ca2+/calmodulin-induced constitutive CaMK-II activity (20, 21), suggests significant cell cycle-dependent activation of CaMK-II. Further studies are needed to address the mechanisms through which CaMK-II subsequently mediates its effects on PMCA4 expression.

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REFERENCES

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19. Jayaraman T, Ondriasova E, Ondrias K, Harnick DJ, and Hanson PI and Schulman H.


