

Calcium/calmodulin-dependent protein kinase II- δ isoform regulation of vascular smooth muscle cell proliferation

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House SJ, Ginnan RG, Armstrong SE, Singer HA. Calcium/calmodulin-dependent protein kinase II- δ isoform regulation of vascular smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 292: C2276–C2287, 2007. First published January 31, 2007; doi:10.1152/ajpcell.00606.2006.—There is accumulating evidence that Ca²⁺-dependent signaling pathways regulate proliferation and migration of vascular smooth muscle (VSM) cells, contributing to the intimal accumulation of VSM that is a hallmark of many vascular diseases. In this study we investigated the role of the multifunctional serine/threonine kinase, calmodulin (CaM)-dependent protein kinase II (CaMKII), as a mediator of Ca²⁺ signals regulating VSM cell proliferation. Differentiated VSM cells acutely isolated from rat aortic media express primarily CaMKII γ gene products, whereas passaged primary cultures of de-differentiated VSM cells express primarily CaMKII δ_2 , a splice variant of the δ gene. Experiments examining the time course of CaMKII isoform modulation revealed the process was rapid in onset following initial dispersion and primary culture of aortic VSM with a significant increase in CaMKII δ_2 protein and a significant decrease in CaMKII γ protein within 30 h, coinciding with the onset of DNA synthesis and cell proliferation. Attenuating the initial upregulation of CaMKII δ_2 in primary cultured cells using small-interfering RNA (siRNA) resulted in decreased serum-stimulated DNA synthesis and cell proliferation in primary culture. In passaged VSM cells, suppression of CaMKII δ_2 activity by overexpression of a kinase-negative mutant, or suppression of endogenous CaMKII content using multiple siRNAs, significantly attenuated serum-stimulated DNA synthesis and cell proliferation. Cell cycle analysis following either inhibitory approach indicated decreased proportion of cells in G1, an increase in proportion of cells in G2/M, and an increase in polyploidy, corresponding with accumulation of multinucleated cells. These results indicate that CaMKII δ_2 is specifically induced during modulation of VSM cells to the synthetic phenotypic and is a positive regulator of serum-stimulated proliferation.

calmodulin kinase II; phenotype modulation

VASCULAR SMOOTH MUSCLE (VSM) cells (VSMCs) in the medial wall of blood vessels are quiescent and differentiated for contraction and regulation of blood vessel tone. However, VSMCs can undergo remarkable changes in phenotype, contributing to the development of atherosclerotic plaques or neointimal hyperplasia following balloon angioplasty and vascular grafting (33). Transition of differentiated VSMCs to a “synthetic” phenotype is characterized by downregulation of smooth muscle (SM)-specific contractile proteins and upregulation of proteins involved in matrix remodeling, DNA synthesis, cell migration, and proliferation (33, 46). A similar mod-

ulation of phenotype is observed in vitro following primary culture of freshly isolated VSMCs (33).

The molecular mechanisms governing phenotypic modulation of VSM are still incompletely understood. Most studies have focused on an identification of environmental factors that trigger changes in phenotype (e.g., growth factors and cytokines, oxidative stress, mechanical stress, and endothelial factors) or on transcriptional regulation of phenotype-specific genes. A few studies have raised the possibility that changes in intracellular signaling components may also contribute to VSM phenotype modulation. For example, cGMP-dependent protein kinase has been shown to be downregulated in synthetic VSMCs, and re-expression promotes characteristics of the differentiated contractile phenotype (20). Subcellular localization of phosphodiesterase 1A, which selectively hydrolyzes cGMP, was recently shown to change upon VSM phenotype modulation in vitro, with a nuclear localization promoting the synthetic phenotype and a cytosolic localization promoting a differentiated contractile phenotype (29). Changes in Ca²⁺-signaling dynamics have been linked to a transition of cerebral VSM to a proliferative phenotype in explant cultures (48). One mechanism may involve differential activation of cAMP-response element-binding protein and nuclear factor of activated T cell transcription factors in response to specific sources or dynamics of the Ca²⁺ signal (2, 9). Other alterations in ion channel expression (19, 30, 47, 48) that may affect Ca²⁺ signaling have also been demonstrated following VSMC culture and conversion to a proliferative phenotype. These studies support the concept that altered patterns of Ca²⁺ signaling may be an important component of VSM phenotype modulation and contribute to the regulation of phenotype-specific cell functions.

The varied actions of Ca²⁺ signals are partly mediated through Ca²⁺/calmodulin (Ca²⁺/CaM)-dependent serine/threonine kinases, including multifunctional CaM-dependent protein kinase II (CaMKII) (42). In mammals, CaMKII subunits are encoded by four differentially expressed genes (α , β , γ , δ) that undergo extensive alternative splicing, resulting in variable domains with demonstrated intracellular targeting and protein interaction functions. For example, the CaMKII δ_3 , or CaMKII δ_B , variant first identified in rat aortic (40) and cardiac cells (10, 40) has been shown to target CaMKII to the nucleus (43). In addition, overexpression of CaMKII α confers cytoplasmic localization, whereas CaMKII β variants have been shown to differentially associate with actin filaments (11). Separate studies identified a cytosolic targeting domain in

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specific γ and δ isoforms (6). Because CaMKII is expressed as large homo- and heteromultimeric holoenzymes, the subunit composition may confer subcellular targeting and specific protein interactions directing enzymatic activity to specific cellular compartments and functions (4, 6, 43).

In the vasculature, differentiated VSMCs express mRNA for both CaMKII γ and δ variants (40, 41), and γ -subunits have been implicated in the control of differentiated contractile function (18, 24, 39). However, cultured arterial VSMCs mainly express the δ_2 isoform (40), and in this setting, CaMKII δ_2 is an intermediate in the Ca²⁺-dependent activation of nonreceptor tyrosine kinases and ERK1/2 (1, 14). CaMKII δ_2 facilitates adhesion-dependent activation of ERK1/2 in cultured VSM through both integrin-dependent and -independent mechanisms (22) and has been shown to be involved in the regulation of cultured VSMC migration (35, 36). CaMKII has been implicated in cell-cycle control in a number of systems, although most studies to date have relied on pharmacological inhibitors of CaMKII, such as KN-62 (49) and KN-93 (45), or overexpression of constitutively active mutants (3, 37). Recently, small-interfering RNA (siRNA) suppression of CaMKII γ was reported to disrupt regulation of mitotic spindles in several human cell lines (17). The function of CaMKII in regulating VSMC proliferation has not been reported, although heparin, a selective inhibitor of VSM proliferation, inhibits serum-stimulated activation and autophosphorylation of CaMKII in VSM (27).

The present studies were designed to evaluate the role of CaMKII in control of VSMC proliferation using molecular approaches to inhibit or suppress endogenous activity. The results demonstrate that the transition of VSMCs from a contractile to synthetic phenotype in primary culture is accompanied by marked and rapid changes in CaMKII isoform expression and that upregulation of the CaMKII δ_2 isoform contributes positively to initiation of VSMC proliferation. Studies in passaged VSMCs confirmed the function of CaMKII δ_2 to positively regulate cell proliferation through effects at the G2/M transition and/or cytokinesis. Overall, the results support the emerging concept that regulation of VSM Ca²⁺ signals and signaling intermediates plays an important role in phenotype modulation and function.

MATERIALS AND METHODS

Antibodies and other materials. Production and specificity of the antibodies used for detection of the δ_2 isoform of CaMKII (40) and pan-CaMKII isoforms (41) were described previously. Anti-peptide antibody corresponding to amino acids 567LNVHYHCSGAPAAPL₅₈₁ of all CaMKII γ subunits was obtained through the services of Quality Controlled Biochemicals (Hopkinton, MA). Monoclonal antibodies specific for β -actin, α -SM actin, calponin, and GAPDH were purchased from Sigma (St. Louis, MO). Polyclonal antibody specific for SM-myosin heavy chain (MHC) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All cell culture media and supplies were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise specified. All other chemicals were purchased from Sigma. Purified recombinant protein standards for CaMKII γ_b , γ_c , and δ_2 were a generous gift from Dr. Roger Colbran (Vanderbilt University).

VSMC culture. VSMCs were obtained from the medial layer of the thoracic aortas of 300–350 g Sprague-Dawley rats (Taconic Farms, Germantown, NY) as previously described (1, 35, 40). Following the removal of the adventitial and endothelial layers, medial VSMCs were enzymatically dispersed and cultured in DMEM-Ham's F-12 medium

with 10% fetal bovine serum (FBS) or 0.4% where indicated. The VSMCs were maintained at 37°C in a 5% CO₂-95% air atmosphere. For studies involving phenotypic modulation, cells were not passaged and were only used through 120 h. Fresh media was added at 72 h. Studies utilizing cultured cells involved cells from passages 3–10 (split twice weekly). The use of experimental animals for these procedures was reviewed and approved by the Albany Medical College Institutional Care and Use Committee.

Western blot analysis. Cells were lysed in 50 mM Tris, 50 mM NaF, 0.1 mM Na₃VO₄, and 0.5% Nonidet P-40 (pH 7.4) and protein quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL). Protein (15 μ g) was mixed with an equal amount of concentrated sample buffer, heated at 95°C for 10 min, and centrifuged at 10,000 g for 2 min at 22°C (Allegra 21R Centrifuge, Beckman Coulter). Proteins were resolved by 10% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. Blots were blocked in 5% nonfat milk-Tris-buffered saline with Tween 20 (TBST) for 30 min at 22°C, incubated with recommended dilutions of primary antibody in TBST for 1 h at 22°C, and washed three times with TBST. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 1 h at 22°C and washed three times with TBST before detection using a commercial chemiluminescence kit (Amersham, Piscataway, NJ) and exposure to X-ray film. Western Blot signals were quantified using a GS700 Scanning Densitometer and Multi-Analyst Software (Bio-Rad, Hercules, CA).

RT-PCR. RNA was extracted and purified using the *mirVana* miRNA Isolation Kit (Ambion, Austin, TX). The RNA concentration was measured by absorbance at 260/280 nm with a Beckman Coulter DU 640 spectrophotometer, and 1 μ g was analyzed on a 1% formaldehyde gel. The gel was imaged using the Gel Doc 2000 (Bio-Rad). RNA (1 μ g) was converted to cDNA using RT and 0.2 μ g random hexamer primers (Invitrogen, Carlsbad, CA) with Ready-TO-Go beads (Amersham). cDNA (60 ng) was amplified on an iCycler (Bio-Rad) using quantitect SYBR Green Master Mix (Qiagen, Valencia, CA) with the standard protocol defining cDNA, primer, distilled H₂O, and Master Mix concentrations. A melt curve was performed to ensure a single product and starting concentrations calculated by the instrument software based on the threshold cycle relative to a standard curve of purified linearized (*Hind*III, New England BioLabs, Ipswich, MA) plasmid for that gene. Values were normalized to GAPDH starting concentrations. Plasmids were generated using standard protocols for TOPO-cloning and chemical transformation (Invitrogen) and purified (Qiagen). Oligo Toolkit (<http://www.operon.com/oligos/toolkit.php>) was used to determine the melting temperature (T_m) for primer and probe sequence and primer-dimerization sequences. The specificity of primer sets was verified by using basic local alignment search tool (BLAST, National Center for Biotechnology). Primer sets and accession numbers used were as follows: for CaMKII δ_2 (X75774₄₀₂₋₆₂₀), the sense primer was 5'-GGAAATGGAATGCCAAAGACAATG-3' and the antisense primer was 5'-CTGTTGACAATTAGAAGACCCAAATG-3'; for CaMKII γ (J04063₄₈₋₁₅₂), the sense primer was 5'-GCCACCTGCACCCGCTTCAC-3' and the antisense primer was 5'-CTCCTGCGTTCGAGTTTCTT-3'; for α -SM actin (X068014₈₋₁₂₁), the sense primer was 5'-GTCGCCATCAGGAACCTCGAGAAC-3' and the antisense primer was 5'-CATCACCAGCAAAGCCCCGCTTAC-3'; for SM-MHC (X16262₁₈₇₆₋₂₀₁₁), the sense primer was 5'-GACACTATGT-CAGGGAAAAC-3' and the antisense primer was 5'-CTTTGTG-CAGGGCTGTGGTTGAC-3'; and for SM-22 α (X71070₁₃₁₋₂₅₀), the sense primer was 5'-GAGCGGCTAGTGGAGTGGATTG-3' and the antisense primer was 5'-GTTCCACCAACTTGCTCAGAATC-3'. GAPDH primers and plasmid containing GAPDH were a gift from Dr. Rebecca S. Keller (Albany, NY). The predicted size of the product was confirmed by running equal amounts of PCR products out on a 10% SDS-PAGE, visualized by staining with ethidium bromide and imaged using the GelDoc 2000 (Bio-Rad).

siRNA targeting CaMKII δ isoforms and adenovirus construction. The siRNA Target Finder and Design Tool (Ambion) was used to select specific sequences targeting the rat CaMKII δ isoforms (GenBank accession no. NM_12519). Potential siRNA targets were subjected to BLAST searches against expressed sequence tag libraries to confirm specificity and multispecies identity. To obtain high efficiency of silencing in VSMCs, adenoviruses expressing short hairpin (sh) green fluorescent protein (shGFP) control and shDELTA, which targets all CaMKII δ isoform variants, were produced using the pSuppressorAdeno system (Imgenex, San Diego, CA) as previously described (22). shTAIL, which targets the alternatively spliced COOH-terminus of CaMKII $\delta_{1-4,9}$, was produced in the same manner with the following target sequence: 5'-AAGCCACCCTGTATTC-CAAAT-3', nt 1,556–1,576. The shDELTA2 construct also targeted a conserved sequence in CaMKII δ variants: 5'ATAAACCAATCCCACTAT-3', nt 1,543–1,562. shDELTA2 was cloned into an adenoviral construct using the AdTrack vector (a generous gift from Dr. Ling-Jun Zhao) (50) and AdEasy system (Stratagene, Cedar Creek, TX). Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Viruses were amplified and purified as previously described (31).

Adenoviral infection. VSMCs at 70% confluence were infected at a multiplicity of infection (MOI) of 50 with purified adenovirus shDELTA2, shGFP, kinase-negative CaMKII δ_2 (36), or β -galactosidase (provided by Dr. Rebecca S. Keller) in the presence of 10% FBS medium and 1 mM antenpedia peptide. For experiments involving shTAIL, shDELTA, and shGFP, a MOI of 100 was utilized. In experiments using freshly dispersed cells, cells were allowed to plate for 1 h and were infected with purified shDELTA2 or shGFP at a MOI of 50 in the presence of 10% FBS medium.

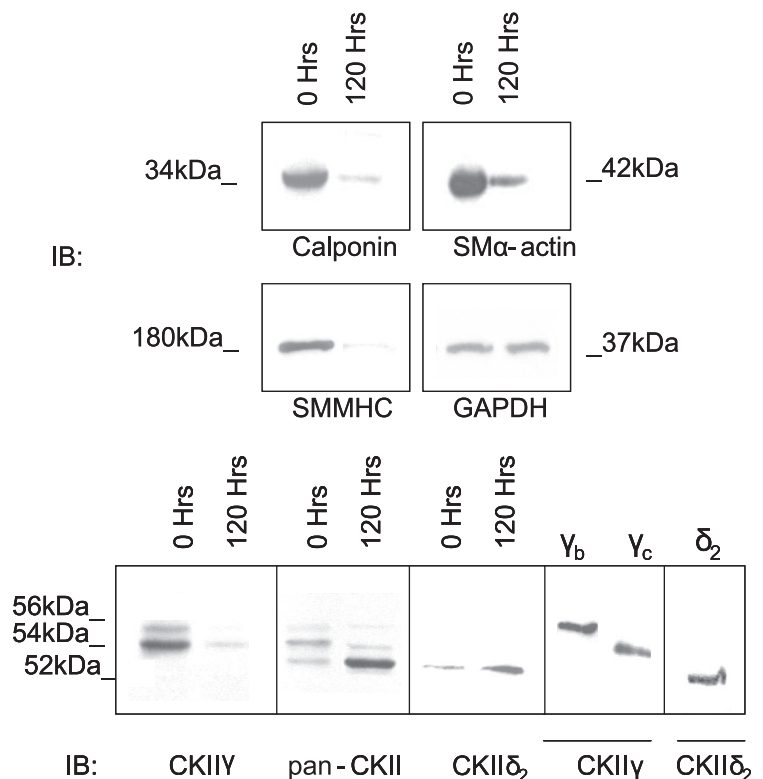
Cell count analysis. Cells were trypsinized at indicated time points, and cell counts were analyzed using a Beckman Particle Counter. DNA proliferation was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation into the DNA of proliferating cells. Following 2 h of incubation into cellular DNA, BrdU was detected by enzyme immu-

noassay in a 96-well format (Roche, Indianapolis, IN). To quantify the percentage of multinucleated cells, VSMCs were seeded on glass coverslips in a temperature- and CO₂-controlled chamber in their standard growth media. At the indicated time points, cells were washed for 5 min in phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 30 min. Coverslips were mounted on glass slides with Vectashield Hard Set Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA). Phase-contrast and fluorescence images were obtained using a Leica DM IRB-inverted microscope (Leica Microsystems). The percentage of multinucleated cells was calculated by dividing the total number of multinucleated cells by the total number of cells. The percentage of infected cells that were multinucleated was calculated by dividing the total number of GFP-labeled cells by the total number of multinucleated cells.

Apoptosis assay. Cells were seeded on glass coverslips and fixed with 4% paraformaldehyde. Cells were permeabilized in 0.01% Triton-X in 0.01% sodium citrate and labeled with TMR red according to package instructions (In Situ Cell Death Detection Kit, Roche). Coverslips were mounted on glass slides with 50% glycerol and analyzed via fluorescence microscopy using an excitation wavelength in the range of 570–620 nm. Positive controls were generated by incubating fixed and permeabilized cells with 3,000 U/ml DNase 1 (Invitrogen) in 50 mM Tris·HCl (pH7.5) and 1 mg/ml BSA for 10 min to induce DNA strand breaks.

Fluorescence-activated cell-sorting analysis. Cells were trypsinized and collected in 12 × 75-mm centrifuge tubes and centrifuged at 1,200 rpm for 4 min at 4°C. Pellets were washed twice with PBS and resuspended in 5 ml PBS. Following centrifugation, cells were resuspended in 0.5 ml PBS and transferred into tubes containing 70% ethanol at 4°C. Cells were fixed for 2 h. The ethanol-suspended cells were centrifuged and resuspended in 5 ml PBS. Cells were suspended in 1 ml propidium iodide/Triton X-100 staining solution with RNase A and kept for 30 min at room temperature. DNA profiles of propidium iodide-stained cells were read on a BD FACSCanto (488–

Fig. 1. Modulation of calmodulin-dependent protein kinase II (CaMKII) isoforms and differentiation markers in freshly dispersed vascular smooth muscle (VSM) cells. VSM cell lysates were prepared 0 and 120 h after dispersion and immunoblotted (IB) with antibodies for VSM differentiation markers calponin, smooth muscle α -actin (SM- α -actin) and smooth muscle myosin heavy chain (SM-MHC) (*top*). CaMKII expression was determined with antibodies recognizing all CaMKII isoforms (pan-CKII) and specifically recognizing CaMKII γ (CKII γ) and CaMKII δ (CKII δ_2) isoforms. Immunoblotting of the VSM lysates for GAPDH was performed to compare protein loading. Purified recombinant CaMKII γ_B , γ_C , and δ_2 protein standards are shown at the right (*bottom*).



633-nm lasers) flow cytometer, and image acquisition and analysis were performed using BD FACSDiva software (BD Biosciences, San Jose, CA). FlowJo software (version 7.1, Tree Star) was used for statistical analysis of DNA profiles.

Statistical evaluations and comparisons. All data are expressed as means \pm SE. Mean values of groups were analyzed using GraphPad PRISM version 4.00 and compared by ANOVA with post hoc comparisons using the Newman-Keuls test. For all comparisons, $P < 0.05$ was considered statistically significant.

RESULTS

CaMKII isoform expression in primary-cultured aortic VSM. We have previously observed marked differences in CaMKII isoform content in differentiated VSM where CaMKII γ isoforms predominate (41) and in cultured VSM where the CaMKII δ_2 isoform predominates (40). The present studies were designed to test the hypothesis that upregulation of the CaMKII δ_2 isoform is functionally important for the modulation of rat aortic VSMCs to a proliferative phenotype during primary culture.

Following 120 h of primary culture, aortic VSMCs significantly downregulate α -SM actin, SM-MHC, and calponin content, two markers of the differentiated contractile phenotype (Fig. 1, top). In addition to the loss of contractile protein markers, by this point in primary culture the cells are also proliferative (see Fig. 3A) and therefore have classical attributes of the synthetic phenotype. Western blot analysis with an antibody recognizing a conserved sequence in all CaMKII isoforms (pan-CKII) indicates a marked shift in isoform composition concurrent with changes in contractile protein expression (Fig. 1, bottom). By blotting the lysates and recombinant isoform controls with custom antibodies specifically recognizing CaMKII γ subunits and δ subunits (see MATERIALS AND METHODS), we have tentatively identified a minor 56-kDa subunit as CaMKII γ_B , a major 54-kDa subunit in rat aortic VSM as CaMKII γ_C , and a 52-kDa subunit as CaMKII δ_2 (40) (Fig. 1). Results with these isoform-selective antibodies clearly indicate a significant loss in γ -subunit content and a gain in δ -subunit content following 120 h of primary culture, with changes quantified in Fig. 2A. Changes in contractile protein and CaMKII isoform content were mirrored by parallel changes in mRNA content as indicated by quantitative RT-PCR (Fig. 2B). Notably, there is a significant decrease in CaMKII γ mRNA and an increase in CaMKII δ_2 mRNA after 120 h of primary culture. Thus acute modulation of VSM from a differentiated to synthetic phenotype includes marked changes in CaMKII isoform expression and content, including upregulation of CaMKII δ_2 .

To better understand the relationship of CaMKII modulation to the onset of VSMC proliferation, isoform content was analyzed at regular intervals beginning 15 h after plating the primary dispersed cells. A volume analysis of Western blots probed with the pan-CaMKII antibody was used to estimate the relative content of CaMKII γ_C (54 kDa) and CaMKII δ_2 (52 kDa). The analysis indicates that the relative decrease in CaMKII γ_C and increase in CaMKII δ_2 protein content are rapid in the onset following the plating of cells with a statistically significant ($P < 0.01$) change in the distribution within 30 h, progressing to a maximal change within 60 h (Fig. 3A). CaMKII isoform modulation coincided closely with an increase in cell proliferation. After an initial loss of cells at

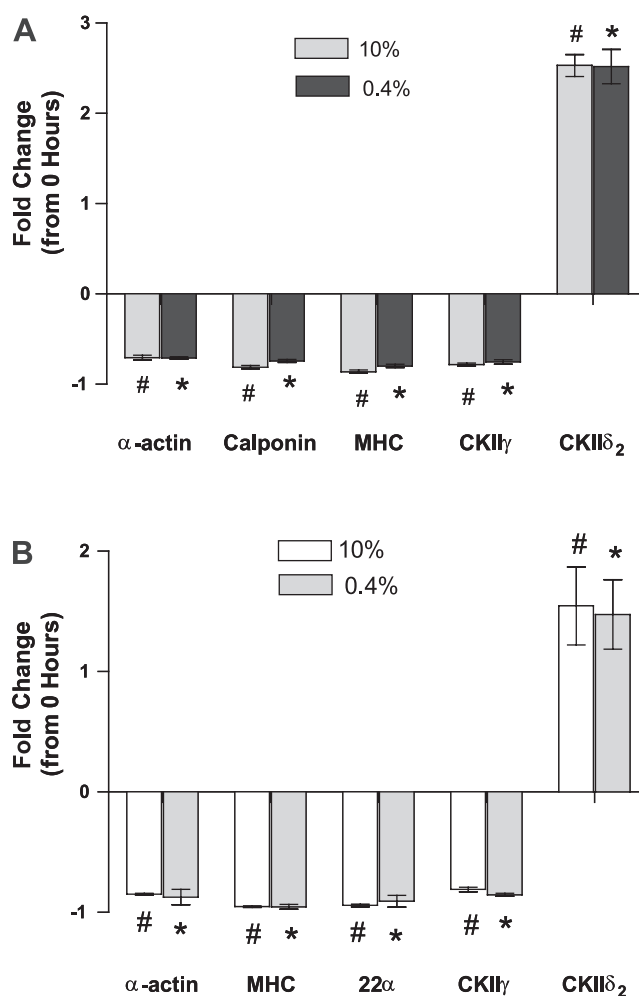
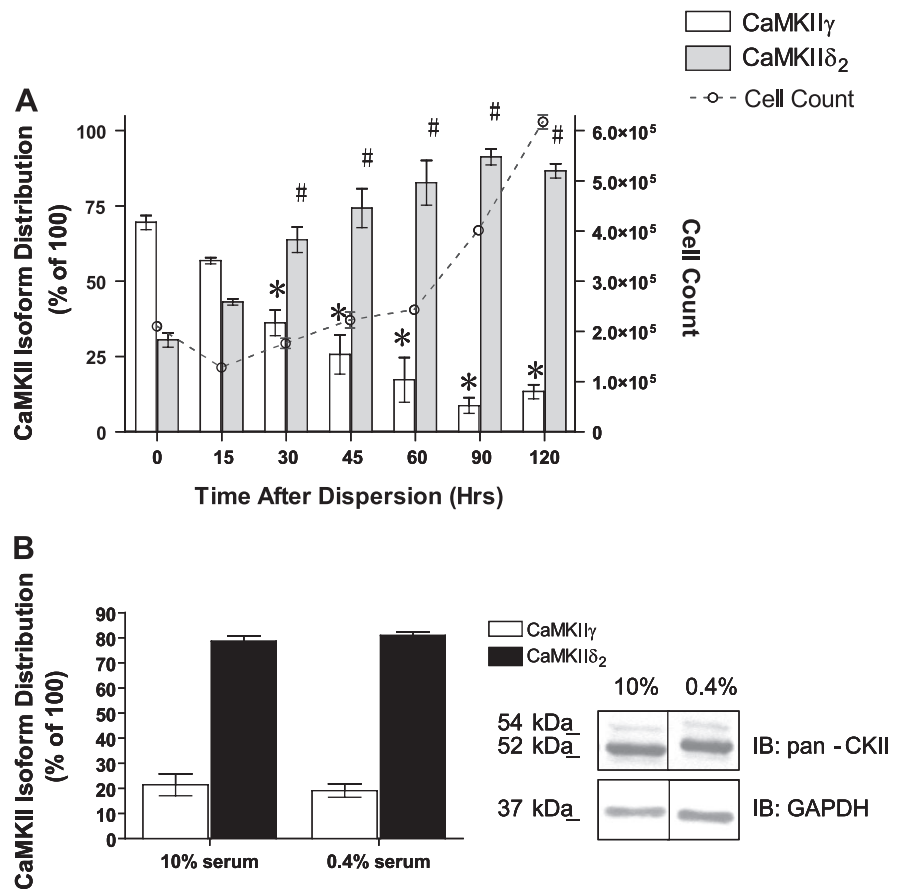


Fig. 2. Quantitation of CaMKII protein and mRNA after VSM dispersion. **A:** equal amounts of protein from VSM cell lysates 0 and 120 h (cultured in 10% or 0.4% FBS) after dispersion were immunoblotted with antibodies selective for calponin, SM- α -actin, SM-MHC, CaMKII γ , and CaMKII δ_2 as described in Fig. 1. The results were quantified by densitometric scanning, normalized to GAPDH, and expressed as a fold change in expression from 0 to 120 h. The fold changes represent means \pm SE; $n = 5$ for cells grown in 10% and $n = 4$ for cells grown in 0.4% media. **B:** CaMKII γ , CaMKII δ_2 , α -actin, MHC, and SM-22 α (22 α) mRNA content from VSM cells at 0 and 120 h (cultured in 10% or 0.4% FBS) after dispersion was determined using quantitative reverse transcriptase PCR. Results are expressed as a fold change in expression from 0 to 120 h. Values represent means \pm SE; $n = 4$ for cells grown in 10% and $n = 3$ for cells grown in 0.4% media. # $P < 0.001$ vs. 10% FBS 0-h control; * $P < 0.001$ vs. 0.04% FBS 0-h control.

15 h due to fractional plating efficiency, a significant increase ($P < 0.05$) in cell number is first detected at 30 h following cell dispersion, and cells begin growing exponentially after the isoform shift has occurred (Fig. 2B). We conclude from these analyses that CaMKII isoform modulation is at least concurrent with and, based on the trend between 0 and 30 h, may precede the onset of cell proliferation following the primary culture of enzymatically dispersed VSMCs.

We considered the possibility that that CaMKII isoform modulation under these conditions might reflect an expansion of a subpopulation of cells with relatively higher CaMKII δ_2 content and lower CaMKII γ content. This was tested by assessing CaMKII isoform distribution following plating of acutely dispersed VSMCs under conditions of 0.4% serum, a

Fig. 3. Time course of CaMKII expression and cell proliferation following VSM cell dispersion. **A:** relative protein expression levels of 54 kDa CaMKII γ C and 52 kDa CaMKII δ ₂ were determined using the pan-CaMKII antibody and expressed as a percentage over the indicated times. Values represent means \pm SE; $n = 4$ (# $P < 0.01$ vs. CaMKII δ ₂ at 0 h; * $P < 0.01$ vs. CaMKII γ at 0 h). Cell proliferation was quantified by cell counts at the corresponding time points following dispersion and plating. Values represent means \pm SE; $n = 5$. Increases in cell number over the minimum 15 h time point were significant ($P < 0.01$) beginning at 30 h. **B:** expression of CaMKII γ C and CaMKII δ ₂ at 120 h after dispersion under growth (10% FBS) or growth arrest (0.4% FBS) conditions was determined by immunoblotting with the pan-CaMKII antibody described previously. Immunoblotting of the lysates for GAPDH was performed to compare equal protein loading. Graph represents quantification of immunoblots from 4 separate experiments. Values represent means \pm SE.



commonly used growth arrest media that did not support proliferation (Fig. 3B). Although the cell number was not quantified in these experiments, the differences in growth under conditions of 10% serum and 0.4% serum were visually obvious with 10% serum-stimulated cultures reaching near confluence by 120 h. Cells were attached and spread in 0.4% serum medium, and a few binucleated cells were observed, perhaps in response to endogenous production of growth factors such as PDGF. After 120 h, there was no difference in relative isoform content under growth or no-growth conditions, indicating that the isoform modulation occurs irrespective of cell proliferation. Under these conditions, we also observed significant changes in SM cell markers both at the level of protein and RNA (Fig. 2, A and B), indicating that the shift from the contractile to synthetic phenotype occurs in the absence of added growth factors.

CaMKII δ ₂ regulates proliferation of freshly dispersed VSMCs. Based on previous literature implicating a positive effect of CaMKII in cell cycle control in other systems (17, 45, 49) and the observed correlation between increased expression of CaMKII δ ₂ and VSMC proliferation in culture, it is logical to hypothesize that this specific isoform is a potential link coupling Ca²⁺ signals to VSMC growth. To suppress the expression of the CaMKII δ ₂ subunit, a siRNA sequence specifically targeting all CaMKII δ subunits was designed and cloned in an adenoviral vector as a shRNA (shDELTA2). The adenoviral vector was used to obtain high-efficiency infection, and the expression of the shRNA constructs and the verification of infection were confirmed through the expression of GFP under

the control of a separate promoter. Approximately 65% of the cells were found to express the GFP tag 75 h after infection with shDELTA2 (Fig. 4A) and compared with a control adenovirus containing an shRNA targeted to GFP, shDELTA2 treatment effectively suppressed accumulation of CaMKII δ ₂ protein in the cells by 70%. The functional consequence of this on cell proliferation in primary culture was assessed by cell counts (Fig. 4B) and BrdU labeling (Fig. 4C). When compared with uninfected or shGFP-infected cells, shDELTA2 significantly inhibited both indexes of cell proliferation. We conclude from this experiment that acute upregulation of CaMKII δ ₂ following VSMC dispersion and primary culture plays an important functional role in facilitating cell proliferation, a characteristic feature of the synthetic VSMC phenotype.

CaMKII δ ₂ regulates proliferation of passaged VSMCs. We have previously shown that passaged rat aortic VSMCs express primarily the CaMKII δ ₂ isozyme (40), a pattern essentially identical to that observed following 120 h of primary culture (Fig. 1). To confirm the conclusions reached using the primary cultured cells, the effects of CaMKII δ gene silencing or inhibition of CaMKII activity were determined in passaged VSMCs expressing the CaMKII δ ₂ isozyme. Infection of growth-arrested passaged VSMCs with adenovirus shDELTA2 at a MOI of 50 resulted in 90% of the cells expressing the GFP tag 96 h after infection and a knockdown of CaMKII δ ₂ protein by \sim 90% by 72 and 96 h as assessed by Western blot analysis with the CaMKII δ tail-specific antibody (Fig. 5A). Quantitative RT-PCR analysis of CaMKII δ gene products confirmed that infection with shDELTA resulted in undetectable δ ₂ mRNA

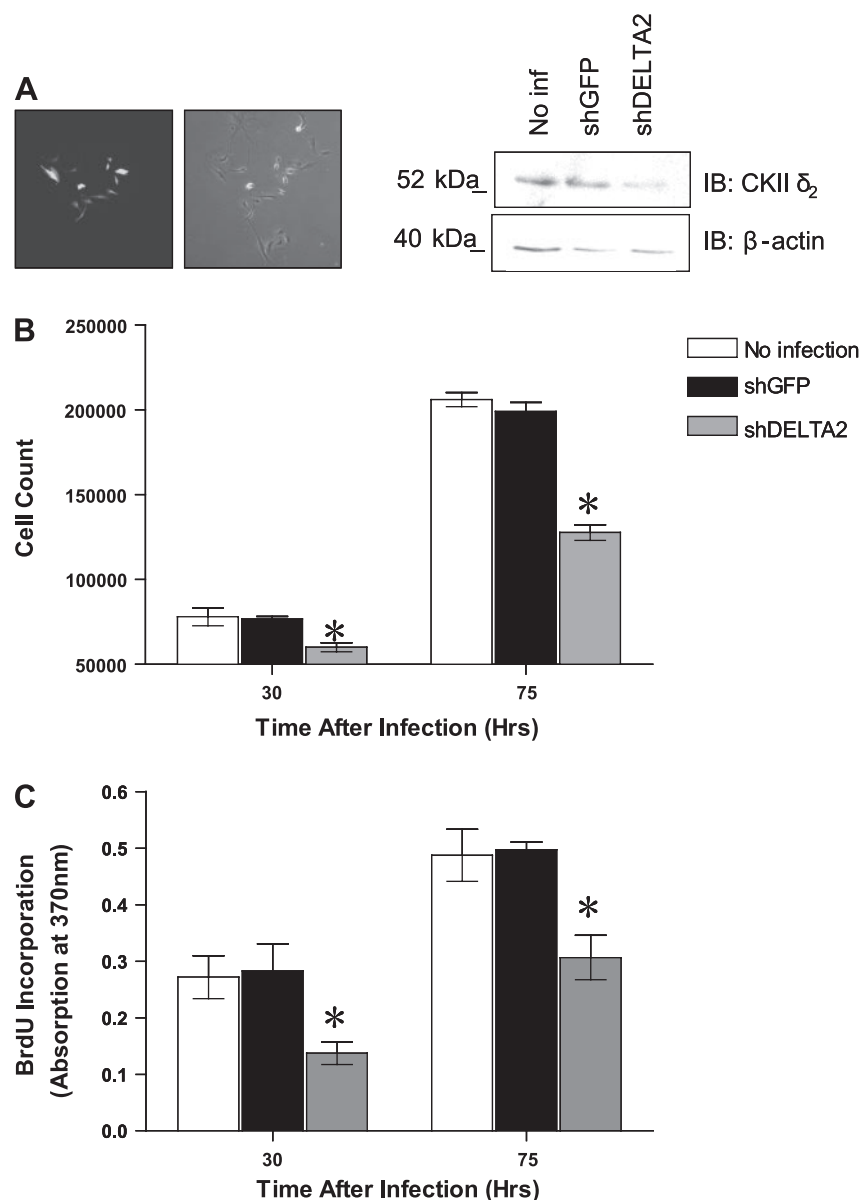


Fig. 4. Prevention of CaMKII δ_2 upregulation in freshly dispersed VSM inhibits cell growth and DNA synthesis. **A**: a representative micrograph 75 h after infection with adenovirus coexpressing short hairpin (sh) RNAi targeted against CaMKII δ_2 (shDELTA2) and green fluorescent protein (GFP) under separate promoters. After 75 h of infection, equal amounts of protein from VSM lysates were immunoblotted for CaMKII δ_2 . Immunoblotting of VSM lysates for β -actin was performed to compare protein loading. Equivalent levels of adenovirus expressing shGFP were used as an infection control. No Inf, no infection. **B**: freshly dispersed VSM cells were infected with shDELTA2 or shGFP as described. At the indicated times, cells were counted with the values representing means \pm SE; $n = 4$ ($*P < 0.05$). **C**: 5-bromo-2'-deoxyuridine (BrdU) incorporation into the DNA of proliferating cells was analyzed by enzyme immunoassay as described in MATERIALS AND METHODS in cells infected with shDELTA2 and shGFP as described in **A**. Values represent means \pm SE; $n = 3$ ($*P < 0.05$).

levels when measured against a standard curve and compared with noninfected and shGFP-infected controls ($n = 3$ experiments, not shown). Suppression of endogenous CaMKII δ_2 using this construct inhibited serum-stimulated cell proliferation (Fig. 5B) and BrdU incorporation (Fig. 5C) by $>50\%$, indicating a positive role for the kinase in regulating passaged VSMC proliferation.

As an alternative molecular approach, the manipulation of endogenous CaMKII δ_2 isoform activity was achieved through adenoviral overexpression of a kinase-negative CaMKII δ_2 mutant (KN δ_2). This construct has been previously described and shown to inhibit endogenous CaMKII activity in cultured VSMCs (36) and was produced by replacing a lysine residue with an alanine in the ATP-binding region, a substitution that has been previously shown to render the kinase inactive in vitro (38). Infection of cultured VSMCs with the adenovirus at a MOI of 50 resulted in a significant expression of the mutant at 48 h ($P < 0.05$) with a further increase in expression at 72 and

96 h when assessed with a CaMKII δ tail-specific antibody (Fig. 5A). Overexpressing the kinase-negative mutant significantly inhibited serum-stimulated VSMC proliferation (Fig. 5B) and BrdU labeling (Fig. 5C). A previous study utilizing KN-93, a pharmacological inhibitor of CaMKII, implicated that CaMKII was necessary for both cell-cycle progression and survival (45). Therefore, apoptosis (number of apoptotic cells/total number of cells) was examined at all time points (24, 48, 72, and 96 h) and was not found to be significantly higher in cells infected with shDELTA2 or KN δ_2 compared with adenovirally infected controls (between 6.4 and 6.9% for 10 separate fields). For positive controls incubated with DNase I (see MATERIALS AND METHODS), the percentage of apoptosis was $94.18 \pm 1.23\%$, $95.47 \pm 1.20\%$, $94.20 \pm 1.07\%$, and $95.24 \pm 1.02\%$, at 24, 48, 72, and 96 h, respectively, where values represent means \pm SE. These results indicate that apoptosis is not contributing to differences in cell proliferation using loss-of-function molecular approaches.

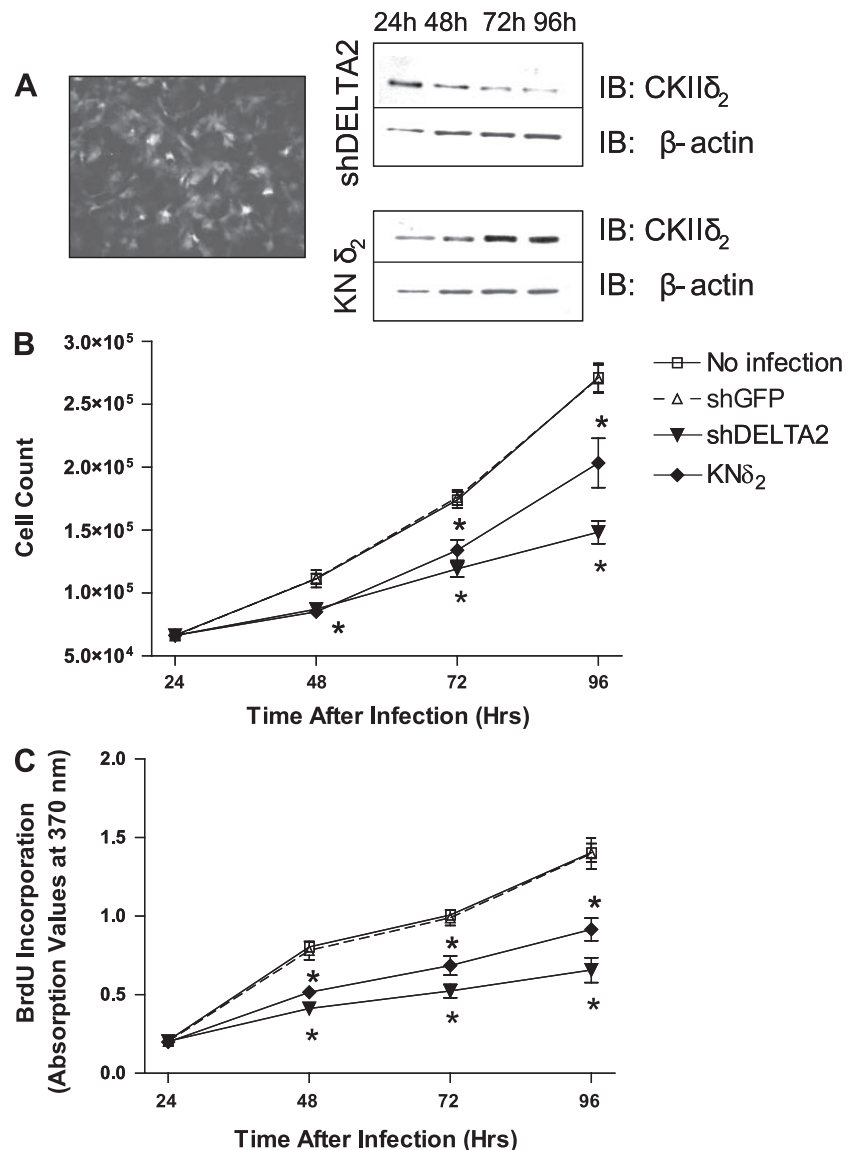


Fig. 5. Effect of CaMKII δ_2 protein suppression on VSM function in passaged VSM cells. **A:** cultured VSM cells were infected with adenovirus expressing shRNA against CaMKII δ_2 (shDELTA2) or overexpressing a kinase inactive CaMKII δ_2 mutant (KN δ_2) and harvested at the indicated times after infection. Levels of CaMKII δ_2 were determined by immunoblotting with the antibody specific for CaMKII δ_2 . Immunoblotting of the VSM lysates for β -actin was performed to compare protein loading. The micrograph represents the infection efficiency of cultured VSM cells with the adenoviral constructs. **B:** cultured VSM cells were infected with shDELTA2, shGFP, or KN δ_2 at equal multiplicity of infection and counted at the indicated times. Values represent means \pm SE; $n = 6$. **C:** BrdU incorporation into the DNA of proliferating cells was analyzed by enzyme at the indicated times by immunoassay. Values represent means \pm SE; $n = 5$ (* $P < 0.01$ vs. shGFP control).

Finally, to further confirm the specificity of the shDELTA2 approach, alternative sequences in the conserved domain of CaMKII δ gene products, and in the unique 21 AA COOH-terminus of CaMKII $\delta_{1-4,9}$ subunits, were targeted using adenoviral short hairpin constructs (denoted shDELTA and shTAIL, respectively). Infection with the alternative constructs produced CaMKII δ_2 suppression (Fig. 6A) comparable with that observed with shDELTA2. Both constructs also significantly inhibited serum-stimulated VSMC proliferation (Fig. 6B) and DNA synthesis (not shown), providing a high degree of confidence in the specificity of the shRNA approach and in the conclusion that CaMKII δ_2 positively regulates VSMC proliferation.

Inhibition of CaMKII δ_2 slows progression of cells through G2-M. A consistent observation in the passaged VSMCs treated with shRNA CaMKII constructs or the kinase-negative CaMKII δ_2 mutant was the presence of an obvious population of multinucleated cells. This is quantified in Fig. 7A in cells fixed and stained with DAPI to identify nuclei. Fields were chosen at random, and both phase-contrast and immunofluo-

rescence micrographs were obtained to quantify the percentage of multinucleated cells (number of multinucleated cells/number of total cells). By 48 h after infection with either the shDELTA2 or kinase-negative CaMKII δ_2 adenovirus, the number of multinucleated cells per field was nearly doubled compared with infection with a shGFP adenoviral control. Since the shDELTA2-infected cells also express GFP, we were able to track infected cells and determine that 65% of the multinucleated cells were also infected, suggesting that the doubling of multinucleated cells in the culture can be accounted for entirely by multinucleated cells infected with shDELTA2 and presumably deficient in CaMKII δ_2 protein.

Flow cytometric analysis was used to investigate the effect of CaMKII δ_2 silencing or inhibition on VSMC cycle progression. Cells were infected with shGFP as a control, with shDELTA2 or kinase-negative CaMKII δ_2 mutant for 48 h, and subsequently growth arrested for 1 day. Following growth arrest, 10% FBS growth media was added to the cells, and flow cytometric analysis using propidium iodide was performed after 1 and 24 h. A general finding was that CaMKII δ_2

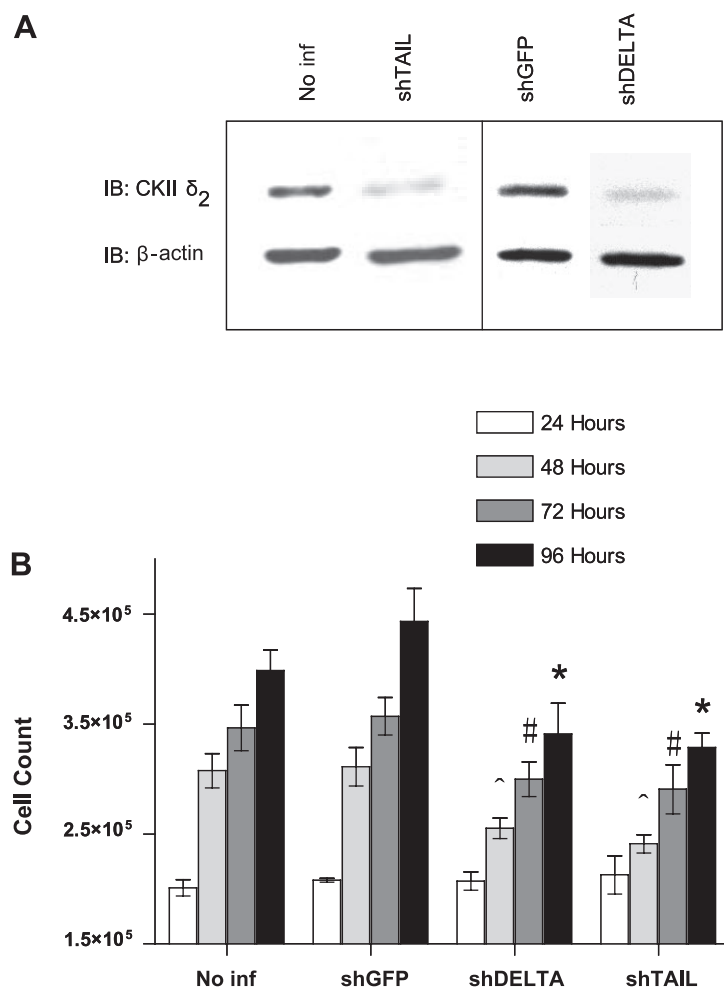


Fig. 6. CaMKII δ ₂ suppression by multiple shRNAs inhibits cell proliferation. **A**: cultured VSM cells were infected with alternative adenovirus constructs encoding shRNA targeting CaMKII δ (shDELTA) and the CaMKII δ ₂ COOH-terminus (shTAIL). Levels of CaMKII δ ₂ expression were determined by immunoblotting with antibody specific to CaMKII δ ₂. Levels of β -actin were determined to compare protein loading. Adenovirus encoding shRNA targeted to GFP was also used as a viral control. **B**: the cultured VSM cells were infected with adenovirus as in **A** and counted at the indicated times. Values represent means \pm SE; $n = 3$ ($^{\wedge}P < 0.05$ vs. 48-h shGFP control; $\#P < 0.05$ vs. 72-h shGFP control; $*P < 0.05$ vs. 96-h shGFP control).

silencing with shDELTA2 or overexpression of the kinase-negative CaMKII δ ₂ mutant reproducibly increased the number of cells in G2/M and also increased the number of polyploidy cells (Fig. 7B). The results of five such experiments are quantified in Table 1. Significant decreases in G1- and S-phase cells after CaMKII δ ₂ manipulation appear to be accounted for entirely by an increase in cells at G2/M and polyploidy cells. Similar results were obtained when CaMKII δ ₂ expression was silenced with shDELTA and shTAIL (not shown). The results of the cell cycle analyses strongly suggest that inhibition of VSMC proliferation following suppression of CaMKII δ ₂ or inhibition of CaMKII δ ₂ activity results from a delayed progression through G2/M and/or inhibition of cytokinesis.

DISCUSSION

Changes in the source and dynamics of Ca²⁺ signals have recently been shown to contribute to the modulation of VSMCs from a quiescent contractile phenotype to a proliferative phenotype in organ culture (48) and in vivo in response to injury (21). A significant finding in the present study is a rapid change in the isoform expression pattern of the multifunctional serine/threonine kinase CaMKII, a major Ca²⁺ signal effector, in VSMCs undergoing phenotypic modulation in primary culture. CaMKII γ isoforms, which have been previously linked to the regulation of contractile activity in VSM (18, 24, 39), were

found to be rapidly downregulated in primary culture, whereas the CaMKII δ ₂ isoform, previously linked to ERK signaling (1, 22), activation of nonreceptor tyrosine kinases (13, 14), and VSMC migration (23, 35, 36), is upregulated. Experiments using molecular approaches to silence CaMKII δ gene expression or to inhibit activity of the endogenous kinase indicated that upregulation of the CaMKII δ ₂ isoform was functionally significant since it proved to be a positive regulator of VSMC proliferation.

Although there are a number of studies in the literature that implicate CaMKII as a regulator of cell proliferation, this is the first study to specifically assess this function in the context of VSMCs, which contribute significantly to the pathophysiology of vascular disease, and the first to specifically consider the effects of the δ ₂ isoform on cell-cycle regulation. Most previous studies have employed the use of pharmacological agents (45, 49), which may have nonspecific cellular effects. In addition, these agents affect all CaMKII isoforms in the cell and therefore do not allow the functions of distinct CaMKII isoforms to be elucidated. To circumvent these problems, loss-of-function molecular approaches involving the use of siRNA technology and overexpression of a kinase-negative CaMKII δ ₂ mutant were employed.

We have known for a number of years that the predominant CaMKII isoform expressed in cultured rat aortic VSMCs is

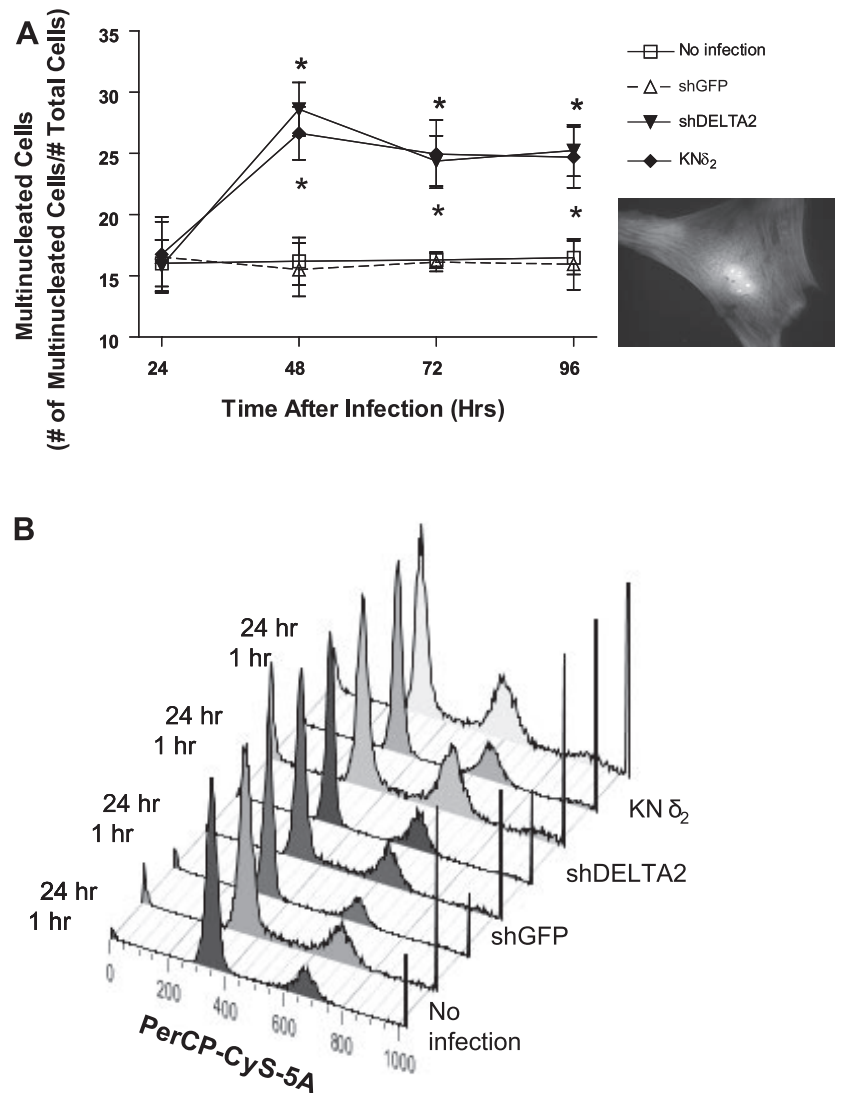


Fig. 7. Inhibition of the cell cycle by shDELTA2 and KN δ_2 . **A:** VSM cells were infected with control adenovirus (shGFP) or adenovirus encoding shRNA targeted against CaMKII δ_2 (shDELTA2) or overexpressing kinase-negative CaMKII δ_2 (KN δ_2) as described in Figs. 4A and 5A. Phase contrast and fluorescent fields were used to determine the percentage of multinucleated cells per field. Values represent means \pm SE; $n = 10$ separate experiments ($*P < 0.01$ vs. shGFP control). The micrograph shows a large binucleated VSM cell infected with shDELTA2 for 96 h. **B:** flow cytometric analysis of DNA content in VSM cells stimulated with serum for 1 and 24 h. Before addition of serum, VSM cells were infected with control adenoviruses encoding shGFP or adenoviruses expressing shDELTA2 of kinase-negative CaMKII δ_2 (KN δ_2) as described in Figs. 4A and 5A.

CaMKII δ_2 (40), and we recently demonstrated that adenoviral-mediated delivery of siRNA (in the form of short hairpin constructs), targeted specifically to CaMKII δ , effectively suppressed expression of endogenous CaMKII δ_2 in these cells (22). Using this approach and multiple siRNAs targeted to sequences common in all CaMKII δ isoforms (shDELTA, shDELTA2) or specifically to δ isoforms (shTAIL) containing an alternatively spliced 21 AA COOH-terminus ($\delta_{1-4,9}$) (44), we showed here that a knockdown of CaMKII δ_2 in the range of 70–90% inhibited, but did not block, proliferation of passaged VSMCs. Although the infection efficiency with adenoviruses in these cells is nearly 100%, because suppression of CaMKII δ_2 was incomplete and there is expected cell-to-cell variability in the effect, it is impossible to conclude from these results whether or not CaMKII δ_2 is required for or, more likely, positively modulates VSMC proliferation. As an alternative molecular strategy, we overexpressed a kinase-negative CaMKII δ_2 mutant that was previously characterized and shown to act as a dominant-negative construct with respect to CaMKII activity both in vitro and in situ (28, 36, 38). This approach also produced comparable inhibition of VSMC proliferation and effects on cell-cycle distribution, strongly sup-

porting the conclusion that CaMKII δ_2 facilitates serum-stimulated VSMC proliferation.

Although most studies in the literature point to a positive role for CaMKII in mediating the cell cycle and proliferation, studies that used a constitutively active mutant of CaMKII α suggest a negative role for CaMKII (3, 37). Interpretation of the latter studies is complicated by potential nonspecific effects of overexpressing a persistently active multifunctional protein kinase and by the fact that CaMKII α was not an endogenous isoform in the cell types studied. To the extent that CaMKII isoforms may be differentially localized affecting substrate specificity, approaches to manipulate the endogenous δ_2 isoform expressed in the cultured VSMCs may be more relevant. Although the δ_2 isoform does not contain a nuclear localization sequence as has been reported for the δ_3 isoform (43), CaMKII δ_2 effects on nuclear cell-cycle regulatory proteins could be dependent on intermediates that translocate to the nucleus. Some insight into mechanisms behind the CaMKII δ_2 isoform regulation of VSM proliferation was provided by flow cytometry where we observed an increase in cells in G2/M population after infection with either shDELTA2 or the kinase-negative CaMKII δ_2 mutant. Patel et al. (34) recently described

Table 1. Cell cycle analysis of VSM cells following suppression of CaMKII δ_2

Sample	G0/G1	S	G2/M	Polyloid
Uninfected control cells				
1 hr	71.68 \pm 1.00	2.14 \pm 0.08	20.4 \pm 1.171	1.34 \pm 0.06
24 h	60.33 \pm 0.73	5.98 \pm 0.19	22.58 \pm 0.54	3.26 \pm 0.23
Control cells infected with adenovirus encoding shGFP				
shGFP, 1 h	69.26 \pm 0.72	2.07 \pm 0.16	21.66 \pm 0.99	2.18 \pm 0.06
shGFP, 24 h	59.62 \pm 0.76	6.06 \pm 0.25	23.12 \pm 0.49	4.47 \pm .027
Cells infected with adenovirus encoding shDELTA2 targeting CaMKII δ				
shDELTA2, 1 h	62.86 \pm 1.73*	1.62 \pm 0.12*	23.12 \pm 0.49*	4.74 \pm 0.27*
shDELTA2, 24 h	49.70 \pm 3.11†	4.15 \pm 0.17†	28.25 \pm 0.86†	5.77 \pm 0.29†
Cells infected with adenovirus encoding kinase-negative CaMKII δ_2				
KN δ , 1 h	63.60 \pm 0.79*	1.43 \pm 0.11*	24.62 \pm 0.15*	4.17 \pm 0.43*
KN δ_2 , 24 h	51.63 \pm 2.30†	3.68 \pm 0.15†	27.56 \pm 0.87†	6.63 \pm 0.33†

Values are means \pm SE; $n = 5$ experiments. Vascular smooth muscle (VSM) cells were infected with adenovirus for 48 h, and serum was starved for 24 h and stimulated with 10% FBS. Flow cytometric analysis of DNA content was determined 1 and 24 h following addition of serum. CaMKII δ_2 , calmodulin-dependent protein kinase II δ_2 ; sh, short hairpin; GFP, green fluorescent protein; KN, kinase-negative; * $P < 0.05$ vs. 1-h ShGFP control; † $P < 0.05$ vs. 24-h shGFP control.

the in vitro phosphorylation of the tyrosine phosphatase Cdc25C, a key mediator of the G2/M checkpoint, and subsequent increase in phosphatase activity by CaMKII. Furthermore, Cdc25C phosphorylation during M phase was inhibited by KN-93, a CaMKII inhibitor (34). Since we consistently observed large multinucleated cells, a separate role for CaMKII δ_2 might be through regulation of cytokinesis. Cytokinesis is dependent on actin/myosin interaction at the cleavage furrow (8). We have previously shown that VSM contraction (18, 39) is positively regulated by CaMKII in differentiated intact SM that mainly express CaMKII γ (24, 41). CaMKII has been shown to be localized in the mitotic apparatus of dividing cells (32), and, specifically, CaMKII γ was reported to be essential for bipolar spindle formation during cell division (17). Since CaMKII isoforms are capable of forming heteromultimers (41, 43), the loss of CaMKII δ_2 might affect the targeting of CaMKII γ to the cleavage furrow and thus adversely affect cytokinesis.

Virtually all VSMC marker genes identified to date are dependent on CARG elements found within their promoter and/or intronic sequence. The transcription factor SRF (serum response factor) is required for subsequent gene transcription and regulation (33). Although the promoter regions of the CaMKII δ and γ genes have not been characterized, it is interesting to note that the CaMKII α promoter contains a CARG SRF-dependent motif (25), potentially allowing SRF-dependent CaMKII gene transcription. However, since the modulation of CaMKII and SM cell markers occurred in the absence of serum mitogens (15), other possibilities such as loss of endothelial factors, changes in matrix interactions, mechanical stresses, and oxidative environment should be considered as potential factors involved in regulating the changes in phenotype in the primary cultured VSMCs (33).

Further questions revolve around how CaMKII γ and δ_2 isoforms are regulated at the transcriptional and posttranslational level and whether ion channel changes in phenotypically modulating SM cells are important in this process. Insight into this may be provided by a recent genomewide analysis of repressor element-1 silencing transcription factor (REST) target genes, which identified several ion channels that are transcriptionally repressed by REST (5). Furthermore, Cheong et al. (7) recently studied the downregulation of REST in

synthetic SM cells as a switch enabling potassium channel expression and subsequent VSMC proliferation. REST may play an active role in the suppression of CaMKII δ_2 in vivo since the δ_2 gene contains several putative REST sites that are conserved across the human and mouse genomes. Loss of REST, which occurs when human saphenous VSMCs are cultured (7), may therefore allow the upregulation of CaMKII δ_2 observed in cultured synthetic VSMCs. Indeed, a significant loss of REST protein and RNA coincides with changes in CaMKII isoform expression in primary cultured VSMCs (data not shown).

In light of the hypothesis that isoform composition regulates the subcellular localization and therefore the functions of CaMKII, it is possible that γ -subunits have a role in regulating differentiated function in medial SM cells by targeting kinase activity to the contractile apparatus (24), whereas δ_2 -subunits may target the kinase in ways that support proliferative and migratory capabilities of dedifferentiated VSMCs. In other words, CaMKII isoform content may be an important component of VSMC phenotype. An alternative and/or complementary hypothesis is that the CaMKII isoform composition may be an important determinant of VSMC phenotype, perhaps by modulating expression of VSM contractile proteins. Although not studied here, it is plausible that CaMKII may regulate VSMC phenotype at the level of gene transcription. CaMKII has been shown to positively mediate the activation of the DNA-binding protein SRF in vitro by phosphorylating SRF on S103 (26). Conversely, in skeletal muscle, CaMKII has been reported to phosphorylate SRF on T160 within the MADS box (DNA-binding domain) (12) and thus negatively affect DNA binding. Given the results of the present study, it would appear that the function of specific CaMKII isozymes in regulating VSMC gene transcription merits investigation.

In summary, we have demonstrated the dynamic regulation of the CaMKII isoform content as a function of VSMC phenotype and we have specifically demonstrated that upregulation of the CaMKII δ_2 isoform positively regulates serum-stimulated VSMC proliferation. Overall, the results of this study are consistent with a more general model whereby alterations in Ca²⁺ signals (21, 48), and as shown here Ca²⁺-signaling pathways, regulate VSM phenotype and proliferative responses. Because increases in VSMC ploidy similar to those

observed here following suppression of CaMKII δ_2 have been linked to vascular remodeling associated with hypertension (16), it may be interesting to consider the role of CaMKII isoform modulation as a factor contributing to hypertensive disease. Given previous studies linking CaMKII to activation of ERK and tyrosine kinase-signaling pathways (1, 14, 22) and regulation of cultured VSMC migration (35, 36), we propose that CaMKII isoform modulation may also have important functions in the response of VSMCs to injury. In vivo work utilizing a rat carotid artery balloon angioplasty model supports this hypothesis since CaMKII δ_2 is significantly upregulated in the medial wall 3 days after balloon injury and is the main isoform expressed in the neointima (unpublished results). Future studies are needed that are aimed at preventing the up-regulation of δ_2 in the rat carotid artery and assessing its effect on neointimal formation.

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GRANTS

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