THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 47, pp. 33519–33529, November 22, 2013

CaMKIIδ-dependent Inhibition of cAMP-response Element-binding Protein Activity in Vascular Smooth Muscle*

Received for publication, June 4, 2013, and in revised form, October 4, 2013 Published, JBC Papers in Press, October 8, 2013, DOI 10.1074/jbc.M113.490870

Yongfeng Liu, Li-Yan Sun, Diane V. Singer, Roman Ginnan, and Harold A. Singer[1]

From the Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208

Background: CaMKII is implicated in both positive and negative regulation of cAMP-response element-binding protein (CREB) activity.
Results: CaMKIIδ increases CREB-Ser^{142} phosphorylation and inhibits binding of CREB to *Sik1* and *Rgs2* promoters in vascular smooth muscle (VSM).
Conclusion: In VSM CaMKIIδ negatively regulates CREB activity and target gene transcription by phosphorylating CREB-Ser^{142}.
Significance: Negative regulation of CREB by CaMKIIδ is a mechanism mediating Ca^{2+} signal-dependent regulation of gene transcription in VSM.

One transcription factor mediator of Ca^{2+}-signals is cAMP response element-binding protein (CREB). CREB expression and/or activity negatively correlates with vascular smooth muscle (VSM) cell proliferation and migration. Multifunctional Ca^{2+}/calmodulin-dependent protein kinases, including CaMKII, have been demonstrated to regulate CREB activity through both positive and negative phosphorylation events *in vitro*, but the function of CaMKII as a proximal regulator of CREB in intact cell systems, including VSM, is not clear. In this study, we used gain- and loss-of-function approaches to determine the function of CaMKIIδ in regulating CREB phosphorylation, localization, and activity in VSM. Overexpression of constitutively active CaMKIIδ specifically increased CREB phosphorylation on Ser^{142} and silencing CaMKIIδ expression by siRNA or blocking endogenous CaMKII activity with KN93 abolished thrombin- or ionomycin-induced CREB phosphorylation on Ser^{142} without affecting Ser^{133} phosphorylation. CREB-Ser^{142} phosphorylation correlated with transient nucleocytoplasmic translocation of CREB. Thrombin-induced CREB promoter activity, CREB binding to *Sik1* and *Rgs2* promoters, and *Sik1*/*Rgs2* transcription were enhanced by a kinase-negative CaMKIIδ_2 (K43A) mutant and inhibited by a constitutively active (T287D) mutant. Taken together, these studies establish negative regulation of CREB activity by endogenous CaMKIIδ-dependent CREB-Ser^{142} phosphorylation and suggest a potential mechanism for CaMKIIδ/CREB signaling in modulating proliferation and migration in VSM cells.

Vascular smooth muscle (VSM)[2] cells comprise the medial layer of blood vessels providing mechanical support and a means for adjusting vascular wall tone and diameter. However, VSM cells are not terminally differentiated and can switch to a proliferative/synthetic phenotype in response to changes in local environment that accompany a number of chronic vascular diseases as well as acute and chronic vascular injuries (1, 2). Identifying transcriptional and post-transcriptional regulatory processes governing VSM phenotype plasticity are therefore of considerable basic interest and clinical relevance.

One key signal in VSM is free intracellular Ca^{2+} ($[Ca^{2+}]_i$), which is well known to regulate differentiated contractile function, as well as synthetic phenotype proliferation and migration (3). $[Ca^{2+}]_i$ signals also regulate a number of transcription factors, including type II HDAC-MEF2, calcineurin/nuclear factor of activated T cells, and cyclic AMP response element-binding protein (CREB) (4–6). CREB, in addition to its defining regulation by cAMP-dependent protein kinase (PKA), has long been known to be regulated by $[Ca^{2+}]_i$ signals via multifunctional Ca^{2+}/calmodulin-dependent protein kinases including CaMKIV and CaMKII (7). Expression of CREB is down-regulated in several vascular diseases, such as hypertension, dyslipidemia, and atherosclerosis, as well as in response to vascular injury (8), suggesting a vasculoprotective effect of CREB (8). Conversely, CREB activation has also been reported to be positively related to thrombin-induced proliferation (9) and TNFα-induced migration in the smooth muscle cells (10).

Several phosphorylation sites in CREB have been demonstrated to regulate transcriptional activity. Ser^{133} is the primary target of PKA and phosphorylation is required for CREB activation, whereas phosphorylation of Ser^{142} by CaMKII has been reported to inhibit CREB activity by interfering with CREB dimerization and protein interactions to form an active promoter complex (11). *In vitro* studies using purified brain CaMKIV and CaMKII have clearly demonstrated that CaMKIV phosphorylates CREB on Ser^{133}, whereas CaMKII has equal affinity for Ser^{133} and Ser^{142} (12). These results were confirmed using transient expression assays and constitutively active CaMKIV and CaMKII constructs. In the case of CaMKII overexpression, inhibitory phosphorylation on Ser^{142} overrides the activating phosphorylation on Ser^{133} (11, 12). Yet in intact neural cells, CaMKII activation has been positively associated with CREB activation (13–15). Based on these studies, it appears that

* This work was supported, in whole or in part, by National Institutes of Health Grants RO1-HL4942 and RO1-HL092510 from the National Heart, Lung and Blood Institute (to H. A. S.) and the Albany Medical College Candice Weir Fund.
[1] To whom correspondence should be addressed: 47 New Scotland Ave., Albany, NY 12208. Tel.: 518-262-8102; Fax: 518-262-8101; E-mail: SingerH@mail.amc.edu.
[2] The abbreviations used are: VSM, vascular smooth muscle; CREB, cAMP response element-binding protein; ANOVA, analysis of variance; HDAC, histone deacetylase; MEF, myocyte enhancing factor.

endogenous CaMKII could be either a positive or negative regulator of CREB, perhaps in a tissue or context-specific manner. Despite the widespread importance of CREB as a transcription factor, there is very little information regarding the importance of negative CREB regulation mediated by CaMKII-dependent Ser^{142} phosphorylation in any intact cellular system.

Pathways regulating CREB activity in response to Ca^{2+}-dependent stimuli in VSM are incompletely understood, and what little is known has been inferred via indirect pharmacological approaches that suggest a net positive function for CaM kinases in regulating CREB (16–18). In this study, we demonstrate for the first time in VSM cells that stimulation with Ca^{2+}-dependent stimuli thrombin and ionomycin resulted in transient CREB-Ser^{142} phosphorylation that preceded the activating phosphorylation on Ser^{133}. Multiple gain- and loss-of-function approaches indicated a specific function for endogenous CaMKIIδ in regulating VSM CREB-Ser^{142} phosphorylation, nucleocytoplasmic translocation, promoter activity, and endogenous target gene CREB binding and transcription. Taken together, these studies definitively establish negative regulation of CREB activity by endogenous CaMKIIδ-dependent phosphorylation of CREB-Ser^{142} and suggest a potential mechanism for CaMKII/CREB signaling in modulating proliferation and migration in VSM cells.

EXPERIMENTAL PROCEDURES

Materials—The antibody specific for phospho-Ser^{142}-CREB was purchased from GeneScript and the antibodies against total CREB, phospho-Ser^{133}-CREB, histone H3, and α-tubulin were purchased from Cell Signaling. The antibodies against β-actin and GAPDH were purchased from Sigma. Polyclonal antibodies against *pan*-CaMKII, CaMKIIδ$_2$, and phospho-Thr^{287}-CaMKII were raised in rabbits as described previously (19, 20). Adenoviruses expressing kinase-negative CaMKIIδ$_2$ (K43A), constitutively active CaMKIIδ$_2$ (T287D), and GFP were described previously (21, 22). The plasmid expressing luciferase under a promoter construct containing four CREB binding site repeats was purchased from Affymetrix. Thrombin was purchased from Sigma; and KN93 and ionomycin were purchased from Calbiochem. The cell culture media and all immunoblotting supplies were purchased from Invitrogen and Bio-Rad Laboratories, respectively.

VSM Cell Dispersion and Culture—Primary cultures of VSM cells were obtained from rat aorta as previously described (23). In brief, Sprague-Dawley rats (150–200 g) were sacrificed using CO_2, and following brief digestion with collagenase the medial layer of the aorta containing VSM cells was freed from the adventitial layer and intima containing endothelial cells was disrupted mechanically. Medial layer VSM cells were isolated by enzymatic digestion with collagenase and elastase and cultured in DMEM/F-12, supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. Cells were passaged when ~80% confluence was reached. Experiments were performed with VSM cells from passages 3 to 7. The biochemical and growth properties of these and similar VSM cell cultures have been extensively characterized (5, 19–23). All animal protocols were reviewed and approved by the Albany Medical Center Institutional Animal Care and Use Committee.

Luciferase Reporter Assay—VSM cells (1×10^6) were electroporated with plasmids expressing CREB luciferase reporter (2 μg) and TK-*Renilla* (0.1 μg) and incubated for a total of 72 h prior to cell lysis. Adenoviruses encoding kinase-negative CaMKIIδ$_2$ (K43A) mutant, which acts as a dominant-negative with respect to kinase activity, or constitutively active CaMKIIδ$_2$ (T287A) mutant (22) were transduced for 48 and 24 h prior to cell lysis, respectively. CREB luciferase reporter activity was stimulated by thrombin (0.5 nM) or forskolin (5 μM) for 6 h prior to lysis. The level of promoter activity was determined by measuring firefly luciferase signal intensity normalized over the internal control TK-*Renilla* luciferase signal intensity using protocols provided in the Dual Luciferase Assay System (Promega).

Quantitative PCR—VSM cells were seeded onto 35-mm culture dishes (0.5×10^5 cells) and treated with 0.5 μM thrombin and 5 μM forskolin for 6 h. RNA was isolated following the manufacturer's instructions for the RNeasy® mini kit (Qiagen). The concentration of extracted RNA was determined using Nanodrop (Thermo). RNA was reverse transcribed to cDNA using the Quantitech® reverse transcription kit (Qiagen). The quantitative RT-PCR was carried out on an Mx3000P QPCR System using IQ™ SYBR® Green Supermix (Bio-Rad). The primers used for *Sik1*/*Kid2*, *Rgs2*, and internal control *Gapdh* were listed as follows, *Sik1* forward, 5′-TCCAAACACCTTCGTTCTCTG-3′, and reverse, 5′-CGATCCCATTACAGCCCAG-3′; *Rgs2* forward, 5′-CAAAGTGCCATGTTCCTGGCTG-3′, and reverse, 5′-AAGTAGCTCAAACGGGTCTTC-3′; *Gapdh* forward, 5′-TCGTCTCATAGACAAGATGGT-3′, and reverse, 5′-GTAGTTGAGGTCAATGAAGGG-3′.

Cell Stimulation and Western Blot—VSM cells were washed twice with pre-warmed Hanks' balanced salt solution and placed on a warm plate (37 °C) for 30 min prior to stimulation with either 50 nM thrombin or 0.5 μM ionomycin. At appropriate times cells were lysed by addition of 3× Laemmli sample buffer. Lysates were heated at 95 °C for 5 min and then resolved using standard 10% SDS-PAGE. Resolved proteins were electrophoretically transferred onto nitrocellulose membranes (GE Healthcare), the membranes blocked with 5% nonfat milk or 5% BSA in Tris-buffered saline supplemented with 1% Tween 20 (TBST) for 1 h, followed by primary antibody incubation for 1 h or overnight at 4 °C and secondary antibody incubation for 1 h. After washing three times with TBST, the membranes were incubated with SuperSignal® chemiluminescent substrate (Thermo) for 5 min. Gel images shown were generated by the Fuji LAS4000 and quantified with Multi Gauge software.

siRNA Electroporation—VMS cells (1×10^6) were harvested and electroporated with 2 μg of siRNA targeting CaMKIIδ gene products (Smart pool siRNA Thermo Fisher, catalog number L-080171-00-0010) or non-targeting siRNA (Thermo Fisher) using the Amaxa Nucleofector system (Lonza) and the VSM cell-specific D33 program (Lonza Amaxa®). After electroporation, the cells were resuspended in 4 ml of cell culture media and reseeded onto four 60-mm culture dishes. Using previously established conditions (5), the cells were used 48 h following electroporation.

Chromatin Immunoprecipitation (ChIP)—To study the interaction between CREB and gene promoters, a ChIP assay

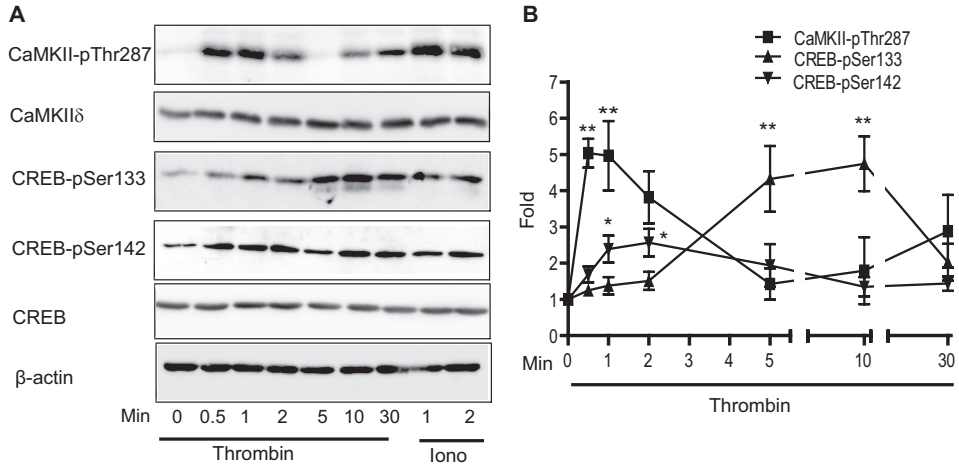

FIGURE 1. **Thrombin-induced phosphorylation of CaMKII and CREB.** *A,* VSM cells were treated with thrombin (50 nM) or ionomycin (*Iono*; 0. 5 μM). Cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-Thr^{287}-CaMKII (*pThr287*), total CaMKIIδ, phospho-Ser^{133}-CREB (*pSer133*), phospho-Ser^{142}-CREB (*pSer142*), and β-actin. *B,* ECL signals were quantified with a FujiLAS 4000. The plot quantifies CaMKII-p287 as an index of CaMKII activation, CREB-Ser(P)^{133} and CREB-Ser(P)^{142}. Data were normalized for loading using β-actin signals and expressed as fold-values compared with unstimulated. Values shown are mean ± S.E., $n = 3$ and were analyzed by one-way ANOVA. *, $p < 0.05$ and **, $p < 0.01$, by Dunnett's Multiple comparison post hoc test.

was performed using the ChIP-IT® Express Kit from Active Motif (53008). 5–6 × 10^6 VSM cells were used for each sample. Following cross-linking and recovery of chromatin following kit protocols, chromatin was sheared in 200 μl of kit shearing buffer using a Bioruptor sonicator at medium power for a total time of 16 min (30 s on and 30 s off for 16 cycles). 50 μl of chromatin lysate was immunoprecipitated overnight at 4 °C with anti-CREB antibody and isolated with magnetic protein G beads. Following cross-link reversal and incubation with proteinase K per kit protocols, immunoprecipitated DNA was analyzed by quantitative PCR. Promoter regions were predicted using PromoSer software (24) and CREB-binding elements were predicted with PROMO 3.0 software (25). Primers flanking a predicted CREB binding site ($^{-1020}$CTGACGTCA^{-1012}) in the rat *Sik1*/*Kid2* promoter were (5′-3′): forward, $^{-1040}$GCGGCTGCTGAGCCCGGT^{-1023}-3′ and reverse, $^{-962}$GGCGGTTGCCAAGAGACTGGAG^{-941}. Primers flanking an *Rgs2* predicted CREB-binding element ($^{-221}$GCCTACGTCA^{-212}) were: forward, $^{-243}$TGTGTGCGCACGAGGATGCGG^{-223} and reverse, $^{-124}$ACCTCTAGAGGGCGCGGATTG^{-104}.

Cell Fractionation—Cell fractionation was performed on VSM cells to separate nuclear and cytosolic fractions using a cell fractionation kit and protocols (Active Motif). In brief, cells were washed with pre-cooled PBS containing phosphatase inhibitors (5 ml), collected with a cell scraper and isolated by centrifugation at 500 × *g* for 5 min at 4 °C. After the resuspension in a kit hypotonic lysis buffer (500 μl), the cells were incubated on ice for 15 min. Detergent (25 μl) was added followed by centrifugation at 14,000 × *g* for 30 s at 4 °C to pellet a crude fraction containing nuclei. The crude supernatant is considered the "cytosolic" fraction and the washed pellet the "nuclear" fraction. The nuclear marker histone H3 and cytosolic marker tubulin were used to verify the fractionation. Proteins in the fractions were by Western blotting.

Statistical Analysis—Quantitative data are presented as mean ± S.E. Student's unpaired *t* test was used in the comparisons between two groups of data and one-way ANOVA for simple comparisons between multiple groups. If found significant by ANOVA, Dunnett's Multiple Comparison or Bonferroni post hoc tests were used to identify between group differences. Two-way ANOVA with a Bonferroni post hoc test was used in Fig. 3 to compare time points across siRNA and siControl groups. Statistics were performed using the GraphPad Prism4 program. "*n*" indicates the number of independent experiments and results were considered to be significantly different if $p < 0.05$. Statistical significance is presented, *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

RESULTS

CREB Phosphorylation in VSM—Thrombin has been proven to increase $[Ca^{2+}]_i$ via activation of membrane PAR receptors (reviewed in Ref. 26), resulting in activation of both CaMKII (27) and CREB (9). CREB Ser^{133} and Ser^{142} are two candidate phosphorylation sites for regulation by Ca^{2+}-dependent pathways with the former leading to CREB activation and the latter to CREB inhibition (12, 28). Addition of the Ca^{2+} ionophore, ionomycin, was used to selectively induce Ca^{2+} influx and stimulate activation of CaMKIIδ in VSM cells. CaMKIIδ activation was indexed by immunoblotting lysates with an antibody recognizing phospho-Thr^{287}, the activation-dependent autophosphorylation site (29) (Fig. 1*A*). Ionomycin (0.5 μM) stimulated CREB phosphorylation 2-fold on both Ser^{133} and Ser^{142}, suggesting either or both phosphorylation events could be mediated by CaMKIIδ (Fig. 1*A*). Addition of the physiological stimulus thrombin (50 nM) biphasically activated CaMKII with peaks between 30–60 s and 30 min, consistent with a previous report (27) (Fig. 1*B*). Thrombin also increased CREB phosphorylation on both Ser^{133} and Ser^{142}, but with distinct time courses. CREB phosphorylation on the inhibitory site Ser^{142} peaked in 2 min, slightly lagging the CaMKII activation time course, whereas phosphorylation on the activating site Ser^{133} lagged considerably with a peak between 5 and 10 min following stimulation. Thus, in VSM cells, activation of CaMKII in response to thrombin correlated temporally with

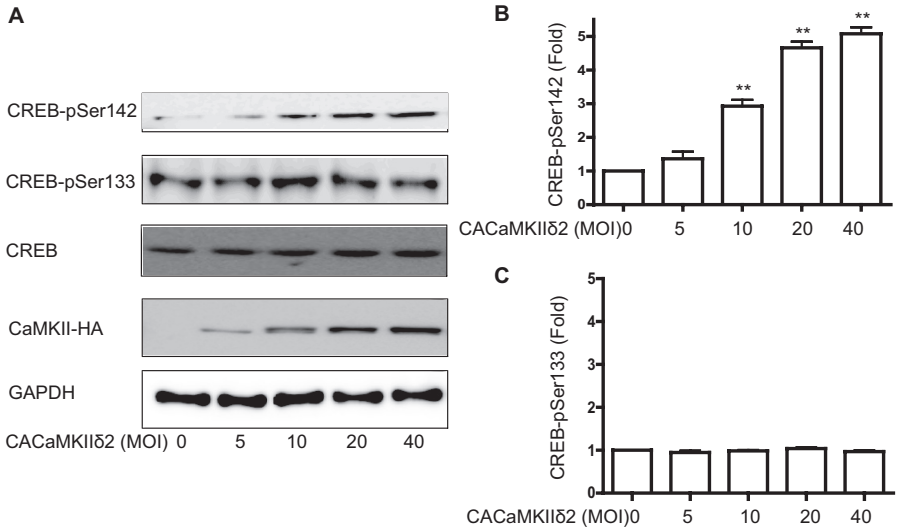

FIGURE 2. **CaMKIIδ selectively phosphorylates CREB-Ser^{142}.** *A*, adenovirus encoding a constitutively active (*CA*) CaMKIIδ2 mutant was transduced in VSM cells for 16 h followed by lysis and immunoblotting for CREB-Ser(P)^{142}, CREB-Ser(P)^{133}, total CREB, and HA tag (CaMKII-HA). *B*, quantification of CREB-Ser(P)^{142} ECL signals. *C*, quantification of CREB-Ser(P)^{133} ECL signals. Data were normalized for loading using GAPDH signals and expressed as fold-values compared with unstimulated. Values shown are mean ± S.E. $n = 3$ and analyzed by one-way ANOVA. *, $p < 0.05$ and **, $p < 0.01$ by Dunnett's post hoc test.

CREB-Ser^{142} phosphorylation compared with CREB-Ser^{133} phosphorylation.

CaMKIIδ Phosphorylates CREB on Ser^{142} in VSM—In vitro studies using purified CaMKII from brain have demonstrated that both CREB Ser^{133} and Ser^{142} are substrates for the kinase (12). The function of CaMKII in regulating endogenous CREB in VSM has only been inferred through pharmacological approaches that do not distinguish between CaMKII and CaMKIV (30). The principal isoform of CaMKII expressed in VSM cells is CaMKIIδ$_2$ (19), or CaMKIIδ$_C$ by alternative nomenclature (31). Transduction of VSM cells with an increasing dose of adenovirus expressing a constitutively active CaMKIIδ$_2$ (T287D) mutant (22) significantly enhanced CREB phosphorylation on Ser^{142} in a dose-dependent manner (Fig. 2, *A* and *B*). However, overexpression of the constitutively active mutant did not affect CREB phosphorylation on Ser^{133} at any of the levels tested. This gain of function experiment indicates a strong specificity of CaMKIIδ for the inhibitory CREB Ser^{142} site in intact VSM cells.

To determine whether endogenous CaMKIIδ regulates CREB phosphorylation on Ser^{133} or Ser^{142}, we used siRNAs specifically targeting CaMKIIδ isoforms to reduce CaMKIIδ expression by ~80–90% (Fig. 3*A*). Consistent with the results in Fig. 1, thrombin stimulated CREB phosphorylation at Ser^{142} (Fig. 3, *A* and *B*) and Ser^{133} (Fig. 3, *A* and *C*) in the presence of control non-targeting siRNA. Suppression of CaMKIIδ expression abolished thrombin-induced CREB phosphorylation on Ser^{142} (Fig. 3*B*), but had no significant effect on thrombin-stimulated CREB phosphorylation on Ser^{133} (Fig. 3*C*). As an alternative approach, we pharmacologically inhibited CaMKII activation in VSM cells using KN93, a selective CaM kinase inhibitor (32). Pretreatment with KN93 (30 μM) for 30 min inhibited thrombin- or ionomycin-induced CaMKIIδ activation (Fig. 4, *A* and *B*) and strongly inhibited CREB phosphorylation on Ser^{142} induced by either stimulus (Fig. 4*C*). Taken together with the gain-of-function results (Fig. 2), these loss-of-function results indicate that endogenous CaMKIIδ mediates CREB phosphorylation on Ser^{142}, but not Ser^{133} in VSM cells.

Additional studies using the MEK1 inhibitor U0126 (5 μM) indicated that essentially complete inhibition of ERK1/2 activation (Fig. 5, *A* and *B*) resulted in a strong inhibition of thrombin- and ionomycin-stimulated CREB-Ser^{133} phosphorylation (Fig. 5*C*), confirming a previous study (9). Residual Ser^{133} phosphorylation in this experiment suggests that another unidentified pathway(s) may also contribute to CREB activation in response to these Ca^{2+}-dependent stimuli.

Thrombin Stimulates Nucleocytoplasmic Translocation of Phospho-Ser^{142}-CREB in VSM—Stevenson *et al.* (33) observed membrane depolarization induced CREB translocation in VSM cells and Garat *et al.* (34) reported nuclear export of CREB in response to sustained PDGF in pulmonary artery smooth muscle cells. The mechanisms signaling nuclear CREB export are not known. Western blotting of nuclear and cytosolic fractions was carried out to assess localization of CREB. Effective fractionation was verified by nuclear histone H3 and cytoplasmic tubulin content, respectively (Fig. 5*A*). Under unstimulated conditions, total CREB and resting levels of phospho-Ser^{142}-CREB were detected in both nuclear and cytosolic fractions. Thrombin stimulation transiently decreased nuclear phospho-Ser^{142}-CREB content by over 50% (Fig. 6*B*), with concomitant increases in the cytosolic fraction (Fig. 6*C*). Phospho-Ser^{133}-CREB increased but remained localized in the nuclear fraction by 5–10 min (Fig. 6, *A* and *D*). Interesting, total CREB also translocated from the nucleus with a time course identical to phospho-Ser^{142}-CREB (Fig. 6, *A* and *B*). CaMKIIδ was detected in nuclear fractions and transiently activated with a peak at 2 min, corresponding temporally with nuclear export of phospho-Ser^{142} and total CREB.

CaMKIIδ Negatively Regulates CREB Reporter Activity and CREB-regulated Genes—The strong correlation between nuclear export of CREB and Ser^{142} phosphorylation by CaMKIIδ suggests one potential mechanism for negative

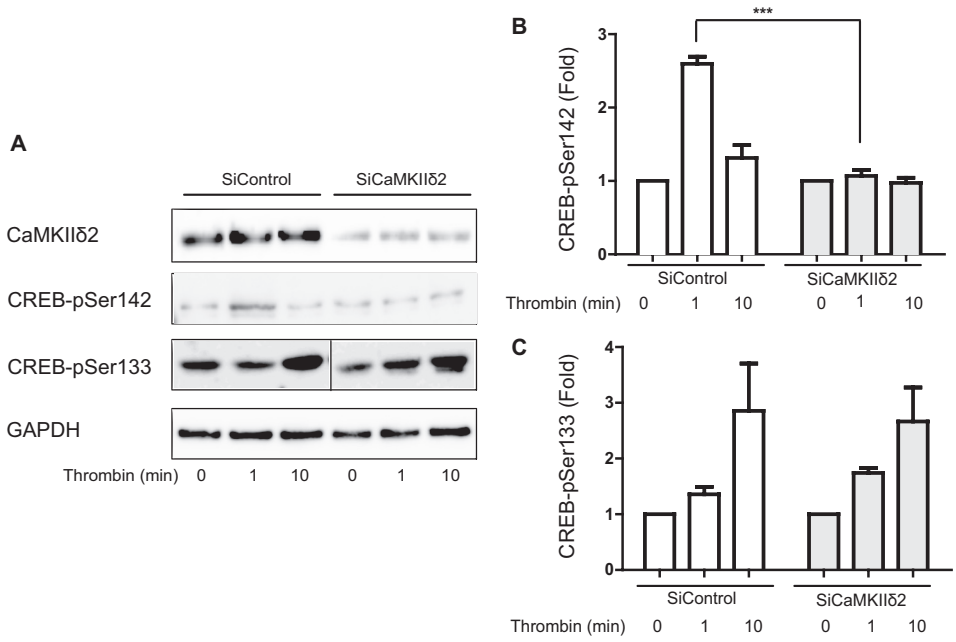

FIGURE 3. **Endogenous CaMKIIδ mediates CREB phosphorylation on Ser^{142}.** *A*, VSM cells were electroporated with non-targeting siRNA (*SiControl*) or siRNA specifically targeting CaMKIIδ (*SiCaMKIIδ*). After 3 days cells were stimulated with thrombin (50 nM) for 1 or 10 min. Cell lysates were resolved by SDS-PAGE and immunoblotted for CaMKIIδ, CREB-Ser(P)^{142}, and CREB-Ser(P)^{133}. *B,* quantification of CREB-Ser(P)^{142} ECl signals. *C,* quantification of CREB-Ser(P)^{133} ECL signals. Data were normalized for loading using GAPDH signals and are expressed as fold-values compared with unstimulated. Values shown are mean ± S.E. $n = 3$. Two-way ANOVA analysis identified significant differences both across time ($p < 0.0001$) and between SiControl and SiCaMKIIδ groups ($p < 0.0001$) in *panel B*, but only across time in *panel C* ($p < 0.0001$). Between groups differences were identified by the Bonferroni post hoc test (***, $p < 0.001$).

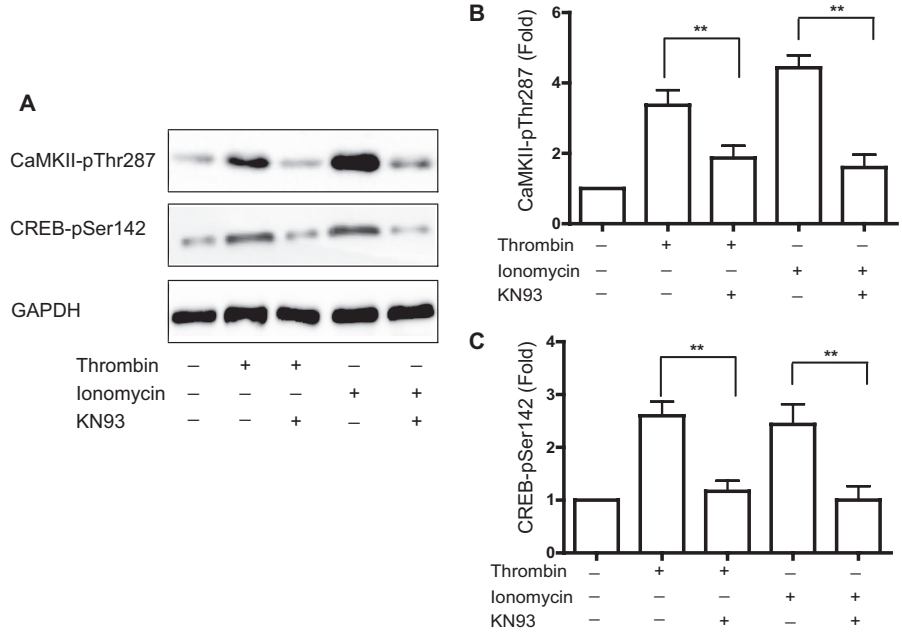

FIGURE 4. **Inhibition of CaMKII activity blocks CREB phosphorylation on Ser^{142}.** *A*, VSM cells were pretreated with KN93 (30 μM) for 30 min and stimulated with thrombin (50 nM) or ionomycin (0.5 μM) for 1 min. Cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-Thr^{287}-CaMKII (*pThr287*), phospho-Ser^{142}-CREB (*pSer142*), and GAPDH. *B*, quantification of CaMKII-Thr(P)^{287} ECL signals. *C,* quantification of CREB-Ser(P)^{142} ECL signals. Data were normalized for loading using GAPDH signals and expressed as fold-values compared with unstimulated. Values shown are mean ± S.E. $n = 3$ and analyzed by unpaired two-tailed Student's *t* test. **, $p < 0.01$.

regulation of CREB activity by CaMKII in VSM. To evaluate the function of CaMKIIδ in regulating CREB activity in VSM, cells were transfected with a luciferase reporter plasmid containing 4 tandem copies of the CREB binding domain. Forskolin, which is a potent activator of the PKA pathway and strongly stimulates CREB activity (7), was used as a positive control. As expected, forskolin (5 μM) strongly increased CREB reporter activity nearly 6-fold, whereas thrombin significantly activated reporter activity by less than 2-fold (Fig. 7, *A* and *B*). Adenoviral overexpression of a kinase-negative CaMKIIδ$_2$ (K43A) mutant, which exerts a dominant-negative effect on endogenous CaMKII activity *in situ* (19), significantly enhanced subsequent throm-

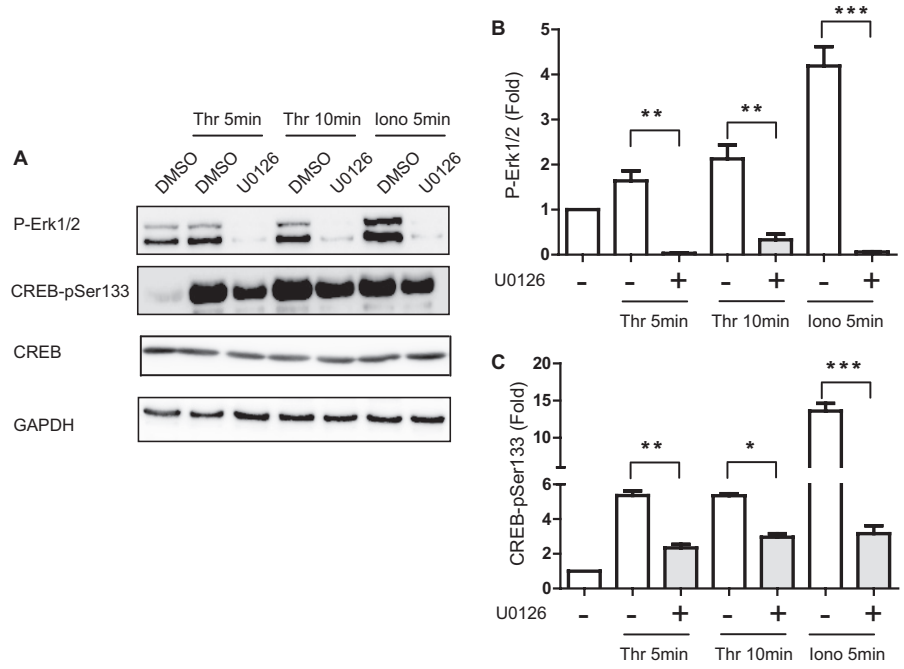

FIGURE 5. **ERK1/2 mediates thrombin-induced phosphorylation of CREB-Ser^{133}.** *A,* cultured VSM cells were pretreated with the MEK inhibitor U0126 (5 μM) or vehicle control (dimethyl sulfoxide, *DMSO*) for 30 min prior to stimulation with thrombin (50 nM) or ionomycin (*Iono*; 0. 5 μM) for the indicated times. Cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-ERK1/2, phospho-Ser^{133}-CREB (*pSer133*), total CREB, and GAPDH. *B,* P-ERK ECL signals, and *C,* CREB-Ser(P)^{133} signals were quantified with a FujiLAS 4000. Data were normalized for loading using GAPDH signals and expressed as fold-values compared with unstimulated. Values shown are mean ± S.E., $n = 3$ and were analyzed by one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Bonferroni post hoc test.

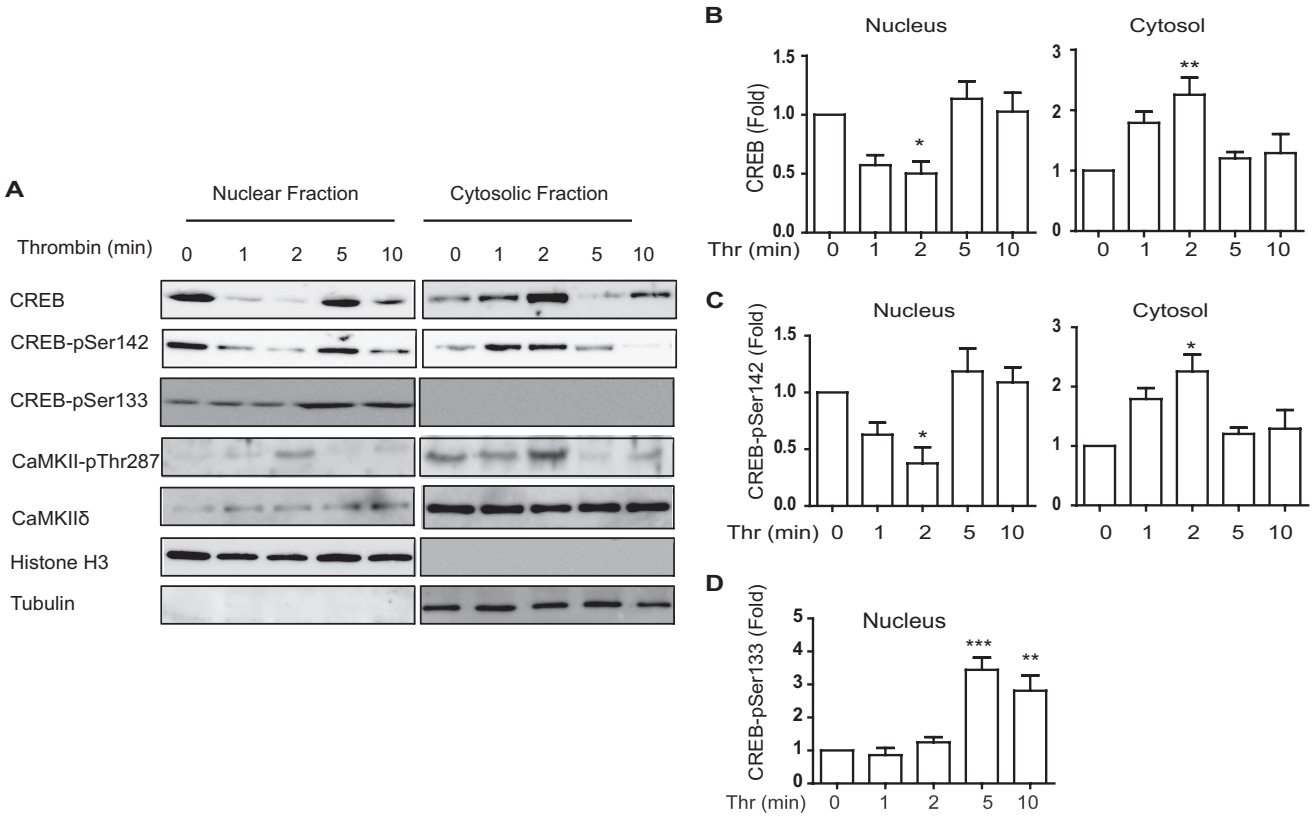

FIGURE 6. **Thrombin-induced CREB translocation.** VSM cells stimulated with thrombin (Thr; 50 nM) were fractionated to separate nuclear and cytosolic fractions. *A,* aliquots of fractions were resolved by SDS-PAGE and immunoblotted for CREB, CREB-Ser(P)^{142}, CREB-Ser(P)^{133}, CaMKIIδ, and CaMKIIδ-Thr(P)^{287}. Histone H3 and -α were used as markers of the nuclear and cytosolic fractions, respectively, and to normalize signals for loading. *B*, quantification of total CREB signals. *C*, quantification of phospho-Ser^{142}-CREB (*CREB-pSer142*) signals. *D*, quantification of phospho-Ser^{133}-CREB (*CREB-pSer133*) signal in nuclear fraction. Signals in *Cytosol* were undetectable. Mean data are expressed as fold of unstimulated at 0 min. Values shown are mean ± S.E., $n = 3$, and were analyzed by one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Dunnett's post hoc test.

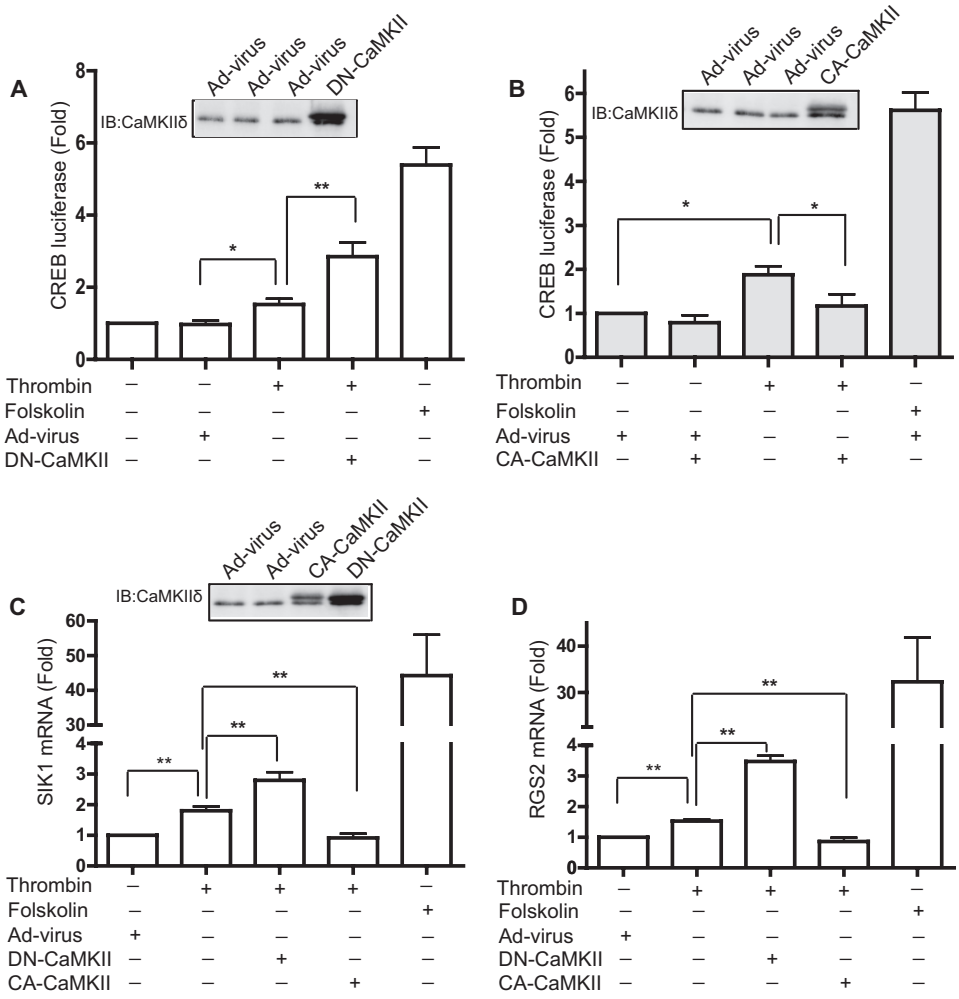

FIGURE 7. **CaMKIIδ inhibits CREB reporter activity and CREB-regulated genes.** *A* and *B*, CREB luciferase reporter stimulated by thrombin (50 nM) or forskolin (5 μM) for 6 h. Adenovirus encoding a dominant-negative CaMKIIδ2 mutant (*DN-CaMKII*) (*A*) or constitutively active CaMKIIδ2 mutant (*CA-CaMKII*) (*B*) was transduced for 48 and 24 h prior to cell lysis, respectively (see *insets* for immunoblots (*IB*) of CaMKIIδ expression). The plotted values are expressed as fold of unstimulated cells transduced with an adenovirus control (*Ad-virus*). *C*, mRNA expression of *Sik1* ($n = 5$), and *D*, *Rgs2* ($n = 4$) was determined by RT-PCR in cells treated as described above. C_t values were normalized to GAPDH. Changes in mRNA in each group are fold-values of the unstimulated control. Values shown are mean ± S.E. and analyzed by one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$, by Bonferroni post hoc test.

bin-induced CREB reporter activity by 3-fold over unstimulated levels (Fig. 7*A*). Conversely, adenoviral transduction and expression of a constitutively active CaMKIIδ$_2$ (T287D) mutant significantly inhibited thrombin-induced CREB reporter activity to nearly basal levels (Fig. 7*B*).

To investigate whether CaMKIIδ-dependent negative regulation of CREB activity extends to endogenous transcriptional targets for CREB in VSM cells, we examined the effect of CaMKIIδ$_2$ mutants on mRNA expression of *Sik1* (salt-induced kinase 1; Fig. 7*C*) and *Rgs2* (regulator of G-protein signaling 2; Fig. 7*D*). SIK1 is endogenously expressed in VSM and contributes to hypertension through regulation of the plasma membrane Na^+,K^+-ATPase (35). In addition, SIK1 has been identified as a transcriptional target for CREB in skeletal muscle cells and to interact with and regulate the type 2 HDAC-MEF2 pathway (36). *Rgs2* contains a conserved CREB binding site in its promoter (37) and functions in an angiotensin II-dependent feedback loop to regulate blood pressure (38). Similar to the luciferase CREB reporter data above, forskolin treatment dramatically increased mRNA levels of *Sik1* and *Rgs2* by 30–40-fold, whereas thrombin treatment induced *Sik1* and *Rgs2* mRNA expression by less than 2-fold. Inhibition of CaMKIIδ activity by overexpressing kinase-negative CaMKIIδ$_2$ significantly enhanced the induction of *Sik1* and *Rgs2* mRNA expression in response to thrombin. Adenoviral introduction of constitutively active CaMKIIδ$_2$ abolished thrombin-induced *Sik1* and *Rgs2* mRNA induction. In contrast to previous reports (16–18), these data suggest that Ca^{2+} signaling mediated by CaMKIIδ *inhibits* CREB activity in VSM cells.

To directly test the effects CaMKIIδ activity on CREB binding to *Sik1* and *Rgs2* promoters, chromatin immunoprecipitation (ChIP) assays were performed using CREB antibody to isolate CREB/CREB-binding element complexes (Fig. 8). Control experiments in unstimulated cells confirmed CREB binding to the targeted binding elements in the *Sik1* and *Rgs2* promoters (Fig. 8*A*). Thrombin stimulation for 10 min increased CREB binding to the promoters in 3 separate experiments. Because the fold-inductions with thrombin were variable between the experiments (2–35-fold for *Sik1*; 3–75-fold for *Rgs2*), effects of overexpressing constitutively active and kinase-negative

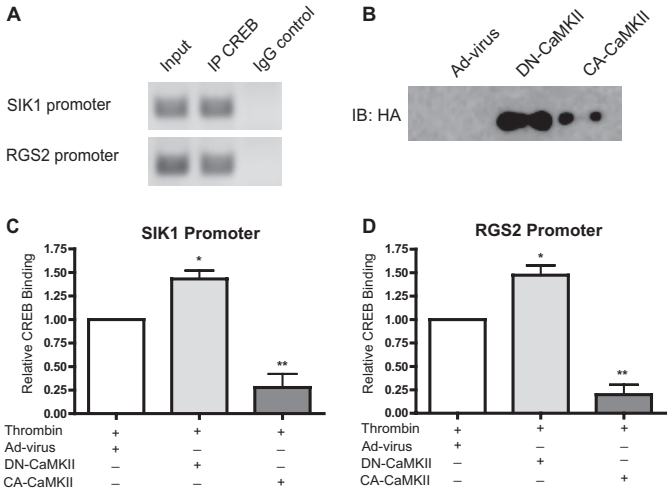

FIGURE 8. **CaMKIIδ inhibits CREB binding to *Sik1* and *Rgs2* promoters.** *A*, ChIP were performed using anti-CREB antibody on unstimulated VSM cells. CREB-binding elements in *Sik1* and *Rgs2* promoters were amplified by quantitative PCR and products resolved on an agarose gel as follows: from sheared chromatin prior to IP (*input*), chromatin immunoprecipitations (IPs) (*IP CREB*), and IP (IgG) control. *B–D*, effect of CaMKII mutant expression on thrombin-stimulated CREB binding to SIK1 (*C*) and RGS2 (*D*) promoters. VSM cells were transduced with adenovirus encoding dominant-negative (DN)-CaMKII (100 multiplicity of infection), constitutively active CA-CaMKII (50 multiplicity of infection), or adenovirus control (*Ad-virus*). Thrombin (50 nM) was added 10 min prior to fixation and processing for ChIP. DN-CaMKII and CA-CaMKII constructs were detected by immunoblotting with anti-HA antibodies (immunoblot (*IB*): HA) in supernatants following ChIP (*B*). C_t values were normalized to thrombin stimulation alone. Values shown are mean ± S.E., $n = 3$, and analyzed by one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$, by Dunnett's post hoc test.

CaMKIIδ$_2$ were normalized to the thrombin signal. Expression of constitutively active CaMKIIδ$_2$ (Fig. 8*B*) was reproducibly effective at blocking thrombin-induced CREB binding on both the *Sik1* (Fig. 8*C*) and *Rgs2* (Fig. 8*D*) promoters, whereas overexpression of kinase-negative CaMKIIδ$_2$ reproducibly enhanced CREB binding.

DISCUSSION

Increasing evidence indicates that Ca^{2+} signaling via CaMKIIδ positively regulates VSM cell proliferation and migration and contributes to vascular remodeling in response to injury or hypertensive disease (38–40). Here, we present evidence that Ca^{2+} signaling via CaMKIIδ mediates thrombin-induced CREB phosphorylation selectively on Ser^{142}, correlating with CREB translocation from the nucleus. One outcome of thrombin-induced CaMKII-dependent signaling in VSM is negative regulation of CREB binding to CREB regulatory elements in specific target genes (in this case *Sik1* and *Rgs2*) and negative regulation of CREB-induced transcriptional activity as indicated by a luciferase reporter assay and transcription of *Sik1* and *Rgs2*. We believe the results of this study provide a definitive answer to the function of CaMKIIδ in regulating CREB activity in VSM cells and correct any potential misinterpretations of experiments that rely solely on pharmacological inhibitors of CaMKII or extrapolations of results from other systems such as neural tissues, which have suggested positive regulation of CREB activity by CaMKII (17, 18, 30).

Early studies demonstrated that purified brain CaMKII phosphorylated recombinant CREB at both Ser^{133} and Ser^{142} in several cultured cell lines (12, 41). Moreover, Wu and McMurray (11) reported that CaMKII-phosphorylated CREB at both Ser^{133} and Ser^{142} in human neuroblastoma and monkey kidney cells. Our experiments, using an adenovirally transduced constitutively active CaMKIIδ$_2$ mutant and multiple molecular and pharmacological loss-of-function approaches demonstrate that in intact VSM cells stimulated by thrombin or a Ca^{2+} ionophore, CaMKII selectively phosphorylates endogenous CREB on Ser^{142}. Lack of CREB Ser^{133} phosphorylation downstream of CaMKII in VSM could be due to isoform-specific effects with CaMKIIδ$_2$ the predominant isoform expressed in VSM cells, compared with CaMKIIα and -β isoforms, which are predominant in neural tissue. Alternatively, unknown factors that are properties of intact VSM cells might restrict CaMKIIδ access to CREB-Ser^{133}.

Most of the positive regulation of CREB via Ser^{133} phosphorylation in this study could be attributed to ERK1/2-dependent signaling as reflected by inhibition of responses by the MEK inhibitor U0126, confirming previous results (9). Although the mechanisms underlying the residual Ser^{133} phosphorylation are not known, we do not expect that Ser^{133} phosphorylation is mediated by CaMKIV in VSM cells, because we are unable to reliably detect either CaMKIV mRNA or protein expression.[3]

Phosphorylation of CREB on Ser^{142} has been shown to attenuate CREB activity, even if Ser^{133} is phosphorylated (11). Consistent with this concept, we observed that expression of constitutively active CaMKIIδ$_2$ nearly abolished the thrombin-induced activation of a CREB reporter, whereas, thrombin-stimulated CREB reporter activity was further enhanced by inhibiting CaMKII activity using dominant-negative CaMKIIδ$_2$. Wu and McMurray (11) presented evidence that CREB Ser^{142} phosphorylation in response to CaMKII activation prevented CREB dimerization and interaction with the CREB-binding protein. There are a few reports of CREB translocation from the nucleus that would also result in decreased activity (33, 34). Membrane depolarization in VSM cells induced elevated intracellular Ca^{2+} and mediated acute nuclear export of CREB (33) and prolonged stimulation with PDGF-BB-induced nuclear export of CREB and subsequently degradation through the ubiquitination pathway (34). Our studies of CREB localization in VSM cells following acute stimulation with thrombin demonstrate a strong correlation between CaMKII-dependent Ser^{142} phosphorylation and nuclear export, a mechanism that could also explain depolarization-induced nuclear export. Even with the relatively brief period of Ser^{142} phosphorylation, the extent of CREB nuclear export is substantial (greater than 50% of total CREB in the nucleus) suggesting a functionally important mode of CREB regulation. However, basal levels of CREB Ser^{142} phosphorylation appear in the nucleus in unstimulated cells, suggesting that mechanisms in addition to Ser^{142} phosphorylation may be required for nuclear export.

Although CaMKIIδ$_2$ is the most abundant isoform in VSM and is localized predominantly in the cytosol, we did detect CaMKIIδ in VSM nuclei and activation of the nuclear kinase following thrombin stimulation. This may be explained by co-

[3] Z. Wang and H. A. Singer, unpublished data.

expression of small amounts of the alternative splice variant CaMKIIδ$_3$ (or CaMKIIδ$_B$) that was first cloned from VSM cells (19). CaMKIIδ$_3$ contains a nuclear targeting domain and when co-expressed with CaMKIIδ$_2$ targets the kinase to the nucleus in proportion to composition of hetromultimeric holoenzymes (42). The small fraction of total and active CaMKIIδ in the nuclear fraction of VSM cells is consistent with the relative expression of the δ$_2$ isoform compared with δ$_3$ in VSM cells (19). How much negative CREB regulation is due directly to CaMKIIδ-mediated phosphorylation of Ser^{142} in the nucleus *versus* indirectly by CREB-Ser^{142} phosphorylation and trapping in the cytosol remains to be determined. We would predict that in other systems such as intact differentiated VSM (19) or heart (43–44) where the nuclear targeting isoform CaMKIIδ$_3$ is expressed more abundantly, this mode of regulating CREB may be even more predominant. Alternatively, conditions of prolonged CaMKII activation occurring under conditions with high frequency Ca^{2+} transients (45) or oxidizing conditions (46–48), may be predicted to promote nuclear CREB export and potentially degradation (34).

Sik1 and *Rgs2* have been reported to be regulated by CREB in multiple cell types, including VSM cells (35–37). In this study, ChIP assays confirmed thrombin-induced CREB binding to an element in the RGS2 promoter previously identified using mutagenesis approaches (36). We also confirmed CREB binding to a predicted element in the *Sik1* promoter (37). Moreover, we extended knowledge of CREB target regulation by demonstrating CaMKIIδ negatively regulated thrombin-induced CREB binding to *Sik1* and *Rgs2* promoters, as well as transcription of the respective mRNAs. It has been reported that SIK1 regulates both the activity and expression of Na,K-ATPase in the renal proximal tubule, and in turn influences sodium and fluid reabsorption (49), which may provide a pathway affecting blood pressure regulation. Xie *et al.* (37) identified a CREB binding site in the RGS2 promoter sequence and demonstrated that angiotensin II-induced CREB activation upregulated *Rgs2* expression in VSM cells with effects on blood pressure homeostasis. CaMKII is also activated in response to angiotensin II stimulation in VSM (5) and is a likely component of angiotensin II-dependent regulation of CREB activity and RGS2 expression.

Our laboratory first reported that CaMKIIδ$_2$ promoted VSM cell migration and proliferation *in vitro* (50) and exerted a positive effect on neointima formation *in vivo* (38). Recent studies using global CaMKIIδ gene knock-out mice have confirmed positive roles of CaMKIIδ$_2$ in regulating the VSM cell proliferation and migration (39, 40). Aside from direct regulation of proteins involved in cell cycle control and cell motility, we have reported that CaMKIIδ$_2$ phosphorylates HDAC4/5, resulting in nucleocytoplasmic shuttling of the HDACs and subsequent de-repression of MEF2 transcriptional activity in VSM cells (5). CREB is a well known downstream effector of the cAMP-PKA signaling pathway and has been shown to exert negative regulation of VSM cell migration and proliferation (34, 51). Negative regulation of CREB by CaMKIIδ as defined in the present study, and subsequent relief of its inhibitory effects could be another mechanism whereby CaMKII affects the phenotype of VSM and contributes to increased VSM cell proliferation and migration.

In summary, we have shown that activation of CaMKIIδ$_2$ in response to Ca^{2+} mobilizing stimuli results in CREB-Ser^{142} phosphorylation and inhibition of CREB activity and transcriptional responses in vascular smooth muscle. In this system, CaMKII has no effect on the activating CREB-Ser^{133} phosphorylation event. For the first time we demonstrate a strong correlation between CREB-Ser^{142} phosphorylation and nuclear export of CREB suggesting one potential mechanism whereby CaMKII negatively regulates CREB activity. Further studies are required to determine the mechanism of CaMKII-dependent nuclear export of CREB and function of the CaMKIIδ$_2$-CREB pathway in VSM cell phenotype control and vascular remodeling contributing to occlusive vascular diseases.

REFERENCES

1. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.* **84,** 767–801
2. Gomez, D., and Owens, G. K. (2012) Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc. Res.* **95,** 156–164
3. Matchkov, V. V., Kudryavtseva, O., and Aalkjaer, C. (2012) Intracellular Ca^{2+} signalling and phenotype of vascular smooth muscle cells. *Basic Clin. Pharmacol. Toxicol.* **110,** 42–48
4. Barlow, C. A., Rose, P., Pulver-Kaste, R. A., and Lounsbury, K. M. (2006) Excitation-transcription coupling in smooth muscle. *J. Physiol.* **570,** 59–64
5. Ginnan, R., Sun, L. Y., Schwarz, J. J., and Singer, H. A. (2012) MEF2 is regulated by CaMKIIδ2 and a HDAC4-HDAC5 heterodimer in vascular smooth muscle cells. *Biochem. J.* **444,** 105–114
6. Wamhoff, B. R., Bowles, D. K., and Owens, G. K. (2006) Excitation-transcription coupling in arterial smooth muscle. *Circ. Res.* **98,** 868–878
7. Shaywitz, A. J., and Greenberg, M. E. (1999) CREB. A stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev. Biochem.* **68,** 821–861
8. Schauer, I. E., Knaub, L. A., Lloyd, M., Watson, P. A., Gliwa, C., Lewis, K. E., Chait, A., Klemm, D. J., Gunter, J. M., Bouchard, R., McDonald, T. O., O'Brien, K. D., and Reusch, J. E. (2010) CREB down-regulation in vascular disease. A common response to cardiovascular risk. *Arterioscler. Thromb. Vasc. Biol.* **30,** 733–741
9. Tokunou, T., Ichiki, T., Takeda, K., Funakoshi, Y., Iino, N., and Takeshita, A. (2001) cAMP response element-binding protein mediates thrombin-induced proliferation of vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **21,** 1764–1769
10. Ono, H., Ichiki, T., Fukuyama, K., Iino, N., Masuda, S., Egashira, K., and Takeshita, A. (2004) cAMP-response element-binding protein mediates tumor necrosis factor-α-induced vascular smooth muscle cell migration. *Arterioscler. Thromb. Vasc. Biol.* **24,** 1634–1639
11. Wu, X., and McMurray, C. T. (2001) Calmodulin kinase II attenuation of gene transcription by preventing cAMP response element-binding protein (CREB) dimerization and binding of the CREB-binding protein. *J. Biol. Chem.* **276,** 1735–1741
12. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8,** 2527–2539
13. Miyamoto, E. (2006) Molecular mechanism of neuronal plasticity. Induction and maintenance of long-term potentiation in the hippocampus. *J. Pharmacol. Sci.* **100,** 433–442
14. Takeda, H., Kitaoka, Y., Hayashi, Y., Kumai, T., Munemasa, Y., Fujino, H., Kobayashi, S., and Ueno, S. (2007) Calcium/calmodulin-dependent protein kinase II regulates the phosphorylation of CREB in NMDA-induced retinal neurotoxicity. *Brain Res.* **1184,** 306–315

15. Wheeler, D. G., Barrett, C. F., Groth, R. D., Safa, P., and Tsien, R. W. (2008) CaMKII locally encodes L-type channel activity to signal to nuclear CREB in excitation-transcription coupling. *J. Cell Biol.* **183,** 849–863
16. Bell, K. F., Bent, R. J., Meese-Tamuri, S., Ali, A., Forder, J. P., and Aarts, M. M. (2013) Calmodulin kinase IV-dependent CREB activation is required for neuroprotection via NMDA receptor-PSD95 disruption. *J. Neurochem.* **126,** 274–287
17. Li, J., Zhao, S. Z., Wang, P. P., Yu, S. P., Zheng, Z., and Xu, X. (2012) Calcium mediates high glucose-induced HIF-1alpha and VEGF expression in cultured rat retinal Muller cells through CaMKII-CREB pathway. *Acta Pharmacol. Sin.* **33,** 1030–1036
18. Sánchez-Muñoz, I., Sánchez-Franco, F., Vallejo, M., Fernández, A., Palacios, N., Fernández, M., and Cacicedo, L. (2010) Activity-dependent somatostatin gene expression is regulated by cAMP-dependent protein kinase and Ca^{2+}-calmodulin kinase pathways. *J. Neurosci. Res.* **88,** 825–836
19. Schworer, C. M., Rothblum, L. I., Thekkumkara, T. J., and Singer, H. A. (1993) Identification of novel isoforms of the delta subunit of Ca^{2+}/calmodulin-dependent protein kinase II. Differential expression in rat brain and aorta. *J. Biol. Chem.* **268,** 14443–14449
20. Van Riper, D. A., Schworer, C. M., and Singer, H. A. (2000) Ca^{2+}-induced redistribution of Ca^{2+}/calmodulin-dependent protein kinase II associated with an endoplasmic reticulum stress response in vascular smooth muscle. *Mol. Cell. Biochem.* **213,** 83–92
21. Ginnan, R., Pfleiderer, P. J., Pumiglia, K., and Singer, H. A. (2004) PKC-δ and CaMKII-δ2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. *Am. J. Physiol. Cell Physiol.* **286,** C1281–1289
22. Pfleiderer, P. J., Lu, K. K., Crow, M. T., Keller, R. S., and Singer, H. A. (2004) Modulation of vascular smooth muscle cell migration by calcium/calmodulin-dependent protein kinase II-δ 2. *Am. J. Physiol. Cell Physiol* **286,** C1238–1245
23. Geisterfer, A. A., Peach, M. J., and Owens, G. K. (1988) Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* **62,** 749–756
24. Halees, A. S., Leyfer, D., and Weng, Z. (2003) PromoSer. A large-scale mammalian transcription start site identification service. *Nucleic Acids Res.* **31,** 3554–3559
25. Messeguer, X., Escudero, R., Farré, D., Núñez, O., Martínez, J., and Albà, M.M. (2002) PROMO. Detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* **18,** 333–334
26. Coughlin, S. R. (2000) Thrombin signalling and protease-activated receptors. *Nature* **407,** 258–264
27. Wang, Z., Ginnan, R., Abdullaev, I. F., Trebak, M., Vincent, P. A., and Singer, H. A. (2010) Calcium/Calmodulin-dependent protein kinase II δ6 (CaMKIIδ6) and RhoA involvement in thrombin-induced endothelial barrier dysfunction. *J. Biol. Chem.* **285,** 21303–21312
28. Kornhauser, J. M., Cowan, C. W., Shaywitz, A. J., Dolmetsch, R. E., Griffith, E. C., Hu, L. S., Haddad, C., Xia, Z., and Greenberg, M. E. (2002) CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. *Neuron* **34,** 221–233
29. Schworer, C. M., Colbran, R. J., Keefer, J. R., and Soderling, T. R. (1988) Ca^{2+}/calmodulin-dependent protein kinase II. Identification of a regulatory autophosphorylation site adjacent to the inhibitory and calmodulin-binding domains. *J. Biol. Chem.* **263,** 13486–13489
30. Cartin, L., Lounsbury, K. M., and Nelson, M. T. (2000) Coupling of Ca^{2+} to CREB activation and gene expression in intact cerebral arteries from mouse. Roles of ryanodine receptors and voltage-dependent Ca^{2+} channels. *Circ. Res.* **86,** 760–767
31. Edman, C. F., and Schulman, H. (1994) Identification and characterization of δB-CaM kinase and delta C-CaM kinase from rat heart, two new multifunctional Ca^{2+}/calmodulin-dependent protein kinase isoforms. *Biochim. Biophys. Acta* **1221,** 89–101
32. Anderson, M. E., Braun, A. P., Wu, Y., Lu, T., Wu, Y., Schulman, H., and Sung, R. J. (1998) KN-93, an inhibitor of multifunctional Ca^{2+}/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J. Pharmacol. Exp. Ther.* **287,** 996–1006
33. Stevenson, A. S., Cartin, L., Wellman, T. L., Dick, M. H., Nelson, M. T., and Lounsbury, K. M. (2001) Membrane depolarization mediates phosphorylation and nuclear translocation of CREB in vascular smooth muscle cells. *Exp. Cell Res.* **263,** 118–130
34. Garat, C. V., Fankell, D., Erickson, P. F., Reusch, J. E., Bauer, N. N., McMurtry, I. F., and Klemm, D. J. (2006) Platelet-derived growth factor BB induces nuclear export and proteasomal degradation of CREB via phosphatidylinositol 3-kinase/Akt signaling in pulmonary artery smooth muscle cells. *Mol. Cell. Biol.* **26,** 4934–4948
35. Popov, S., Silveira, A., Wågsäter, D., Takemori, H., Oguro, R., Matsumoto, S., Sugimoto, K., Kamide, K., Hirose, T., Satoh, M., Metoki, H., Kikuya, M., Ohkubo, T., Katsuya, T., Rakugi, H., Imai, Y., Sanchez, F., Leosdottir, M., Syvänen, A. C., Hamsten, A., Melander, O., and Bertorello, A. M. (2011) Salt-inducible kinase 1 influences Na^+,K^+-ATPase activity in vascular smooth muscle cells and associates with variations in blood pressure. *J. Hypertens.* **29,** 2395–2403
36. Berdeaux, R., Goebel, N., Banaszynski, L., Takemori, H., Wandless, T., Shelton, G. D., and Montminy, M. (2007) SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. *Nat. Med.* **13,** 597–603
37. Xie, Z., Liu, D., Liu, S., Calderon, L., Zhao, G., Turk, J., and Guo, Z. (2011) Identification of a cAMP-response element in the regulator of G-protein signaling-2 (RGS2) promoter as a key cis-regulatory element for RGS2 transcriptional regulation by angiotensin II in cultured vascular smooth muscles. *J. Biol. Chem.* **286,** 44646–44658
38. House, S. J., and Singer, H. A. (2008) CaMKII-delta isoform regulation of neointima formation after vascular injury. *Arterioscler. Thromb. Vasc. Biol.* **28,** 441–447
39. Li, W., Li, H., Sanders, P. N., Mohler, P. J., Backs, J., Olson, E. N., Anderson, M. E., and Grumbach, I. M. (2011) The multifunctional Ca^{2+}/calmodulin-dependent kinase II delta (CaMKIIδ) controls neointima formation after carotid ligation and vascular smooth muscle cell proliferation through cell cycle regulation by p21. *J. Biol. Chem.* **286,** 7990–7999
40. Scott, J. A., Xie, L., Li, H., Li, W., He, J. B., Sanders, P. N., Carter, A. B., Backs, J., Anderson, M. E., and Grumbach, I. M. (2012) The multifunctional Ca^{2+}/calmodulin-dependent kinase II regulates vascular smooth muscle migration through matrix metalloproteinase 9. *Am. J. Physiol. Heart Circ. Physiol.* **302,** H1953–1964
41. Enslen, H., Sun, P., Brickey, D., Soderling, S. H., Klamo, E., and Soderling, T. R. (1994) Characterization of Ca^{2+}/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. *J. Biol. Chem.* **269,** 15520–15527
42. Srinivasan, M., Edman, C. F., and Schulman, H. (1994) Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. *J. Cell Biol.* **126,** 839–852
43. Mayer, P., Möhlig, M., Idlibe, D., and Pfeiffer, A. (1995) Novel and uncommon isoforms of the calcium sensing enzyme calcium/calmodulin dependent protein kinase II in heart tissue. *Basic Res. Cardiol.* **90,** 372–379
44. Zhang, T., and Brown, J. H. (2004) Role of Ca^{2+}/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure. *Cardiovasc. Res.* **63,** 476–486
45. Li, L., Stefan, M. I., and Le Novère, N. (2012) Calcium input frequency, duration and amplitude differentially modulate the relative activation of calcineurin and CaMKII. *PloS One* **7,** e43810
46. Erickson, J. R., He, B. J., Grumbach, I. M., and Anderson, M. E. (2011) CaMKII in the cardiovascular system. Sensing redox states. *Physiol. Rev.* **91,** 889–915
47. Erickson, J. R., Joiner, M. L., Guan, X., Kutschke, W., Yang, J., Oddis, C. V., Bartlett, R. K., Lowe, J. S., O'Donnell, S. E., Aykin-Burns, N., Zimmerman, M. C., Zimmerman, K., Ham, A. J., Weiss, R. M., Spitz, D. R., Shea, M. A., Colbran, R. J., Mohler, P. J., and Anderson, M. E. (2008) A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* **133,** 462–474
48. Shetty, P. K., Huang, F. L., and Huang, K. P. (2008) Ischemia-elicited oxi-

dative modulation of Ca^{2+}/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **283,** 5389–5401

49. Taub, M., Springate, J. E., and Cutuli, F. (2010) Targeting of renal proximal tubule Na,K-ATPase by salt-inducible kinase. *Biochem. Biophys. Res. Commun.* **393,** 339–344
50. House, S. J., Ginnan, R. G., Armstrong, S. E., and Singer, H. A. (2007) Calcium/calmodulin-dependent protein kinase II-δ isoform regulation of vascular smooth muscle cell proliferation. *Am. J. Physiol. Cell Physiol* **292,** C2276–2287
51. Klemm, D. J., Watson, P. A., Frid, M. G., Dempsey, E. C., Schaack, J., Colton, L. A., Nesterova, A., Stenmark, K. R., and Reusch, J. E. (2001) cAMP response element-binding protein content is a molecular determinant of smooth muscle cell proliferation and migration. *J. Biol. Chem.* **276,** 46132–46141