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Calcineurin-mediated pathway involved in the differentiated phenotype of smooth muscle cells

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Abstract

The calcineurin-mediated pathway is involved in skeletal and cardiac hypertrophy and vascular development in vivo, but the relationship between this pathway and the phenotype of smooth muscle cells (SMCs) remains unknown. Using visceral SMCs in culture as a model system of differentiated SMCs, we investigated the role of the calcineurin-mediated pathway in maintaining the differentiated phenotype of SMCs, which depends on the insulin-like growth factor (IGF-I)-triggered activation of the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB(Akt)) pathway. Treatment with calcineurin inhibitors, cyclosporin A or FK506, or the forced expression of the natural calcineurin inhibitor, CAIN, induced SMC dedifferentiation. Notably, suppression of the promoter activities of the SMC molecular markers caldesmon and α1 integrin by blocking the PI3-K/PKB(Akt) pathway was rescued by the forced expression of constitutively active calcineurin Aα, suggesting that the calcineurin-mediated pathway is critical for maintaining the differentiated phenotype of SMCs and works downstream of the PI3-K/PKB(Akt) pathway.

Keywords: Smooth muscle cells; Phenotypic modulation; Caldesmon; α1 Integrin; Calcineurin; Protein kinase B

Recent studies demonstrate that the signaling pathway mediated through calcineurin/nuclear factor of activated T-cells (NFAT) plays a pivotal role in cardiac and skeletal muscle hypertrophy and vascular development [1,2]. Transgenic mice expressing a constitutively active calcineurin or NFATc4 in the heart develop a hypertrophy that induces dilated cardiomyopathy [3]. Treatment of primary cultured cardiomyocytes with a calcineurin inhibitor, cyclosporin A (CsA), prevents the hypertrophy that is induced by angiotensin II or phenylephrine [4]. Similarly, treatment with CsA or expression of CAIN, a natural inhibitor of calcineurin, markedly inhibits skeletal muscle differentiation in culture [5] and the calcineurin/NFATc1-mediated hypertrophy of skeletal muscle triggered by insulin-like growth factor-I (IGF-I) [6,7]. In vivo, NFATc2-deficient mice [8] or mice given CsA [9] show a significant decrease in the size of their myofibers. Mice with null mutations of both NFATc3 and c4, but neither alone, show a disorganization of developing blood vessels, and similar phenotypes are also seen in mice bearing a mutation in the regulatory subunit of calcineurin B [10]. In agreement with these results, the administration of CsA or another type of calcineurin inhibitor, FK506, to pregnant mice results in the identical developmental defects [10]. In passaged vascular smooth muscle cells (SMCs) showing the dedifferentiated phenotype, treatment with CsA or FK506 markedly inhibits the expression of SMC molecular markers, such as smooth muscle α-actin and myosin heavy chain [11]. However, it remains unclear whether the pathway mediated through calcineurin is involved in the determination of the SMC phenotype.

We have established primary culture systems for visceral and vascular SMCs, in which both types of SMCs are cultured on laminin under IGF-I-stimulated conditions and can maintain a differentiated phenotype as defined by a spindle-like shape, ligand-induced contractility, and high expression and isoform conversion of SMC molecular markers [12–16]. In differentiated SMCs, IGF-I solely activates the phosphatidylinositol

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3-kinase (PI3-K)/protein kinase B (PKB(Akt)) pathway, but not mitogen-activated protein kinases (MAPKs) such asextracellular signal-regulated kinase (ERK) and p38MAPK, which play a vital role in the induction of SMC dedifferentiation [14–16]. Thus, the PI3-K/PKB(Akt) pathway is critical for maintaining the differentiated phenotype of visceral and vascular SMCs, and the balance of the strengths between the PI3-K/PKB(Akt) pathway and the two MAPK pathways determines their phenotype [14–16]. To date, the role of the calcineurin-mediated pathway has not been characterized in visceral SMCs. We investigated the effects of CsA, FK506, and CAIN on the differentiated phenotype of visceral SMCs in culture. We used this model system for differentiated SMCs because, as described above, the same signaling pathways regulate the phenotypic determination of visceral and vascular SMCs [15,16]. Here, we show for the first time that the calcineurin-mediated pathway is critical for maintaining the differentiated phenotype of SMCs and that it works downstream of the PI3-K/PKB(Akt) pathway.

Materials and methods

SMC culture. Differentiated SMCs were prepared from 15-day-old chicken embryo gizzards and were cultured on laminin under IGF-I [2 ng/ml]-stimulated conditions as described previously [14]. Ligand-induced contractility was monitored as described in our previous papers [14].

Northern blotting. Total RNAs isolated from the cultured SMCs under the indicated conditions were separated on 1.0% agarose–formaldehyde denaturing gels and transferred to nylon membranes. The blots were hybridized (with ^{32}P-labeled antisense stranded cDNA corresponding to the common region of *h*- and *l*-CaD, CN, and α1 integrin, as described previously [14,17]. To quantify the amount of RNAs in each sample, the membranes were stained with 0.02% methylene blue to visualize the ribosomal RNAs.

Promoter analysis. The CaD promoter plasmid (GP2-luc) was constructed by replacing the CAT gene in GP2CAT [18] with the luciferase gene from pGL3-Basic (Promega). The α1 integrin promoter plasmid was described in our previous reports [17,19]. The expression plasmid of dominant-negative PKB(Akt) was constructed by substituting Lys^{179} from the ATP-binding site with Ala [20]. The Myc-tagged CAIN [5] expression plasmid was constructed in an expression plasmid carrying the cytomegalovirus promoter (pCS2+MT). The Myc-tagged constitutively active calcineurin Aα (amino acids 1-345) expression plasmid was constructed in pCS2+MT. The insert was amplified by PCR using rat calcineurin cDNA that was provided by Dr. T. Kuno (Department of Pharmacology, Kobe University School of Medicine) as a template, as described previously [4]. Differentiated SMCs were co-transfected with 1.0 μg of control plasmid carrying the β-galactosidase gene under the simian virus 40 (SV40) promoter (pSV-β-galactosidase), 2.0 μg of the CaD or α1 integrin promoter plasmid, and the indicated expression plasmid, using Trans IT-LT1, polyamine transfection reagents (Pan Vera Corporation). Two days after transfection, the SMCs were lysed and the luciferase and β-galactosidase activities were measured. The luciferase activities were normalized to the transfection efficiency, which was defined by the β-galactosidase activity.

Immunocytochemistry. SMC cultures under the indicated conditions were washed with PBS(−) and fixed with 3.7% paraformaldehyde for 5 min. After they were washed again with PBS(−), the fixed cells were blocked with PBS(−) containing 2% horse serum, 2% BSA, and 0.1% Triton X-100 for 30 min, and then incubated with anti-calcineurin A antibody (Transduction Laboratories) (1:200 dilution) for 16 h at 4 °C. Subsequently, the cells were labeled with anti-mouse Alexa488-conjugated secondary antibody (1:200 dilution) and 0.1 mg/ml Hoechst for 30 min at room temperature, and then visualized under a Zeiss Axiophoto fluorescence microscope.

Results and discussion

We analyzed the effects of CsA or FK506 on the differentiated phenotype of SMCs as defined by cell morphology, ligand-induced contractility, and the expression of SMC molecular markers. Interestingly, the treatment of differentiated SMCs with CsA [5 μM] resulted in a morphological change from a spindle-like shape to a fibroblast-like one within 3 days. Concomitant with this morphological change, SMCs lost their carbachol (CCH)-induced contractility (Fig. 1A). A similar result was obtained following treatment with FK506 [10 μM] (data not shown).

We analyzed the mRNA expression of the SMC molecular markers CaD, CN, and α1 integrin and performed promoter analyses using the reporter genes for CaD and α1 integrin to monitor the SMC phenotype. We previously demonstrated that isoform conversion from *h*-CaD to *l*-CaD and down-regulation of the expression of CN, α1 integrin, and total CaD at the mRNA level precede changes at the protein level during phenotypic modulation of SMCs [14,21]. Thus, changes in the expression of SMC molecular marker mRNAs are a good indicator of the SMC phenotype. CsA or FK506 dose- and time-dependently decreased the expression of total CaD, CN, and α1 integrin mRNAs, and increased the isoform conversion from *h*-CaD to *l*-CaD (Figs. 1B and C). They also suppressed the promoter activities of both CaD and α1 integrin in a dose-dependent manner (Fig. 1D). These results suggest that the calcineurin-mediated pathway is critical for maintaining the differentiated phenotype of SMCs.

To confirm the direct involvement of calcineurin in maintaining the differentiated phenotype of SMCs, we performed promoter analyses by co-transfection of the CaD or α1 integrin reporter gene with the expression plasmid of a natural calcineurin inhibitor, CAIN [4], in differentiated SMCs (Fig. 2). Expression of CAIN dose-dependently inhibited the promoter activities of CaD (Fig. 2A) and α1 integrin (Fig. 2B), but not of Rous sarcoma virus (RSV) (Fig. 2C). These results indicate that the calcineurin-mediated pathway is directly involved in maintaining the differentiated phenotype of SMCs. We reported that the PI3-K inhibitors, LY294002 or wortmannin, induce SMC dedifferentiation by blocking the IGF-I-triggered PI3-K/PKB(Akt) pathway [14]. In contrast, forced expression of constitutively

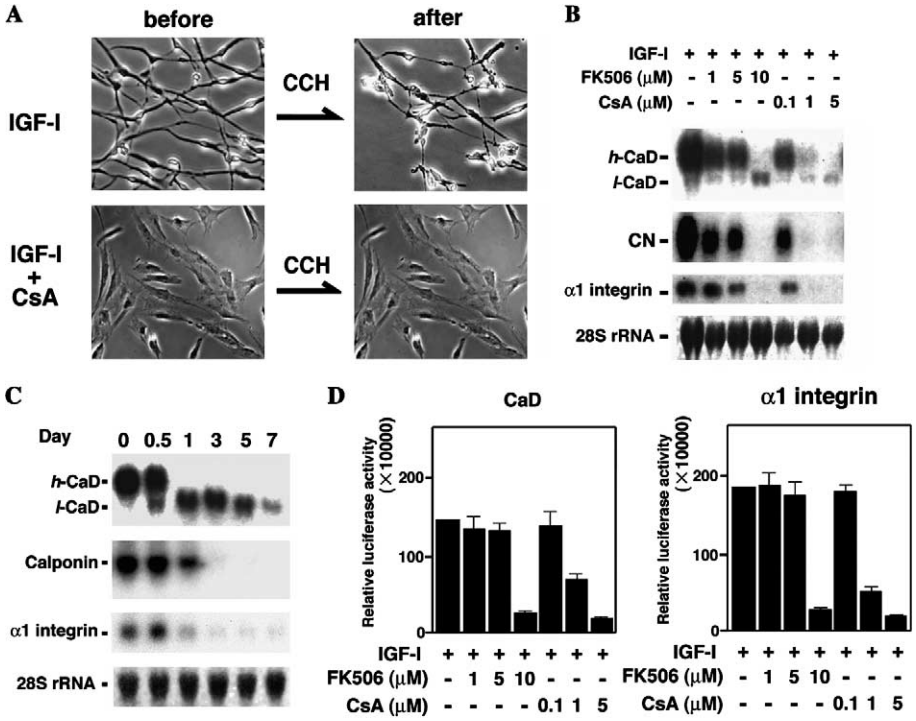

Fig. 1. Effects of CsA or FK506 on the differentiated phenotype of SMCs cultured on laminin under IGF-I-stimulated conditions. SMCs were cultured under the indicated conditions for 3 days. Cell morphology and carbachol (CCH)-induced contractility (A) and the expression of SMC molecular markers such as *h*- and *l*-CaDs, CN, and α1 integrin mRNAs (B) in cultured SMCs treated with the indicated concentrations of CsA or FK506 are shown. The expression of the indicated SMC molecular marker mRNAs was analyzed by Northern blots. CCH-induced contractility was monitored by the addition of CCH for 1 min. Photographs show the cultured SMCs before (left panels) and after (right panels) CCH treatment. Progressive changes in the expression of the SMC molecular marker mRNAs in cultured SMCs treated with CsA [3 μM] are shown (C). These are representative results from four separate experiments. The effects of CsA or FK506 on the promoter activities of CaD and α1 integrin in differentiated SMCs are shown (D). Differentiated SMCs were transfected with the CaD or α1 integrin promoter plasmid and were cultured for 2 days under IGF-I-stimulated conditions in the presence of the indicated concentrations of CsA and FK506. Relative luciferase activities of each of the CaD or α1 integrin promoter are shown. Each value represents the average and SD of at least three independent experiments. The promoter analyses in other figures are presented in the same way.

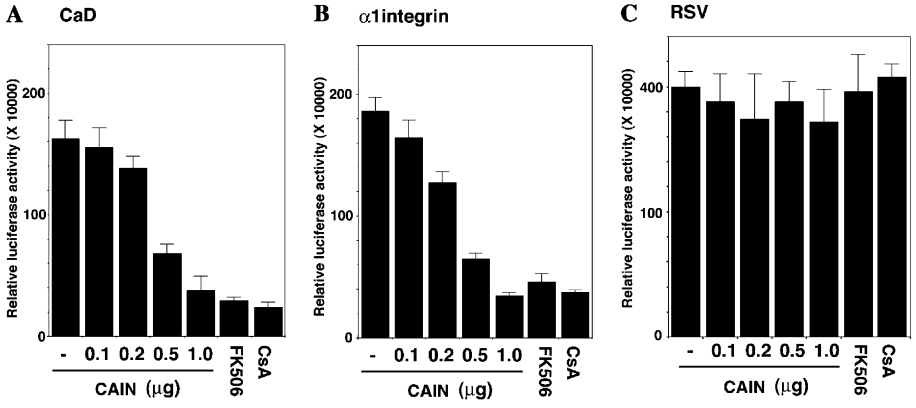

Fig. 2. CAIN inhibited the promoter activities of CaD (A) and α1 integrin (B), but not that of the RSV (C) in differentiated SMCs. The indicated amounts of the CAIN expression plasmid were co-transfected with either the CaD, α1 integrin, or RSV promoter plasmid in differentiated SMCs. As negative controls, differentiated SMCs were transfected with the CaD or α1 integrin promoter plasmid and were cultured in the presence of CsA [3 μM] or FK506 [10 μM].

active PKB(Akt) rescued SMC dedifferentiation triggered by either LY294002 or wortmannin (data not shown). Consistent with these findings, expression of dominant-negative PKB(Akt) suppressed the promoter activities of CaD and α1 integrin in differentiated SMCs, whereas forced co-expression of constitutively active

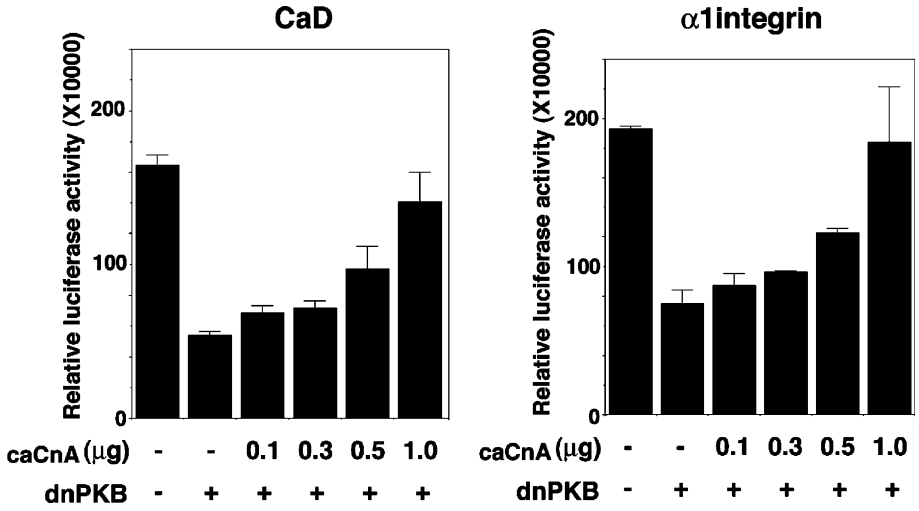

Fig. 3. Forced expression of constitutively active calcineurin Aα rescued the inhibition of CaD or α1 integrin promoter activity by blocking the PKB(Akt)-mediated pathway. The CaD or α1 integrin promoter plasmid was co-transfected with 1 μg of the expression plasmid of dominantly negative PKB(Akt) (dnPKB) and the indicated amounts of the expression plasmid of constitutively active calcineurin Aα (caCnA) or control plasmid in differentiated SMCs.

calcineurin Aα reversed the dominant-negative PKB (Akt)-induced suppression of both promoter activities (Fig. 3). Forced expression of constitutively active calcineurin Aα also overcame the suppression of both promoter activities by treatment with LY294002 or wortmannin (data not shown). These results indicate that calcineurin is a critical signaling molecule that regulates the SMC phenotype downstream of the PI3-K/PKB(Akt) pathway.

We further compared the subcellular localization of the calcineurin A protein between differentiated SMCs and CsA [5 μM]-induced dedifferentiated SMCs (Fig. 4). In this experiment, we analyzed at least 50 cells in 10–20 fields in each of three independent experiments. In differentiated SMCs cultured under IGF-I-stimulated conditions, the calcineurin A protein was exclusively localized to the nuclei. However, almost all of the calcineurin A protein was seen in the cytoplasm and/or the perinuclear region of CsA-induced dedifferentiated SMCs (Fig. 4). These results indicate that the calcineurin A protein translocates from the nuclei to the cytoplasm during the phenotypic modulation of SMCs. Thus, the nuclear localization of calcineurin A is likely to be closely associated with the maintenance of the differentiated SMC phenotype.

Our recent studies using visceral and vascular differentiated SMCs in culture show that stimulation with SMC dedifferentiation factors such as platelet-derived

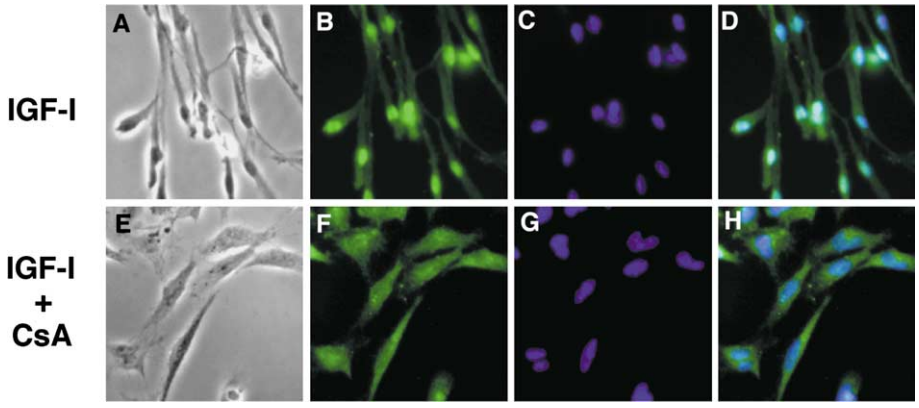

Fig. 4. Subcellular localization of calcineurin A protein in differentiated and CsA-induced dedifferentiated SMCs. SMCs were cultured under IGF-I-stimulated conditions with or without CsA [5 μM] for 3 days. The morphologies of differentiated SMCs (A) and CsA-induced dedifferentiated SMCs (E) are shown (A). Both SMCs were immunostained with an anti-calcineurin A antibody (B and F, green) and a nuclear stain (Hoechst) (C and G, blue). Panels D and H show the merged images of the calcineurin A immunofluorescence and nuclear stain. These are representative results from 10 to 20 fields analyzed for each of three independent experiments.

growth factor-BB (PDGF-BB), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and unsaturated lysophosphatidic acids (LPAs) triggers the coordinated activation of ERK and p38MAPK, which induces SMC dedifferentiation [15,16]. In contrast, CsA or FK506 did not potently activate both MAPKs in differentiated SMCs (data not shown), whereas these calcineurin inhibitors induced SMC dedifferentiation (Fig. 1). As shown in Fig. 3, forced expression of constitutively active calcineurin Aα overcame the suppression of the promoter activities of CaD and α1 integrin by blocking the PKB(Akt)-mediated pathway. These results suggest a novel signaling pathway in which calcineurin is downstream of the PI3-K/PKB(Akt) pathway in differentiated SMCs cultured under IGF-I-stimulated conditions.

CsA is known to stimulate expression of TGF-β1 both in vitro and in vivo [22]. In our previous study, we demonstrated that TGF-β1 is one of potent SMC dedifferentiation factors [14]. Taken together with this result, our present data suggest that in addition to the blocking of the PI3-K/PKB(Akt) and the downstream calcineurin pathways, CsA-induced expression and secretion of TGF-β1 may be involved in SMC dedifferentiation. To date, the downstream target of calcineurin has been unclear in differentiated SMCs. The NFAT is a possible candidate, because calcineurin associated with NFAT translocates to the nucleus [1,2] and the association of NFATc4 and GATA-4 [4], NFATc1 and GATA-2 [7], or NFATc1 and GATA-6 [11] in cardiac, skeletal, and dedifferentiated vascular SMCs is involved in the transcription of their respective molecular marker genes. In addition, glycogen synthase kinase-3 (GSK3) phosphorylates conserved serine residues in the amino terminus of NFAT that are target sites of calcineurin, thus promoting NFATs exit from the nucleus [23], and GSK3 is a target of PKB(Akt) whose activity is inhibited after phophorylation by PKB(Akt) [24]. We, however, think that a direct pathway between PKB(Akt) and calcineurin may function in the IGF-I signaling that is critical for maintaining the differentiated phenotype of SMCs, rather than such an indirect one.

We recently demonstrated that the visceral SMC-specific homeoprotein family, Barx1b, and serum response factor coordinately activate the promoter of β-tropomyosin in visceral SMCs [25]. Further, we found that the triad of Nkx-3.2, serum response factor, and GATA-6 transactivated the promoters of CaD, α1 integrin, and SM22α in vascular SMCs [19]. However, the relationships among calcineurin and these transcriptional mechanisms remain unclear. Further studies will be necessary to reveal the calcineurin-mediated pathway and its downstream transcriptional machinery in IGF-I signaling in differentiated SMCs.

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