Nuclear translocation of calcineurin Aβ but not calcineurin Aα by platelet-derived growth factor in rat aortic smooth muscle

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Submitted 24 March 2005; accepted in final form 9 February 2007

Am J Physiol Cell Physiol 292: C2213–C2225, 2007. First published February 15, 2007; doi:10.1152/ajpcell.00139.2005.—Calcineurin is a heterodimer composed of a 58- to 61-kDa catalytic A subunit and a 19-kDa Ca^{2+}-binding regulatory B subunit (25, 30). The catalytic A (CnA) subunit is encoded by three separate genes giving rise to CnAα (PPP3CA), CnAB (PPP3CB), and CnAg (PPP3CC) isoforms. In addition, these isoforms show alternative splicing that results in at least two splice variants per isoform (30). So far, the α- and β-subunits have been found to be coexpressed in all tissues examined to date (30, 34), whereas the γ-subunit is restricted to specialized organs like the testis (30). Despite an 85% homology between CnAα and CnAB, different substrate affinities and catalytic activities are displayed by these two isoforms (26). Enzyme specificity may also result from the distinct isoform-specific distribution that has been observed both within and between tissues (30, 34). For example, CnAα expression is more abundant than CnAB in the brain, heart, and kidney, whereas this pattern is reversed in T cells, the thymus, and the spleen (16).

One of the main targets of calcineurin is a family of transcription factors known as the nuclear factor of activated T cells (NFAT), which is composed of four well-characterized members: NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), and NFATc4 (NFAT3) (14). Once activated, calcineurin binds to and dephosphorylates NFAT, causing its translocation to the nucleus (14, 37). The immunosuppressants cyclosporin A (CysA) and FK506 block this translocation step by inhibiting calcineurin activity (14). A number of immunosuppressants cyclosporin A (CysA) and FK506 block this translocation step by inhibiting calcineurin activity (14).

Calcineurin is a ubiquitously expressed serine/threonine protein phosphatase that is activated by a rise in intracellular Ca^{2+} (30, 34). This phosphatase regulates several cellular functions, including cell cycle regulation, cell growth, microtubule assembly, and ion channel activity and, more recently, has been implicated in cardiac and skeletal muscle hypertrophy (25, 34). In smooth muscle, comparatively little is known about calcineurin function, although recent evidence suggests that it is required for the normal development of the vasculature (12) and is a major regulator of L-type Ca^{2+} channels (32), ATP-sensitive K^+ channels (42), and Ca^{2+}-activated Cl^- channel (13, 20) activity.

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C2214  CALCINEURIN A ISOFORMS IN VASCULAR SMOOTH MUSCLE

MATERIALS AND METHODS

RASM cells (RASMCs) were dissociated enzymatically from the thoracic aorta of male Sprague-Dawley rats (200–250 g) using a modified method (42). For culturing, RASMCs were resuspended in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen) and grown at 37°C in a humidified atmosphere of 5% CO₂. Only cells between passages 4 and 9 were used. A smooth muscle phenotype was confirmed as previously described (5).

RT-PCR of CnA isoforms. Total RNA from rat thoracic aorta denuded of the endothelium and adventitia was isolated according to the manufacturer’s instructions using the Epicentre Master Pure RNA isolation kit (Epicentre Technologies). cDNA was synthesized using 4 µg of the total RNA and oligonucleotide primers as per the manufacturer’s instructions. PCRs were performed using the following sets of oligonucleotide pairs designed against brain sequences. For rat CnAα (Genbank Accession No. X57115), the forward primers were 5'-ACGGTTGGTTGTCTCAGAGA-3' (nt 866–886) and 5'-GAG-GCCTGTTCTGGCAG-3' (nt 928–944) and the corresponding reverse primers were 5'-GAGTGAAGATTGTCCAGTT-3' (nt 997–1018) and 5'-GTTGGCTCGTCAAATCCTAT-3' (nt 1412–1433), respectively. For human CnAβ (Genbank Accession No. M29551), the forward primer was 5'-CGAGGATTTCTCTCCACACT-3' (nt 1518–1538) and the reverse primer was 5'-TGCGGTGTTCAGAGATTGA-3' (nt 1627–1646). Primers were also designed for rat CnAβ (Genbank Accession No. D90036) around known splice sites reported for rat (31) and human CnAβ (23), with the forward primer being 5'-GCTCATGAAAGCTCAAGATGC-3' (nt 904–923) and the reverse primer being 5'-GACATAGTGGTGCCAGTCC-3' (nt 1592–1610). Amplification was performed using 2 µl of the RT reaction product under the following conditions: 94°C for 2 min; 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension for 5 min at 72°C. PCR products were separated by electrophoresis on a 4% agarose gel and detected by the SYBR green fluorescent stain.

Western blot analysis. Thoracic aorta denuded of endothelial and adventitial layers were washed in cold Tris-buffered saline and snap frozen in liquid nitrogen. Two frozen aortas were placed in 400 µl of cold lysis buffer containing 50 mMol/l Tris-HCl (pH 7.5), 2 mMol/l EGTA, 0.5 mMol/l DTT, 1 mMol/l PMSF, and a Complete protease inhibitor pill (Roche Biologicals) and homogenized on ice for 30 min using a glass tissue grinder (Anachem, Bedford, UK). Frozen rat brains were treated similarly. Lysates were then centrifuged at 1,000 g for 5 min at 72°C. PCR products were separated by electrophoresis on a 4% agarose gel and detected by the SYBR green fluorescent stain.

Secondary antibody alone. No CnA bands were observed if blots were probed with the secondary antibody alone.

Confocal immunofluorescence. RASMCs were seeded at a density of 0.5 × 10⁶ cells/ml on poly-L-lysine-coated eight-chambered slides (BD Biosciences, Oxford, UK), grown for 24 h in DMEM containing 10% FBS, and growth arrested by a 48-h incubation in DMEM alone or a 72-h incubation in DMEM containing 0.1% FBS. The immunostaining procedure was carried out using a modified method (6). Briefly, freshly dispersed (see above) or cultured RASMCs were washed at room temperature with HBSS. All subsequent steps were carried out at 4°C unless otherwise stated. RASMCs were fixed with 4% formaldehyde in PBS and permeabilized with either 0.2% Triton X-100 in PBS for 20 min or 0.3% Nonidet P-40 and 1% glycerol in PBS for 10 min. Subsequently, cells were incubated with control blocking solution (2% BSA, 5% goat serum, and 0.1% Triton X-100 in PBS) for 1–2 h. Primary and secondary antibodies were prepared in blocking solution at the dilutions indicated. For double staining, the primary antibody was applied for either 1 h when anti-CnA (1:100), goat anti-NFATc1 (K-18, sc-1149, 1:100, Santa Cruz Biotechnology), or goat anti-NFATc3 (A-17, sc-22914, 1:200, Santa Cruz Biotechnology) antibodies were used or overnight for mouse monoclonal anti-NFATc3 (1:50) antibody (F-1, sc-8405, Santa Cruz Biotechnology). Secondary antibodies were always applied for 1 h at room temperature, which was goat anti-rabbit Alexa 488 (1:200, Molecular Probes, Leiden, The Netherlands) for the CnA isoform experiments and mouse anti-goat or goat anti-mouse IgG FITC-conjugated antibodies for NFATc1 and NFATc3 (1:250, Molecular Probes). To identify cellular nuclei, the fluorescent nucleic acid binding dye TO-PRO-3 (1:300, Molecular Probes) was added with the secondary antibody. For triple labeling, RASMCs were first probed overnight with anti-NFATc3 primary antibody followed by an incubation with goat anti-mouse IgG antibodies for NFATc3. RASMCs were then probed for CnAβ as described above except that the secondary antibody used was Texas red-conjugated goat anti-rabbit IgG antibody (Alexa 594, 1:250, Molecular Probes). After a final step of washing, slides were air dried and mounted with Vectashield (Vector Laboratories). Slides were viewed and analyzed at ×60 magnification (water-immersion lens) using a Bio-Rad Radiance 2000 laser-scanning confocal Nikon TE1000 microscope (Hemel Hempstead, UK). Green fluorescence of FITC/Alexa 488 and blue fluorescence of TO-PRO-111 were measured at an excitation/emission wavelengths of 492/520 or 644/657 nm, respectively. No fluorescence was detected in the presence of either primary or secondary antibodies alone, confirming the specificity of immune staining. Images of RASMCs were taken at a focal plane taken from the middle of the cell using a z-stack of 10 images with 0.1 µm spacing. Nuclear localization was quantified using Bio-Rad LaserPix 4.0 software. Computer visualization of cyto was taken to mean positive localization of either CnA or NFAT within the nucleus or negative if a blue signal was observed. The percent nuclear localization refers to the ratio of the integrated cyan signal to the total blue signal in the unmerged image. In colocalization experiments, yellow reflects positive colocalization of both CnA and NFATc3, which was assessed by measuring the colocalization coefficient of the unmerged images of green (FITC/c3) with red (Texas red, Cnbα/c3).

NFATc3-CnA binding assays. Human NFATc3 cDNA (Genbank Accession No. BC001050) was mutated using PCR for in-frame ligation following the codon for amino acid 420 with glutathione-S-transferase (GST). The first 420 amino acids of NFATc3 contains the binding regions for CnA (14). A COOH-terminal GST-NFATc3 1–420 fusion protein was generated using the Gateway system (Invitrogen), bacterial expression, and purification with GSH-Sepharose. The NH2-terminal fragments of CnAα (Genbank Accession No. X57115) and CnAβ (Genbank Accession No. D90036) were generated using PCR for in-frame ligation following the codon for amino acid 350 (CnAα350) or amino acid 360 (CnAβ360) with a hexa-His affinity tag. The NH2-terminal catalytic domain of CnA has previously been shown to contain the NFAT binding region (9, 28).
COOH-terminal His-CnA350 and His-CnAβ360 fusion proteins were generated using the Gateway system, bacterial expression, and purification with Ni-Sepharose. CnA350 and CnAβ360 binding to GST-NFATc3 was assayed by incubating 2 μg of each CnA protein with increasing amounts (0.5–12.5 μg) of GST-NFATc3 and 50 μl GSH-Sepharose in a total volume of 100 μl. The reaction [PBS (pH 7.4) and 0.1% Tween 20] was gently mixed at 4°C for 2 h and microfuged for 10 s at 1,000 g, and the supernatant was removed. Beads were washed three times with binding buffer, and, after the final wash, beads were boiled in 50 μl SDS-PAGE sample buffer. The beads were pelleted, and 30 μl of the supernatant were separated by SDS-PAGE (10% gels), followed by Western blotting onto nitrocellulose sheets. Blots were probed with anti-CnAα or anti-CnAβ antibodies (Chemicon) at 1:5,000 dilutions, followed by horseradish peroxidase-conjugated secondary antibody (1:50,000, Chemicon). CnA protein bands were visualized with ECL Advance (Amersham), or anti-CnAα or anti-CnAβ antibodies (Chemicon) at 1:5,000 dilutions, followed by horseradish peroxidase-conjugated secondary antibody (1:50,000, Chemicon). CnA protein bands were visualized with ECL Advance (Amersham), and images were collected using a charge-coupled device camera-based detection system (Epi Chem II, UVP Laboratory Products). The collected TIFF images were inverted and analyzed using Adobe Photoshop.

Cell proliferation. RASMCs were plated onto six-well plates at a density of 2 × 10⁴ cells/ml and grown in DMEM containing 10% FBS for 24 h. Prior to drug treatment, cells were growth arrested by incubating them in serum-free DMEM for 48 h. Following this, media were replaced with DMEM containing human PDGF-BB alone or in combination with CysA, cypermethrin, forskolin, adenosine cyclic (hydrogenphosphorothioate)triethylammonium (cAMPS-Sp), or ethanol (cypermethrin), or distilled water. Aliquots were kept at −20°C, and, on the day of the experiment, solutions were diluted to give the desired concentration.

RESULTS

CnA isoform expression in native RASM. Analysis of total RNA was performed using primers that specifically amplify CnAα or CnAβ isoforms. With primers designed against different conserved regions in the catalytic A subunit, single bands were detected (Fig. 1, A and B) and shown to be CnAα (153 and 506 bp) and CnAβ (129 bp) by sequence analysis of the PCR products (not shown). With the use of primers flanking known splice sites, three distinct bands were detected for CnAβ (Fig. 1B). The upper two products shown in Fig. 1B are predicted from full-length CnAβ and a splice variant previously identified with a 30-bp deletion (23, 31). The third product shown in Fig. 1B may correspond to novel splice variants that we have identified with a 95-bp deletion that may or may not contain the 30-bp deletion as well (unpublished observations). To assess protein expression, Western blot analysis was performed in native RASM homogenates, with purified CnA isoforms and rat brain samples used as positive controls (Fig. 1B). Protein samples were probed using Upstate Biotechnology anti-CnA antibodies raised against different COOH-terminal sequences in rat CnAα and CnAβ. With the use of crude and cytosolic protein from the RASM and rat brain and aortic homogenates (20 μg protein/lane except where indicated).

Statistical analysis. Values are given as means ± SE, and n indicates the numbers of cells or experiments. Statistical significance was assessed using Student’s t-test or one-way ANOVA with Bonferroni post hoc test. P values of <0.05 were considered statistically significant.

A

![Image](http://ajpcell.physiology.org/)

B

![Image](http://ajpcell.physiology.org/)

Fig. 1. RNA and Western blot analysis of calcineurin A (CnA) isoforms. A: RT-PCR using RNA isolated from rat aorta. Amplification of cDNA was performed using specific primers for CnAα and CnAβ designed around conserved regions and known splice sites in CnAβ. Expected product sizes for CnAα and CnAβ are shown with possible splice variant sizes given in parentheses for CnAβ (right). No PCR product was obtained if murine Moloney leukemia virus reverse transcriptase was omitted from the reverse transcription step (lane 4 at left). B: protein expression in different cellular fractions of CnAα (top) and CnAβ (bottom) isoforms confirmed by Western blotting of rat brain and aortic homogenates (20 μg protein/lane). Controls are purified CnA proteins (0.125 μg protein/lane except where indicated).
brain, bands of ∼57–60 kDa were observed that ran at a similar mobility to purified CnAα and CnAβ protein. The density of these bands was much greater in the rat brain, indicating a higher level of CnA expression compared with smooth muscle. Both CnA isoforms were essentially undetectable in the particulate fraction from the RASM, whereas distinct bands were visible in this fraction from the brain. Similar results were observed in at least nine independent experiments. However, a faster migrating band of ∼53 kDa was detected with the anti-CnAβ antibody in the RASM but not in the brain (Fig. 1B, bottom). The specificity of the antibodies was confirmed by the lack of cross reactivity when purified CnAα was probed with the anti-CnAβ antibody and CnAβ was probed with the anti-CnAα antibody (Fig. 1B).

**Differential distribution of CnAα and CnAβ in RASM.** We investigated the expression pattern of CnAα and CnAβ isoforms in both freshly dispersed and cultured RASMCs. Under control/unstimulated conditions, the CnAα isoform appeared to be uniformly distributed in the cytoplasm and nuclei of freshly dispersed cells bathed in HBSS (Fig. 2A, top). In contrast, the CnAβ isoform was localized almost exclusively in the cytoplasm, being mainly distributed in the perinuclear area with minimal distribution in the nuclei (Fig. 2A, bottom). Consistent with the Western blot analysis data, we did not detect membrane staining of either isoform. We also determined the isoform distribution in growth-arrested, cultured RASMCs. We found that the expression pattern was similar to freshly dispersed cells (Fig. 2B) with some differences. There appeared to be less cytoplasmic immunostaining of CnAα, and staining was punctate and more diffuse, whereas the opposite appeared true for CnAβ, as cytoplasmic staining appeared more intense. When nuclear localization of each CnA isoform with TO-PRO-3 was assessed (Fig. 2C), nuclear localization of CnAβ was estimated to be <12% in both cell preparations. However, nuclear accumulation of CnAα compared with CnAβ was substantially greater (P < 0.001) in freshly dispersed cells, averaging 54.4 ± 5.7% (n = 21), which was higher (71.8 ± 2.5%, n = 17, P < 0.001) in cultured cells.

**Effect of PDGF-BB on cellular localization of CnA isoforms.** To elucidate the effect of receptor activation on CnA distribution and NFAT translocation, we investigated the response of the potent vascular smooth muscle mitogen PDGF-BB. This agent activates a tyrosine kinase-linked receptor to raise intracellular Ca2+ levels in the rat aorta (3) and causes NFAT translocation in visceral smooth muscle (35). We found that nuclear distribution of CnAα did not significantly change in response to a 30-min application of PDGF-BB in cultured RASMCs (Fig. 3). Furthermore, nuclear accumulation was not altered by a 30-min incubation with CysA (Fig. 3B) nor following a 48-h treatment, with nuclear localization being 68.3 ± 0.9% (n = 48) under control conditions versus 68.8 ± 0.9% (n = 46) after CysA (1 μM). In contrast, a significant increase in nuclear accumulation of CnAβ was induced by PDGF-BB after 15 min of application, which increased further at 30 min and a little more at 60 min (Fig. 4A). Immunofluorescent images showed that PDGF-BB consistently caused CnAβ to accumulate into discrete areas within the nucleus (Figs. 4B and 5B), akin to recent data showing tiny clusters of calcineurin associated with nucleoli of developing rat skeletal muscle cells (38). Moreover, CysA (1 or 10 μM), applied 20 min before and in the presence of PDGF, significantly attenuated ligand-induced translocation of CnAβ to the nucleus (Fig. 4, B and C). Similar quantitative and qualitative effects to PDGF-BB were also observed with UTP (Fig. 4), another vasoconstrictor and inducer of cell growth in the rat aorta (15).

It is well known that calcineurin and cAMP have antagonistic effects on cellular function in many cell types (25), which may relate in part to inhibition of calcineurin-NFAT complex formation (33). Therefore, we investigated the effects of the adenyl cyclase activator forskolin (1 μM) on CnAβ-induced translocation. When RASMCs were treated with forskolin before and in the presence of PDGF or UTP, CnAβ nuclear localization was close to levels seen in unstimulated cells (Fig. 4, B and D).

**Effect of NFAT inhibitors on nuclear translocation of CnAβ.** We assessed the role of two novel inhibitors of calcineurin-NFAT signaling on PDGF-BB-induced translocation of CnAβ. The first agent used was A-285222, a bis-trifluoromethylpyrazole belonging to a novel class of immunosuppressive agents that inhibit nuclear import of NFAT and cytokine production in T cells (7, 39). The second NFAT inhibitor used was INCA-6, a VIVIT-like compound that binds to calcineurin with high affinity and specifically disrupts its interaction with NFAT, blocking both dephosphorylation and nuclear export in T cells (29). Both compounds, however, can be distinguished from calcineurin inhibitors like CysA since they do not inhibit phosphatase activity directly or prevent the dephosphorylation of other well-known calcineurin substrates like the RII subunit of PKA (29, 39). In such experiments, we found that A-285222 (10 μM) and INCA-6 (3 μM) had little effect on the nuclear distribution of CnAβ in growth-arrested cells, although these agents fully inhibited its nuclear accumulation induced by PDGF-BB (Fig. 5).

**Cellular translocation of NFATc3 but not NFATc1 with PDGF-BB.** Experiments were performed to determine which NFAT isoform might translocate with CnAβ. We chose to investigate NFATc1 and NFATc3, since CysA-sensitive nuclear accumulation of these isoforms has been reported with receptor activation in smooth muscle (11, 35, 44). In unstimulated cells, cytoplasmic staining of both NFATc1 and NFATc3 was evident, although NFATc1 staining was most intense around the perinuclear region (Fig. 6). Some basal nuclear staining was also observed with NFAT antibodies, although accumulation was significantly higher (P < 0.001) for NFATc3 than for NFATc1 (Fig. 6). Moreover, nuclear accumulation of NFATc3, but not NFATc1, was substantially increased in the presence of PDGF-BB (Fig. 6B), and this was prevented by prior treatment with 1 μM CysA (Fig. 7A). In experiments where cells were simultaneously stained with NFATc3 and CnAβ antibodies, colocalization of these proteins was extremely high in the nucleus and immediately outside the nucleus (Fig. 7B), with 86.7 ± 1.6% of nuclear and 45.2 ± 10.0% of cytosolic CnAβ staining colocalizing with NFATc3 in the presence of PDGF-BB (n = 47 cells). Interestingly, cytosolic colocalization was much higher in control cells (91.4 ± 0.7%, n = 23), suggesting that PDGF-BB also caused translocation of NFATc3 that was independent of CnAβ movement. Although strong nuclear staining was consistently observed for CnAα, overall this was associated with significantly less NFATc3 colocalization (Fig. 7C) and was not altered by PDGF-BB, averaging 27.6 ± 1.1% (n = 27) in control cells versus 27.9 ± 0.9% (n = 18) with PDGF-BB.
Binding of CnA and CnAβ to a GST-NFATc3 fusion protein. To study the interaction of NFATc3, a GST-NFATc3 1–420 fusion protein was generated from human NFATc3 cDNA (cf. Ref. 9). For the two CnA isoforms, we used recombinant calcineurin containing the catalytic domain but lacking the calcineurin B-binding helix, the calmodulin binding region, and the autoinhibitory domain. With the use of this approach, it has previously been shown that human CnAα (1–347) binds either NFATc1 or NFATc2 as avidly as full-length calcineurin that has first been activated with Ca\(^{2+}\) and calmodulin (9). Increasing amounts of the GST fusion protein were used to precipitate an equivalent amount (similar molar ratios) of the calcineurin and to quantify the extent of binding by immunoblot analysis. To study the interaction of NFATc3, a GST-NFATc3 1–420 fusion protein was generated from human NFATc3 cDNA (cf. Ref. 9). For the two CnA isoforms, we used recombinant calcineurin containing the catalytic domain but lacking the calcineurin B-binding helix, the calmodulin binding region, and the autoinhibitory domain. With the use of this approach, it has previously been shown that human CnAα (1–347) binds either NFATc1 or NFATc2 as avidly as full-length calcineurin that has first been activated with Ca\(^{2+}\) and calmodulin (9). Increasing amounts of the GST fusion protein were used to precipitate an equivalent amount (similar molar ratios) of the calcineurin and to quantify the extent of binding by immunoblot analysis.
ratio) of CnAα350 or CnAβ360 on GSH-Sepharose beads (Fig. 8). Western blotting of bound CnA was visualized using NH2-terminal isoform-specific antibodies (Chemicon). GST-NFATc3 1–420 did not appear to significantly bind CnAα350 at a ratio of 0.2:1, whereas binding of CnAβ360 was apparent. Increasing amounts of GST-NFATc3 1–420 only weakly increased CnAα350 binding, whereas that of CnAβ360 markedly increased at a 1:1 ratio. This occurred under conditions where immunoblotting of the purified CnA fragments showed almost equivalent protein signals on the blots. The anti-CnA antibodies did not pull down GST if added alone. Curiously, binding of CnAα350 but not CnAβ360 declined at the highest ratio.

**DISCUSSION**

We show here, for the first time, that CnA α- and β-isosforms are expressed in RASM and have distinct subcellular patterns of expression, which differ from the brain. Furthermore, we demonstrate that the β-isosform is likely to regulate the nuclear import/export of NFAT in response to growth-promoting and -inhibitory agents, most likely through NFATc3, because the latter is heavily colocalized with CnAβ and this isoform appears to preferentially bind NFATc3.

Western blot experiments revealed that both CnA isoforms are expressed mainly in the cytosolic fraction of RASM with little evidence for membrane localization. This contrasts with our results in the brain, where the distribution was equally split between cytosolic and particulate fractions. The latter agrees well with a previous study (19) showing 58% of total CnAα and 47% of total CnAβ protein is associated with the particulate fraction in the rat cerebrum and other studies (30, 34) showing a high localization of calcineurin to the cytoskeleton. Interestingly, Western blots of RASM lysates show two distinct bands that migrate at 60 and 54 kDa but only the higher band in the brain. Most nucleotide sequences of known CnAβ splice variants predict proteins of 61–57 kDa (25, 30). However, we have identified a number of novel CnAβ splice variants including one with a 95-bp deletion (928–1022 bp in sequence D90036; unpublished observations) that theoretically could account for the 54-kDa immunoreactive band. Likewise, two GenBank sequences predicted by automated computational analysis of annotated genomic sequences (XM-508174 and XM-857434) would also give rise to...
A complex with NFAT (14, 34). Our results suggest that CnA interacts with calcineurin to translocate from the cytosol to the nucleus by forming complexes, with the respective subcellular distribution being reported in rat and mouse neuronal and retinal cell types, with the distinct isoform-specific subcellular distribution being unknown but may result from binding to specific A-kinase anchoring proteins or direct tethering to subcellular structures (25). In subsequent studies (24, 40), distinct subcellular distribution was reported in rat and mouse neuronal and retinal cell types, with the α-isofrom being predominately nuclear and the β-isoform being predominately cytosolic. What gives rise to the distinct isoform-specific subcellular distribution is unknown but may result from binding to specific A-kinase anchoring proteins or direct tethering to subcellular structures (25). In this regard, it is worth noting that the NH2- and COOH-termini of CnA isoforms share very little homology so that these domains may be involved in the spatial targeting of calcineurin (24, 25).

In immunohistochemical experiments, we found clear differences in the subcellular distribution of CnA isoforms, where the α-subunit was expressed in the cytosol and the nucleus and the β-subunit was essentially cytosolic. Early work reported a similar localization of the α- and β-subunits in the rat brain, with both isoforms being highly enriched in cytoplasmic fractions and only a small amount (20%) located in the nuclei (19). In subsequent studies (24, 40), distinct subcellular distribution was reported in rat and mouse neuronal and retinal cell types, with the α-isoform being predominately nuclear and the β-isoform being predominately cytosolic. What gives rise to the distinct isoform-specific subcellular distribution is unknown but may result from binding to specific A-kinase anchoring proteins or direct tethering to subcellular structures (25). In this regard, it is worth noting that the NH2- and COOH-termini of CnA isoforms share very little homology so that these domains may be involved in the spatial targeting of calcineurin (25, 30).

It is well known that a rise in intracellular Ca2+ causes calcineurin to translocate from the cytosol to the nucleus by forming a complex with NFAT (14, 34). Our results suggest that CnAα is unlikely to be the major stimulus facilitating NFAT translocation, since this isoform displayed a predominantly nuclear localization in cultured RASMCs and PDGF-BB failed to increase the nuclear accumulation of CnAα. In contrast, CnAβ exhibited essentially no nuclear localization until cells were exposed to PDGF-BB or UTP. CysA abrogated this nuclear accumulation, as did NFAT inhibitors, further confirming the activation of calcineurin. A previous Western blotting study (21) in cultured RASMCs has reported increases in nuclear calcineurin protein with angiotensin II over a similar time frame, although the specific CnA isoform involved was not investigated. The observed equipotency of both UTP and PDGF at inducing CnAβ translocation in RASM is, however, different from those in previous studies. Thus, UTP along with endothelin were the most potent agonists at inducing the nuclear accumulation of NFATc3 in the cerebral artery, whereas PDGF-BB had no effect in this tissue. Such tissue specificity in agonist-mediated NFAT nuclear translocation may relate to different receptor densities between tissues and/or in cell culture or receptors coupling to different second messenger pathways. Although PDGF and UTP act via receptor tyrosine kinases (2) and purinergic receptors coupled to various phospholipases (15), respectively, both mitogens elevate intracellular Ca2+ in the rat aorta (1, 3) and, hence, would be predicted to activate calcineurin.

The identity, distribution, and function of NFAT isoforms in smooth muscle are still controversial. For example, NFATc3 was the dominant isoform found in native smooth muscle and primary cultured RASMCs, with expression presiding predominately in the cytosol (11, 22, 35). In contrast, NFATc4 appears the only isoform significantly expressed in cultured human pulmonary arterial smooth muscle cells (43). However, other studies (3, 44) in cultured RASMCs have detected significant expression of

54- to 55-kDa proteins. We cannot at this stage rule out the possibility that abnormal migration might result from some unexplained artifact or from posttranslational modification of CnA, although, so far, this has only been reported for the calcineurin B subunit (30). Furthermore, proteolysis is unlikely to explain the existence of the lower band, since the known tryptic digests for the COOH-terminal antibody we used (Upstate Biotechnology) would give rise to bands ranging from 9 to 16 kDa, with the bulk of the catalytic subunit being resistant to proteolysis (18, 25).

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Fig. 5. Nuclear factor of activated T cells (NFAT) inhibitors block PDGF-BB-induced nuclear accumulation of CnAβ. A: immunofluorescent images of RASMCs stained with CnAβ in the absence (left) and presence (right) of PDGF-BB with and without either A-285222 (A-285; 10 μM) or inhibitor of NFAT-calcineurin association 6 (INCA-6; 3 μM) as indicated. The NFAT inhibitors were given 30 min prior to treatment with PDGF-BB. C: summarized data showing percentages of nuclear localization following various treatments. Numbers within the bars refer to the numbers of cells. ***P < 0.001 compared with control.
NFATc1 and NFATc2, but little NFATc3, with NFATc1 showing both cytosolic and nuclear accumulation (3, 44). In our study, we found NFATc1 to be predominately cytosolic, whereas NFATc3 displayed a small but significant amount (~20%) of nuclear accumulation in growth-arrested cultured RASMCs. Moreover, nuclear accumulation of CnAα was ~20% higher in cultured compared with freshly isolated cells, also consistent with our finding of some nuclear colocalization of CnAα (~27%) with NFATc3. The predominant nuclear localization previously reported for NFATc2 and the lack of NFATc4 expression in RASM

NFATc1 and NFATc3, but not NFATc1, translocates to the nucleus in the presence of PDGF-BB. A and B: immunofluorescent images of RASMCs stained with NFATc1 (A) or NFATc3 (B) under control conditions and after a 30-min incubation with 10 ng/ml PDGF-BB. C: summarized data showing percentages of nuclear localization. Numbers within the bars refer to the numbers of cells. ***P < 0.001.
suggest that NFATc2 may indeed reside in the nucleus with CnAβ. The functional significance of this remains unclear, but the presence of significant nuclear CnAα in freshly isolated RASMCs may advocate their involvement in maintaining a differentiated smooth muscle phenotype. Moreover, nuclear export of CnAα was not promoted by CysA, suggesting that cellular targeting of CnAα is regulated by different import and export mechanisms to CnAβ.

The lack of NFATc1 and CnAα translocation and the observed strong nuclear colocalization of CnAβ with NFATc3

(3, 44) suggest that NFATc2 may indeed reside in the nucleus with CnAα. The functional significance of this remains unclear, but the presence of significant nuclear CnAα in freshly isolated RASMCs may advocate their involvement in maintaining a differentiated smooth muscle phenotype. Moreover, nuclear export

Fig. 7. Nuclear localization of NFATc3 is dependent on calcineurin. Immunofluorescent images of RASMCs stained with NFATc3 after a 30-min application of 10 ng/ml PDGF-BB are shown. Cells were pretreated for 1 h with 1 μM CysA (A) or were concurrently stained with TO-PRO-3 and either CnAβ (B) or CnAα (C). Yellow staining in the merged images represents nuclear colocalization of NFATc3 with CnA.

Fig. 8. Unequal binding of CnAα and CnAβ to NFATc3. GSH-Sepharose beads containing glutathione-S-transferase (GST)-NFATc3 1–420 were incubated with CnA350α or CnA360β. Bound CnA proteins were detected by 10% SDS-PAGE and immunoblot (IB) analysis with anti-CnAα (left) or anti-CnAβ (right) antibodies. As a negative control, 10 μg GST was added alone with the primary antibodies.
following treatment with PDGF-BB suggest that NFATc3 is a critical downstream effector of calcineurin-mediated stimulation in response to vascular mitogens. Interestingly, both NFATc3 and CnAβ had a similar uneven distribution within the nucleus and were concentrated together in punctate dots (Fig. 6). The significance of this remains unknown, although punctate intranuclear distribution with different NFAT isoforms has been reported (33, 37) and recent data have shown tiny clusters of calcineurin associated with nucleoli of developing rat skeletal muscle cells (38). Furthermore, increases in nuclear NFATc3 levels after 30 min have been described in ileal and portal vein smooth muscle for PDGF-BB (8, 35). Our data do not concur with Western blot data in RASMCs, where the same agonist caused NAFTc1 translocation but had no effect on NFATc3 distribution (44). The reason for this discrepancy remains unclear but could relate to the different time points at which translocation was measured in this study (30 min) compared with 2 h by Yellaturu and colleagues. However, they did report a threefold increase in nuclear NFATc3 protein levels following a 2- to 8-h treatment with PDGF-BB, suggesting a role for this isoform in cell proliferation to this mitogen (44). However, our results are reminiscent of previous findings where receptor stimulation appears associated with selective movement of only one NFAT isoform (17, 44). Thus, NFATs appear under the control of different signaling pathways and are probably differently activated due to localized Ca2+ signaling and/or spatial location (11, 14). Indeed, specific cellular functions for different NFATs appear an emerging theme for this family of transcription factors (14, 34, 41). One intriguing finding is that there appears to be movement of NFATc3 into the nucleus independent of CnAβ. Since continued dephosphorylation appears necessary for NFAT to be retained in the nucleus (14), this suggests the involvement of another phosphatase or the existence of a novel splice variant of CnA. Alternatively, CnA (α or β) may promote the movement of NFAT through its indirect activation of protein phosphatase 1 (25). Indeed, phosphatase activity in RASMCs shows a high level of okadaic-sensitive (~55%) activity (unpublished observations). Clearly, future experiments will be required to distinguish between these possibilities. However, NFAT can be activated in T cells through a CysA-resistant pathway, although the exact nature of the pathway remains unclear (10).

The structurally unrelated calcineurin inhibitors CysA and cypermethrin both completely inhibited PDGF-induced cell proliferation, as did the NFAT inhibitors A-285222 and INCA-6, suggesting that both calcineurin and NFAT are crucial for the stimulation of growth through this receptor pathway. Likewise, in cultured RASMCs, CysA inhibited NFAT-mediated transcription induced by PDGF (3) as well as thymidine incorporation and cell proliferation (44). Although calcineurin has many cellular substrates that may be involved in mediating the response to growth factors, our data with NFAT inhibitors suggest that calcineurin dephosphorylation of NFAT alone is sufficient to drive the proliferative response to PDGF. This contrasts with data in fibroblasts showing that PDGF-induced growth was blocked by CysA and FK506, but not by cypermethrin, suggesting the involvement of a calcineurin-independent pathway, at least in this cell type (27).

The fact that both CnAβ and NFATc3 isoforms were responsive to mitogenic signals and heavily colocalized in the nucleus makes it conceivable that they promote cellular growth and proliferation. Moreover, CnAα350 showed only weak binding to the COOH-terminal GST-NFATc3 fusion protein, whereas CnAβ360 appeared to bind strongly. While it is unclear if such preferential binding occurs in vivo, such findings are supported by functional data elsewhere. For example, hearts from CnAβ−/− or NFATc3−/− mice failed to undergo hypertrophy in response to aortic banding or infusion of angiotensin II or isoproterenol (4, 41). Thus, both CnAβ and NFATc3 work via a common signaling pathway. Moreover, cardiac hypertrophy is associated with increased message and protein levels for CnAβ, whereas those of CnAα remain unaltered (36). Similarly, we found CnAβ staining to increase and CnAα staining to decrease in cultured compared with freshly isolated RASMCs. However, our results do not rule out the possibility that CnAα is involved in PDGF-mediated effects on vascular growth. Indeed, CnAα activity may increase through changes in nuclear Ca2+ or this subunit may regulate ion channel function to enhance Ca2+ entry (13, 20, 32, 42) and thus promote growth indirectly. Future experiments involving subtype selective inhibition of CnA as well as NFAT isoforms will be required to determine their respective roles.

In our studies, the PKA activators forskolin and cAMPS-Sp also inhibited cell growth and CnAβ translocation stimulated...
by PDGF-BB. It is tempting to speculate that the cAMP/PKA pathway opposes cell growth by preventing CnAβ-induced NFATc3 translocation. NFAT must be dephosphorylated by calcineurin for nuclear translocation to occur. However, the maintenance of NFAT in the nucleus requires continuous calcineurin signaling to counteract nuclear export kinases like PKA, glycerone synthase kinase 3β, and JNK, which phosphorylate NFAT at multiple sites and oppose translocation (14). Thus, a reduction in cell growth may be brought about by PKA-induced phosphorylation of NFAT directly (33) or indirectly by inhibition of calcineurin activity through lowering intracellular Ca2+ (e.g., increased movement of Ca2+ into internal pools) (2), which are both effects leading to retention of NFAT in the cytosol. Such a mechanism may represent a novel pathway whereby cAMP-elevating agents regulate vascular smooth muscle cell proliferation.

In conclusion, we showed that not only do CnA isoforms have differential cellular distribution but that after stimulation of aortic smooth muscle cells with PDGF, CnAβ heavily colocalized with NFATc3 in the nucleus. Inhibition of calcineurin or NFAT activity prevented cell growth, and, thus, we postulate that CnAβ, through its interaction with NFATc3, is an important initiator of vascular smooth muscle proliferation, although at this stage we cannot rule out a contribution from CnAα.

ACKNOWLEDGMENTS

We thank Dr Kelly (University College London) and Dr. William Hatton (Reno, NV) for the technical assistance in confocal microscopy and Maggie Elorza (Reno, NV) for obtaining and culturing the aortic smooth muscle cells in some experiments.

GRANTS

This study was funded by Medical Research Council (MRC) UK Grant G117/440 (to L. H. Clapp) and by National Institute of Neurological Disorders and Stroke Grant NS-36318 (to B. A. Perrino). L. H. Clapp was an MRC G117/440 (to L. H. Clapp) and by National Institute of Neurological Disorders and Stroke Grant NS-36318 (to B. A. Perrino).

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