Expression of the antigen-regulated, cyclopisin A-sensitive nuclear factor of activated T cells (NFAT) is not restricted to lymphoid cells, as thought initially, but the physiological inducers of NFAT-mediated transcription in non-lymphoid cells are unknown. Here, cultured vascular smooth muscle cells (VSMC) are shown to express two isoforms of the NFAT family endogenously, which are localized differentially in cells under resting conditions. Using a retroviral NFAT-specific luciferase reporter, we show that VSMC support previously unrecognized complexities in NFAT-mediated transcription, including evidence for negative regulation by Ca^{2+} signaling and positive regulation through co-activation of adenylyl cyclase and Ca^{2+} mobilization. The VSMC mitogen platelet derived growth factor-BB (PDGF-BB) induces NFAT-mediated transcription in VSMC. Thrombin and angiotensin II, which activate Go_{q}-coupled receptors, are significantly weaker inducers of NFAT-mediated luciferase expression than is PDGF-BB. However, co-stimulation studies show that Go_{q} receptor agonists augment the NFAT-mediated transcriptional response to PDGF-BB. This synergy can be explained in part by augmented intracellular Ca^{2+} transients elicited by multiple agonist challenges. These data indicate that agonists for phospholipase C-coupled receptors stimulate NFAT-mediated transcription in VSMC differentially, and that NFAT can function to integrate co-activating signals in the extracellular environment.

Transcription mediated by the nuclear factor of activated T lymphocytes (NFAT) is regulated tightly in response to elevations of both intracellular calcium ion (Ca^{2+}) and diacylglycerol second messengers following activation of phospholipase C (PLC) (1). Increased intracellular Ca^{2+} stimulates calcineurin-dependent dephosphorylation of cytoplasmic NFAT, leading to its nuclear translocation (2–5). Macrolide immunosuppressive agents, such as cyclopisin A (CsA) and FK506 (6), block this step. In the nucleus, NFAT forms a heteromeric transcriptional co-activator complex with API proto-oncogene partners that are co-induced downstream of receptor activation (3). This multimeric NFAT complex participates in the transactivation of several cytokine genes by interactions with purine-rich genomic NFAT-responsive enhancer elements (NFRE) (7).

The first evidence for the existence of NFAT was derived from analysis of antigen-responsive enhancer elements in the cytokine IL-2 gene promoter in lymphocytes (8). A growing body of data now suggests that NFAT is expressed more widely but the picture is far from complete. To date, NFAT expression or function has been described in several types of non-lymphoid cells, including mast (9), endothelial (10), and neuronal cells (11). Each of the mRNAs for the known NFAT isoforms are expressed in distinct tissue-specific patterns (12). However, the precise non-lymphoid cell types that express NFAT isoforms in vivo are largely unknown. For instance, it remains unclear whether NFAT is expressed to some degree in all cells, or if its expression in non-lymphoid tissues is restricted to certain subpopulations of cells within tissues.

The presentation of foreign antigen on lymphocytes provides the best understood physiological inducer of NFAT-mediated transcription. The existence of NFAT in non-immune cells suggests that it can be directed to regulate gene expression by other physiologic stimuli. Physiological agonists for receptors that activate PLC signaling, such as hormones, neurotransmitters, and growth factors, provide obvious candidates for this function (11, 13). The extent to which such stimuli can trigger NFAT-mediated transcription in non-lymphoid cells that express endogenous NFAT isoforms needs to be determined. Additionally, it is not known if NFAT transactivation properties in non-lymphoid cells differs from that in the better understood lymphocyte context.

Vascular smooth muscle cells (VSMC) respond to a diverse group of extracellular signals to progress through phenotypic modulation, a process that occurs in the development and progression of cardiovascular diseases such as hypertension and atherosclerosis (14). Changes in gene expression patterns underlie this transition, which serves to regulate processes involved in extracellular matrix remodeling, VSMC growth, and medium; PBS, phosphate-buffered saline; LTR, long terminal repeat; GM, growth medium.
migration. Cultured VSMC serve as a useful system to understand the molecular and cellular consequences of VSMC activation, and are helpful in elucidating the genes, gene expression control pathways, and the extracellular signals associated with VSMC activation. We speculated that NFAT might be expressed in cultured VSMC and may participate in facilitating the VSMC activated state in response to mitogenic signals. The experiments described herein represent the first demonstration that NFAT is expressed in VSMC and provide evidence that PDGF-BB, which activates a tyrosine kinase-linked receptor, can serve as a physiological inducer of NFAT-mediated transcription in VSMC. This response is synergized by co-stimulation of VSMC with thrombin and angiotensin II, which activate PLCβ activity by coupling through the Gq class of heterotrimeric GTP-binding proteins. However, these latter agonists are weaker inducers of NFAT-mediated transcription than is PDGF-BB.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—The following plasmids used in this study were received as gifts: the retroviral plasmid pLNCX, from A. D. Miller (Baylor College of Medicine, Houston, TX); pSH210 and pSH211, plasmids conferring human IL-2 promoter activity, from S. Gerard (St. Jude Children’s Research Hospital, Memphis, TN); K100 and NFATc2, from G. Crabtree (Stanford, CA); and plasmid pFPAt/luc, from R. Bram (Memphis, TN). A mouse monoclonal antibody (IgG1, clone 7A8) against NFATc1 was purchased from Affinity Bioreagents, Inc. (Golden, CO). The monoclonal antibody (GHS-G1-G9) against NFATc2 was a gift from G. Crabtree. Mouse IgG in clarified ascites was purchased from Sigma. Secondary antibodies were bought from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). The tyramide-green immunochemical reporter was purchased from NEN Life Science Products. Except where noted all cell culture media, antibiotics and molecular biology supplies were purchased from Life Technologies, Inc. Fetal bovine and calf serum were obtained from Atlanta Biologicals (Norcross, GA). Luciferin and additional salts and buffers used were purchased from Sigma. The retroviral plasmids used in this study were subcloned and maintained in Escherichia coli Top10F’ (Invitrogen, Inc., Carlsbad, CA) in 100 μg/ml ampicillin plus 12.5 μg/ml tetracycline. The VSMC used in this study were derived from dissociated rat thoracic aorta (passages 10 to 25) and are maintained as a continuous primary cell line in DMEM, 4.5 g/ml glucose, 10% heat-inactivated calf serum with 100 units/ml each penicillin and streptomycin (15). Amphotropic (ATCC CRL-11554) retroviral producer cells were obtained from the American Type and Culture Collection (Rockville, MD). Angiotensin II was purchased from Sigma, purified human thrombin was provided generously by S. Krishnaswamy (Dept. of Medicine, Emory University, Atlanta, GA), and PDGF-BB was purchased from Calbiochem, Inc. (San Diego, CA).

**Retroviral Reporter Plasmids—**pTJM9 was created by placing a new promoter of interest into the cytomegalovirus promoter in HindIII- and ClaI-digested pLNCX (16) using the oligonucleotides 5′-AGCTTC-CATTGTGCTGGTCGACGCGTCCAGCACAATGGAT and 5′-GCAGTCGTGTGGCACGGTACGACACATG. The cytomegalovirus promoter in pTJM9 was removed by digestion with BanHI and HindIII, and the plasmid was closed by blunt-end ligation after Klenow treatment, the fragment ends were ligated with non-cohesive BstXI adapters (5′-CTGGCCGC and 5′-CGCCGCGACACA) and subsequently cloned into pTJM12 digested with BstXI. The resulting construct, pKα9, can accept promoters in the unique BanHI and HindIII (5′ to 5′) sites just proximal of the luciferase 5′ end, and transcribes luciferase off of the opposite strand as that for the viral 5′-LTR promoter. Plasmid pKα7 contains a ∼250-base pair BanHI-HindIII fragment with an NFAT enhancer triplex linked to the minimal IL-2 promoter fragment excised from pFPAt/luc (19), which was cloned into the same sites in pKα8. Plasmid pKα9 contains a ∼130-base pair BanHI-HindIII fragment for the minimal IL-2 promoter, without the NFAT enhancer, amplified from pNFAT/luc by PCR using the primers 5′-CTCCGCCATCTAGTGAATACAACTTACGACAC and 5′-GGCAAGCTTGGAGTTGAGGTTACTGTGA. To create a reporter plasmid bearing a mutation in the NFAT binding site (mutations shown in bold letters), the oligonucleotides F87 (5′-GATCCAGGATCTGACCTGCTTTCGATACAAGG) and F88 (5′-GATCCGCTTGTCTTGGATTAGCATGTCCT) were concatenated by brief ligation before adding BanHI digested pKα9 to the ligation reaction. pKA10 represents a clone derived from this procedure that was identified by sequencing as having an enhancer region identical that in pKα7 except for conversion of the NFAT binding site from GGGAAA to CTTGAC. **Retroviral Production and Infection—**Retroviruses were prepared by transducing virus-free amphotropic producer cells (20). Bing-CAS9 cells (ATCC CRL-11554) were maintained expanded stocks without selective antibiotics for less than 30 days in growth medium (GM) consisting of DME, 4.5 g/ml glucose, 10% fetal bovine serum, and a mixture of 100 units/ml penicillin and 100 units/ml streptomycin in a humidified 5% CO2 atmosphere at 37 °C. To produce infectious retroviral supernatants, the cells at 50–80% confluence in GM were transfected with the retroviral plasmids using calcium phosphate and 25 μl chloroquine for 6–12 h before refeeding with 25 ml of GM. Twenty-four hours after initiating the transfection, the GM was aspirated and replaced with 9 ml of fresh GM before placing the dishes in a humidified 5% CO2 atmosphere at 32 °C. Previous work has shown this lower temperature enhances retroviral titers (21). The supernatant containing retroviral particles was harvested after 24 h, and twice more at 12–18 h intervals thereafter replenishing with 9 ml of GM each time. Each collected supernatant (9 ml) was filtered through sterile 0.45-μm syringe-tip cellulose acetate disk, aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C. VSMC (∼20% confluent) grown on 6 × 35-mm multwell plates were infected with retroviruses by adding thawed retroviral supernatant (2 ml with 8 μg/ml Polybrene) to each well and spinning the cells at 2,500 rpm for 30 min at 32 °C in a Beckman model GS-6R refrigerated centrifuge in a swinging bucket rotor before placing in a 5% CO2 incubator at 37 °C. This infection protocol was repeated twice more at 8–12 h intervals. Forty-eight hours following the last infection, the cells were treated with 400 μg/ml G418 in normal growth VSMC medium (Life Technologies, Inc.). Within 7 days of selection, the surviving cells from each well were combined, expanded, and used subsequently for further study without subcloning and maintained in the absence of G418. Because cell death during selection of retrovirus-infected cultures was minimal compared with that in retrovirus-naïve cells treated in parallel with 400 μg/ml G418, transduction efficiency was estimated in all cases to be nearly quantitative.

**Cell Treatments and Luciferase Assays—**Recombinant VSV selected for reporter retroviruses were grown to confluence in 24-well plates for 3 days in growth medium lacking G418 and then for an additional 24 h in medium lacking serum before stimulation with drugs and agonists. The drugs were prepared as 10-fold concentrated stocks in phosphate-buffered saline, pH 7.4 (PBS), and added as aliquots directly to the cells to begin stimulation. These were performed in a humidified 5% CO2 atmosphere at 37 °C for the indicated time before the cells were lysed and extracts were prepared for luciferase determinations, as described previously (22). **Western Blot Analysis—**To prepare control cell extracts expressing defined NFAT isoforms, COS-7 cells were transfected by the DEAE-dextran/chloroquine approach with the vector pSH107c, encoding human NFATc1; or the vector pSH210, encoding human NFATc2 (23); or pSH211, encoding human NFATc3 (20). All cells were infected with retroviruses by adding thawed retroviral supernatant (2 ml with 8 μg/ml Polybrene) to each well and spinning the cells at 2,500 rpm for 30 min at 32 °C. Cells were cultured for 6–12 h before incubation in a 1:100-fold stock solution. The cells received either CsA (1 μM final concentration) or its vehicle. After 15 min, the media was aspirated and replaced with 9 ml of fresh GM containing 0.2 TIU/ml aprotinin, 0.1 mM leupeptin, 0.2 mM sodium orthovanadate, 5 μg/ml phosphatase A, and 5 μg/ml soybean trypsin inhibitor). Insoluble debris was removed by centrifugation at 10,000 × g at 4 °C and discarded. Protein concentrations in the supernatants were determined using a kit purchased from Bio-Rad, and γ globulin as standard. Samples were dissolved in SDS loading buffer, heated for 5 min at 95 °C before separation on 10% SDS-polyacrylamide gels, and transferred to Immobilon P membranes that were blocked in 0.5% nonfat dried milk and incubated overnight with a 1:1000 dilution of either the anti-NFATc1 or anti-NFATc2 monoclonal antibodies. The blots were developed using a chemiluminescent detection kit (Phototope-HRP, New England Biolabs, Natick, MA) using 1:20,000 horseradish peroxidase (HRP)-labeled anti-mouse IgG secondary.

**Immunohistochemistry—**VSMC (7 × 105 cells) were plated in 35-mm dishes 24 h prior to drug treatments, and changed to serum-free medium 1 h before the addition of drugs. Cells were then incubated in 2.5% CO2 in cell culture medium containing 0.1% Tween 80 for 1–2 h before fixation with a 100% concentrated stock solution. Ionomycin was added to the cells directly from a 100-fold stock solution. The cells were then treated with 25 μg/ml chloroquine for 6–12 h before inspection under a fluorescence microscope.
4°C in PBS. Antibody staining was performed at room temperature within 1 week of these treatments. The samples were first blocked against avidin/biotin using the AVB kit (Vector Laboratories), according to directions supplied by the manufacturer. After rinsing with PBS, nonspecific protein binding was blocked by incubating the cells with PBS containing 2% horse serum, 0.2% Tween 20, and 0.5% Triton X-100 for 30 min at 22°C. All primary antibodies were diluted in this blocking solution at a 1:250 dilution and applied to the cells for 60 min at 22°C. Following three washes with PBS containing 0.2% Tween 20, the cells were incubated for 60 min with a 1:10,000 dilution of biotinylated anti-mouse IgG (Jackson Immunonochemicals, Inc., West Grove, PA). After washing as above, antibody binding was visualized using a catalyzed reporter deposition kit (T.S.A. Direct Green, NEN Life Science Products according to the manufacturers directions but with a 1:100 dilution of the streptavidin HRP conjugate. After the final rinse, the cells were incubated for 10 min at 22°C with a Hoechst stain (H33258) in PBS, then washed three times with PBS. After coverslipping using Vectashield, all analyses and photography were performed on a Zeiss Axiosvert microscope equipped with an Optronics video camera, which was in line with a Power Macintosh 7600 computer equipped with NIH Image.

**Fura-2 Assays**—Confluent 10-cm plates of VSMC were washed three times with Ca2+/Mg2+-free Hank’s balanced salt solution (HBSS) and treated with 1 mg/ml type-1 collagenase (178 units/ml; Worthington Biochemical, Inc., Freehold, NJ) at 37°C. The dispersed cells were collected, washed in Ca2+/Mg2+-replete HBSS (Ca2+/Mg2+-HBSS), counted, and then resuspended to 3–4 × 106 cells/ml in HBES-buffered DMEM (pH 7.4) containing 0.5 mg/ml bovine serum albumin (DMEM+BSA). Fura-2/AM (1 μM stock in Me2SO) was added to a final concentration of 2 μM, and the cells were incubated for 15 min at 37°C in the dark. The cells were brought to a final volume of 40 ml in DMEM+BSA, spun at 500 rpm for 10 min at 4°C in a benchtop centrifuge, and the cell pellet was resuspended in 10 ml of Ca2+/Mg2+-HBSS. After counting, the cells were diluted into 3-ml aliquots containing 2 × 107 cells/ml. Just prior to assay, each aliquot was centrifuged briefly and the pellet resuspended gently in 1 ml of Ca2+/Mg2+-HBSS that was added to stirred cuvettes containing 2 ml of pre-warmed HBES-HBSS. A Agonist was added from 100-fold concentrated stocks, and the excitation ratio (340/380 nm) was measured for emission intensity at 510 nm on a Perkin Elmer LS50 spectrometer.

**Gel Mobility Shift Assays**—VSMC, or control 293 cells transected with pSH210, were treated with drugs as described in legend to Fig. 5 before nuclei were harvested as described previously (15). Nuclear extracts were prepared by gently resuspending the nuclei in 0.5 volumes of salt buffer (20 mM HEPES, pH 7.9 at 4°C, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 20 mM KCl). An equivalent volume of high salt buffer (low salt buffer except with 0.8 M KCl) was next added dropwise with gentle mixing, and the nuclei were incubated on ice for 30 min. After centrifugation in a Beckman TLS55 rotor at 25,000 × g for 30 min at 4°C, the supernatant was dialyzed against 500 volumes of a buffer identical to the low salt buffer above except containing 100 mM KCl. Aliquots of the dialyzed sample were frozen at −80°C until use. Binding assays were performed at 22°C for 30 min in a 20-μl volume composed of nuclear extract (containing 10 μg of VSMC nuclear protein or 1 μg of 293/NFATc1 nuclear protein), 4.5 μg of bovine serum albumin, 2 μg of poly(dI-dC), 12 μM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 80 mM KCl, 13% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The probe used in binding assays (wtNFRE) was derived from the distal NFRE in the human IL-2 gene and formed by the oligonucleotides D79 (5′-GAAAAGAAAAAACTGTCTT-TCATACAGAGCGGTT) and D80 (5′-GACGCTTCTTGTATGAAAACAGT- GTTTTTCCTCCTTT), which were end-labeled with 32P using T4 polynucleotide kinase. Unlabeled competitor probes used in binding assays include mutNFRE, which was formed by annealing oligonucleotides F57 and F88 (see above), and the wtNFRE composed of oligonucleotides D79 and D80. After the binding incubation period, the samples were loaded onto Tris-glycine polyacrylamide gels, resolved by electrophoresis, and subjected to autoradiography.

**RESULTS**

Western blot analysis indicates that a protein in VSMC reacts with monoclonal antibody 7A6 (23), which was prepared against a polypeptide from the NFATc1 isomorf (Fig. 1). The antibody specificity for the NFATc1 isoform is indicated by immunoreactivity in extracts of COS-7 cells transfected with a human cDNA for NFATc1, but not in extracts transfected with sham plasmid or with a plasmid encoding the NFATc2 isoform (23). The heterogeneous mobility of the immunoreactive VSMC protein is typically seen in NFAT-expressing cells, and might result from either alternate exon usage or various post-translational modifications, including phosphorylation (24). No other currently available antibody directed against the three other NFAT isoforms (12, 23, 25) is compatible with our Western blot protocol, including the GH8-G1-G9 monoclonal antibody directed against NFATc2 that is described below (data not shown).

Immunohistochemical analysis of cultured VSMC provides evidence that, in addition to NFATc1, NFATc2 is not only expressed in these cells, but has a subcellular pattern of expression distinct from that of NFATc1. In unstimulated cells, NFATc1 immunoreactivity is predominantly cytoplasmic, but occurs in a clustered pattern, consistent with vesicular localization (Fig. 2a). This basal pattern contrasts sharply with the more pronounced nuclear localization observed after a 10-min exposure to 1.0 μM ionomycin (Fig. 2c). Treatment of the cells with 1 μM CsA before and during challenge with ionomycin prevents ionomycin-induced nuclear translocation of NFATc1 (Fig. 2e). This calcium-dependent, CsA-sensitive nuclear transport is a characteristic property of NFAT (2, 24, 26). In striking contrast, NFATc2 immunoreactivity is predominantly nuclear in unstimulated cells (Fig. 2g), and this localization is unaffected by ionomycin treatment (Fig. 2i) or by ionomycin treatment in the presence of CsA (Fig. 2k). The specificity of these antibodies was confirmed in immunohistochemical control experiments, in which 293 cells were transfected with plasmids encoding each of the four major NFAT isoforms. Furthermore, there is no detectable staining in VSMC incubated with non-immune IgG as a negative control (data not shown). From these controls and the staining shown in VSMC, these data indicate that localization of various endogenously expressed NFAT isoforms differs in VSMC, suggesting they may be under control of different regulatory processes.

In order to study control of NFAT-mediated transcription in VSMC, a retroviral-based luciferase transcriptional reporter system was developed to take advantage of the higher efficiency of retroviral gene transfer than is possible in VSMC using plasmid transfection approaches. The retroviral NFAT/ luciferase reporter vector pKA7 is depicted in Fig. 3A. This vector contains a luciferase coding sequence under the control of a minimal human IL-2 promoter with an upstream triplex of the distal IL-2 gene NFRE. These elements were derived from a plasmid NFAT-responsive reporter vector whose fidelity has been characterized extensively, by ourselves and others (13, 19).
A neomycin resistance gene is expressed from the viral 5'-LTR promoter, and selection for cells resistant to this antibiotic ensures that all cells in the culture have been infected with a retrovirus, which is important for comparing responses among the various control reporter vectors. In preliminary experiments, we found that NFAT-luciferase reporter vectors are substantially more responsive to stimuli when the orientation of luciferase transcription is directed toward the retroviral 5'-LTR, rather than when transcription of the two promoters are driven off of the same strand (data not shown). The control reporter vectors are shown in Fig. 3B and include a promoterless luciferase (pKA8), a luciferase driven by a minimal IL-2 promoter but lacking an NFRE (pKA9), and a minimal promoter with an NFRE possessing mutations that disrupt the NFAT-binding site within the NFRE (pKA10).

The data shown in Fig. 3C indicate that highly inducible and synergistic luciferase expression consistent with NFAT-mediated transcription occurred only in VSMC infected with retrovirus prepared from plasmid pKA7, and that no responses were observed in cells infected with any of the other reporter viruses. Stimulation for 4 h with 100 nM PMA alone yielded a response that was 28 ± 1-fold over basal (mean ± S.E., n = 3), whereas the response to 1 μM ionomycin (1.3 ± 0.2-fold; mean ± S.E., n = 3) was no different from that of vehicle. Co-stimulation of VSMC with ionomycin and PMA resulted in a synergistic response that was 96 ± 10-fold over basal (mean ± S.E., n = 3), and was consistent with the known co-dependence of NFAT-mediated transcription upon simultaneous Ca²⁺ and protein kinase C signaling (27). The lack of response in VSMC harboring virus prepared from pKA10, which has mutations in the NFAT binding site in the NFRE, indicates that luciferase activity transcribed from the pKA7 vector is strictly dependent upon the binding of a protein to the well characterized NFAT binding site in the NFRE.

NFRE DNA binding assays were performed using nuclear extracts prepared from both VSMC and from 293 cells transfected with a plasmid encoding NFATc1 (Fig. 4). Both high and low mobility complexes (HMv and LMv, respectively) in VSMC extracts bound to this probe. Binding in the LMv was enhanced in extracts from cells co-stimulated with ionomycin and PMA, compared with either vehicle or drug treatment alone (compare lanes 4 to lanes 1, 2, or 3). We interpret the enhanced presence of the LMv in co-stimulated cell extracts as indicative of an assembly of proteins, which is consistent with the formation of an NFAT transcriptional complex. As shown by comparing lanes 5, 6, and 7 to lanes 9, 10, and 11 in Fig. 5, lower concentrations of the NFRE containing an intact NFAT binding site (wtNFRE) competed more effectively for LMv binding than do lower concentrations the mutant NFRE (mutNFRE). Since the mutNFRE competitor contains mutations only in the NFAT binding site, and not the AP1-like site of the NFRE, it is also capable of inhibiting both LMv and HMv binding to the labeled probe, albeit at higher concentrations. However, the NFRE binding complexes are difficult to study at the relatively low levels of endogenous NFAT expression that occur in VSMC. The use of relatively high levels of nuclear proteins necessary in the binding assays precludes a definitive evaluation of what proteins in VSMC bind to this probe.

For this reason, we also studied binding in extracts of 293 cells transfected with an NFATc1 expression vector, which is also shown in Fig. 4. No binding activity was observed in extracts prepared from untransfected 293 cells, whether or not they were treated with drugs (data not shown). However, both
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**FIG. 4.** Nuclear protein binding to a probe prepared from the human IL-2 gene distal NFRE. VSMC were treated for 30 min with vehicle (lane 1), 1.0 μM ionomycin (lane 2), 100 nM PMA (lane 3), or with both drugs together (lanes 4–12). Nuclear extracts were incubated with 32P-labeled wtNFRE probe. Competition experiments included increasing concentrations of either unlabeled wtNFRE (lanes 4–8) or mutNFRE (lanes 9–12), which contains a mutation in the NFAT binding site in the NFRE. The concentrations of competitors used are approximately 3 nM (lanes 4 and 9), 10 (lanes 5 and 10), 30 nM (lanes 6 and 11), and 100 nM (lanes 7, 12, 14, and 15), whereas the concentration of the labeled probe was estimated to be approximately 1 nM. The binding in lanes 13–15 represents that in extracts prepared from 293 cells transfected with an NFATc1 expression plasmid and treated for 30 min with ionomycin and PMA. This experiment was repeated twice with similar results.

**FIG. 5.** Dose relationships for luciferase induction in VSMC by ionomycin and PMA. VSMC infected with virus prepared from the NFAT-responsive luciferase vector pKA7 were incubated with the indicated concentrations of drugs for 4 h before assessing luciferase levels. The data are a dose response to ionomycin alone (open circles), a dose response to PMA alone (open triangles), an ionomycin dose response in the presence of 100 nM PMA (closed triangles), and the dose response to ionomycin in the presence of 1 μM CsA and 100 nM PMA (closed circles). Each point represents the mean ± S.E. of three separate experiments performed in duplicate, and all treatments were performed in parallel.

![Luciferase Level fold-over-basal](image)

**FIG. 6.** Forskolin-stimulates NFAT-mediated transcription in VSMC in the presence of Ca2+ ionophore. VSMC infected with virus prepared from the NFAT-responsive luciferase vector pKA7 were treated for 5 h with the indicated concentrations of drugs before assessing luciferase levels. Each curve represents an ionomycin dose response in the presence of 10 μM forskolin (closed circles), 100 nM PMA (closed diamonds), 10 μM forskolin and 1 μM CsA (open squares), or 10 μM forskolin in the presence of 100 nM PMA (open circles). Each point represents the mean ± S.E. of three independent experiments performed in duplicate.

As shown in Fig. 5, stimulation of VSMC that were infected with retrovirus prepared from the pKA7 plasmid with PMA alone induces a luciferase response dose-dependently, representing a 21-fold maximal effect at 100 nM. Although ionomycin itself is a very weak activator, it synergized markedly the luciferase response of 100 nM PMA in a dose-dependent manner. A maximally effective concentration of CsA (1 μM) blocked completely this synergy between 100 nM PMA and ionomycin. This particular observation is characteristic of an NFAT-mediated transcriptional response and indicates that the retroviral reporter cassette mimics NFAT-mediated transcription faithfully. Although the 20-fold induction of luciferase in response to 100 nM PMA in the presence of low concentrations of ionomycin was unaffected by CsA treatment, it was decreased in the presence of CsA with increasing ionomycin concentrations. This indicates that the NFAT transcriptional response to PMA alone is regulated negatively by Ca2+, a feature that is revealed only when CsA blocks nuclear import of NFAT.

The influence of cAMP elevation on NFAT transcriptional activity in VSMC was investigated by examining the effects of forskolin on luciferase expression (Fig. 6). Unlike for PMA, forskolin (10 μM) alone did not stimulate luciferase above basal levels in VSMC infected with the NFAT reporter virus. However, luciferase levels were induced synergistically by increasing doses of ionomycin in the presence of 10 μM forskolin. The maximal response was 45 ± 6-fold over basal (mean ± S.E., n = 3) at 10 μM forskolin with 100 nM ionomycin. Since this effect was completely abolished in the presence of 1 μM CsA, it suggests that forskolin synergies with ionomycin represents NFAT-mediated transcription. At maximally effective concentrations of ionomycin, 10 μM forskolin co-treatment with 100 nM PMA gave a 111 ± 6-fold over basal response (mean ± S.E., n = 3), which was not different from the maximal effect of ionomycin and PMA treatment. However, the ionomycin dose-effect curve in the presence of forskolin and PMA was shifted leftward relative to that in the presence of PMA alone, indicating forskolin and PMA have either additive or synergistic effects on luciferase transcription at submaximal concentrations of ionomycin.

It is of interest to understand what physiologic activators of VSMC might be capable of controlling NFAT-mediated transcription in VSMC. Fig. 7 shows dose-dependent luciferase induction by the mitogen PDGF-BB in cultures of VSMC infected with the NFAT reporter virus. PDGF-BB activated a...
tyrosine kinase receptor leading to activation of signaling molecules including PLCγ (28). The luciferase induction in the KA7 cells was blocked by 1 μM CsA, indicating the response to PDGF-BB was also NFAT-mediated. We also sought to determine if agonists for Gαq-coupled receptors, which activate PLCβ isofoms, could stimulate NFAT-mediated transcription by stimulating NFAT reporter virus infected VSMC with angiotensin II and thrombin. As shown in Fig. 8, 100 nM angiotensin II alone induced NFAT-mediated transcription approximately 1.6 ± 0.2-fold over basal (mean ± S.E., n = 3), whereas the response to 100 nM thrombin was 7.4 ± 0.8-fold over basal. These responses were significantly less than those elicited by the growth factor PDGF-BB (24 ± 2-fold over basal). Co-stimulation with PDGF-BB and either angiotensin II (38 ± 6-fold over basal), or thrombin (43 ± 5-fold over basal), or both of these Gαq-coupled receptor agonists (49 ± 6-fold over basal) yielded a greater response than the sum of the agonists alone, indicating NFAT-mediated transcription in VSMC is synergized modestly by multiple agonist stimuli.

Recent studies in T cells have suggested that Ca2+ transient duration is a critical determinant for committing NFAT to transcription (26). The data shown in Fig. 9 compare the Ca2+ transients elicited in VSMC by agonists alone and in combination, using Fura-2 spectrofluorometry. The Ca2+ transients elicited by the Gαq-coupled receptor agonists angiotensin II and thrombin peak within 30 s after agonist addition and are for the most part exhausted an additional 30 s later. These contrast with the Ca2+ transient elicited by PDGF-BB, which is slower in onset but significantly more prolonged than for the Gαq-coupled receptor agonists. The response to the simultaneous addition of agonist mixtures behaved as a composite of their individual effects. In particular, the rise of intracellular Ca2+ elicited by co-stimulation of VSMC with PDGF-BB and angiotensin II was more rapid than that seen with PDGF-BB alone, and more sustained above basal levels than that seen with angiotensin II alone. The transient was even greater when the cells are stimulated with all three agonists. This augmented release of intracellular Ca2+ by two or more agonists provides one reasonable explanation for the ability of Gαq-coupled receptor agonists to induce a synergistic effect on NFAT-mediated transcriptional responses from the growth factor alone. However, although angiotensin II mobilized Ca2+ more robustly than did thrombin, the latter was a stronger activator of NFAT-mediated transcription (see Fig. 8). Thus, the amplitude of the immediate-early Ca2+ transient elicited by Gαq-coupled receptor activation is not a sufficient predictor of agonist induction of NFAT-mediated transcription in VSMC.

DISCUSSION

The use of a heterologous promoter reporter in this study, reconstructed from a T-cell-derived NFAT-responsive gene, was necessary because the genes which might be regulated by NFAT in VSMC are currently unknown. To our knowledge, the use of retroviral-based inducible promoter system to study the control of transcription by cell surface signaling has not been reported previously. This study shows the promise of this approach for analysis of transcriptional function in cultured cells that are otherwise difficult to transfect with plasmid DNA. The pattern of luciferase expression in response to PMA, ionomycin, and CsA in VSMC from this reporter vector is consistent with that known for PLC-mediated NFAT induction in other cells (2, 3, 19, 27), and serves to demonstrate that the retroviral reporter vector mimics NFAT-mediated transcriptional responses with fidelity.

With the exception of antigen receptors on lymphocytes, little is known about how NFAT-mediated transcription might be controlled by endogenous receptors for physiological agonists. This report shows that growth factors can serve as strong physiological activators of NFAT-mediated transcription in VSMC, whereas in comparison, agonists of Gαq-coupled receptors are relatively weaker activators of NFAT. Previous studies have shown that NFAT-mediated transcription can be strongly induced in both PC12 pheochromocytoma and Jurkat T cells transfected with recombinant Gαq-coupled muscarinic receptors (13, 29). On the basis of these previous observations, we initially predicted that angiotensin II and thrombin treatment would be more powerful inducers of NFAT-mediated transcription in VSMC than what is in fact observed. There are several potential explanations for why this is so. First, various cell phenotypes may differ in receptor-mediated control of capacitive Ca2+ entry. A proposal from previous studies suggests that a requirement for prolonged cytosolic Ca2+ transients in NFAT-mediated signaling may provide an important mechanism to prevent NFAT from committing to gene induction in response to the relatively minor and the brief Ca2+ transients elicited by various stimulators of cell surface receptors in the course of normal cellular function (26). The present results provide support for this hypothesis in that PDGF-BB, which alone elicits a more sustained Ca2+ transient than either angiotensin II or thrombin, is a stronger activator of NFAT-
mediated transcription than the latter agonists. Consistent with this is our observation that co-stimulation of the cells with PDGF-BB and one or more agonists results in both more pronounced \( \text{Ca}^{2+} \) release and also augmented NFAT-mediated transcription.

The observation that thrombin is a stronger inducer of NFAT-mediated transcription but a weaker inducer of \( \text{Ca}^{2+} \) release than is angiotensin II (Fig. 8) suggests additional complexities are responsible for dictating NFAT responsiveness to receptor stimulation. Differential regulation of the receptors at the cell surface may in part explain how angiotensin II and thrombin elicit different magnitudes of NFAT responsiveness, perhaps reflecting that angiotensin II receptors desensitize more quickly than do thrombin receptors. Alternatively, angiotensin II may be a weaker activator than thrombin of the signaling pathways responsible for inducing AP1 complexes, the NFAT transcriptional partner. Another possibility for the differential responsiveness to the two agonists is that thrombin and angiotensin II receptors may be expressed on distinct classes of cells in this primary culture, which differ in their intrinsic degree of NFAT-responsiveness.

Current evidence indicates that both nuclear import and export of NFAT can be regulated dynamically (24, 26). Signals downstream of \( \text{G}_{\alpha} \)-coupled receptor activation may influence both of these processes differentially and explain in part how these agonists are weaker inducers of NFAT-mediated transcription. In T cells, relatively profound and sustained cytosolic \( \text{Ca}^{2+} \) transients such as those that occur following antigen receptor engagement appear to be necessary to activate calcineurin and counterbalance the effects of processes that effect nuclear export of NFAT (30). The short duration \( \text{Ca}^{2+} \) transients elicited by agonists of \( \text{G}_{\alpha} \)-coupled receptors may provide a signal to stimulate the export of nuclear NFAT, a signal which we suggest would serve to compete against the effect of \( \text{Ca}^{2+} \) on the calcineurin-dependent nuclear import pathway. One basis for this postulate is the observation shown in Fig. 5 in which PMA-stimulated luciferase levels are reduced dose-dependently by ionomycin in the presence of CsA. Presumably, the 20-fold transcriptional response following PMA treatment in the absence of \( \text{Ca}^{2+} \) ionophore reflects that some level of NFAT pre-exists in the VSMC nuclei. Our data showing nuclear localization of NFATc2 under basal conditions (Fig. 2) is consistent with these functional responses and supports this interpretation. CsA treatment does not lower the effect of PMA stimulation alone, as anticipated since CsA only serves to block the calcineurin-mediated nuclear import of NFAT. However, since increasing ionomycin doses in the presence of CsA lead to a reduction in the responsiveness to PMA, this result at face value indicates that a \( \text{Ca}^{2+} \) signal can negatively regulate NFAT-mediated transcription in VSMC. One postulate is that this might involve activating some process stimulating the nuclear export of NFAT. As shown in Fig. 2, brief ionomycin stimulation in the presence of CsA does not yield any obvious export of the NFATc2 immunoreactivity from the nucleus, which argues against this possibility. However, it would be premature to rule out this possibility for now given the relatively static nature of this experiment. The possibility remains that the rate of nuclear export of either an NFAT isoform or a crucial component of the complex necessary for NFAT-mediated transcription is enhanced by \( \text{Ca}^{2+} \) signaling alone or by other kinases activated in this experiment.

We speculate that the \( \text{Ca}^{2+} \) signals of shorter duration elicited by activation of the \( \text{G}_{\alpha} \)-receptors may preferentially activate this putative negative regulatory processes, whereas activation of calcineurin, dephosphorylation of NFAT and its subsequent nuclear import require \( \text{Ca}^{2+} \) transients of longer duration than these agonists can achieve alone. This notion is consistent with recent studies providing evidence that a nuclear kinase activity is involved in re-phosphorylating NFAT and exporting it to the cytosol as a means for terminating its transcriptional activity (31). Although protein kinase A (PKA) and glycogen synthase kinase-3 have been implicated as the major NFAT kinases in Jurkat T-cells, calmodulin-dependent kinases appear to have some NFAT nuclear export activity as well in an heterotopic expression system (30).

The novel finding shown here that ionomycin and forskolin act synergistically to stimulate NFAT-mediated transcription suggests that a down stream effector of cAMP-dependent signaling may be capable of partnering with NFAT isoforms on the NFRE. To our knowledge, co-activation of NFAT-mediated transcription in other cells through simultaneous stimulation of PKA and \( \text{Ca}^{2+} \) pathways has not been reported. In general,
the effects of stimulating PKA activity in lymphoid cells is held to be immunosuppressive, although a recent study both provides and extensively documents evidence suggesting otherwise (32). The synergy between ionomycin and forskolin in VSMC suggests that PKA activation is capable of bypassing the protein kinase C arm of the signaling pathway. NFAT has been shown to cooperate with ATF-2/Jun dimer binding to a closely spaced Ca2+-responsive element in the tumor necrosis factor α promoter to mediate transcription in response to Ca2+ ionophore, but it is unknown to what degree Ca2+-dependent processes are involved in this effect (33). Because little is understood generally about how Ca2+-regulated transcription factors function in VSMC, discovering whether NFAT assembles with PKA-regulated transcription factors of the CREB/ATF family (34) on the enhancer used in these studies in the VSMC nuclear context is of interest. Alternately, PKA may induce other AP1 proteins via CREB-mediated effects and these, rather than CREB/ATF proteins, assemble with NFAT (35, 36). No matter which of these possibilities proves true, these observations highlight the attractiveness of NFAT-mediated transcription in VSMC as a model for understanding mechanisms for integration of multiple co-activating cell surface signals into defined gene expression control regimes.

The present finding that NFAT is expressed and regulated by physiological agonists in VSMC follows a recent observation of NFAT expression in cultured vascular endothelial cells (10), which together may have important implications. Atherosclerotic vascular lesions are composed of VSMC resembling those which together may have important implications. Atherosclerosis.

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