MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5915–5923 Vol. 16, No. 10
0270-7306/96/$04.00+0

Voltage-Insensitive Ca^{2+} Channels and Ca^{2+}/Calmodulin-Dependent Protein Kinases Propagate Signals from Endothelin-1 Receptors to the c-*fos* Promoter

YUAN WANG AND MICHAEL S. SIMONSON*

Department of Medicine, Division of Nephrology, School of Medicine, Case Western Reserve University, and University Hospitals of Cleveland, Cleveland, Ohio 44106

Received 18 January 1996/Returned for modification 19 March 1996/Accepted 29 July 1996

Endothelin-1 (ET-1) triggers poorly understood nuclear signaling cascades that control gene expression, cell growth, and differentiation. To better understand how ET-1 regulates gene expression, we asked whether voltage-insensitive Ca^{2+} channels and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) propagate signals from ET-1 receptors to the c-*fos* promoter in mesangial cells. Ca^{2+} influx through voltage-insensitive Ca^{2+} channels, one of the earliest postreceptor events in ET-1 signaling, mediated induction of c-*fos* mRNA and activation of the c-*fos* promoter by ET-1. A CaMK inhibitor (KN-93) blocked activation of the c-*fos* promoter by ET-1. Ectopic expression of CaMKII potentiated stimulation by ET-1, providing further evidence that CaMKs contribute to c-*fos* promoter activation by ET-1. The c-*fos* serum response element was necessary but not sufficient for CaMKII to activate the c-*fos* promoter. Activation of the c-*fos* promoter by ET-1 and CaMKII also required the FAP *cis* element, an AP-1-like sequence adjacent to the serum response element. Thus, voltage-insensitive Ca^{2+} channels and CaMKs apparently propagate ET-1 signals to the c-*fos* promoter that require multiple, interdependent *cis* elements. Moreover, these experiments suggest an important role for voltage-insensitive Ca^{2+} channels in nuclear signal transduction in nonexcitable cells.

Endothelins (ETs) are a family of paracrine and autocrine regulatory peptides related to sarafotoxin snake venom peptides (57). ET-1, a 21-amino-acid endothelium-derived peptide, maintains vascular homeostasis and regulates vasoconstriction and cell growth (28, 30, 42, 56, 57). ET-1 has also been implicated in compensatory remodeling of vascular cells in response to injury (3, 7, 11, 27, 43) and in the development of several tumors (34, 55). Gene targeting experiments demonstrate an essential role for ETs in development of neural crest-derived cell lineages, pharyngeal arch tissues, and the great vessels derived from cephalic and cardiac tissues (2, 21, 24, 25). Thus, the ability to activate new programs of gene expression is fundamental to the biological actions of ET-1. Binding of ET-1 to its heterotrimeric G protein-coupled receptors stimulates a complex network of transmembrane signals (28, 30, 44), but it is not yet clear how these postreceptor signals control gene expression.

One of the earliest ET-1 postreceptor signals is Ca^{2+} conductance through voltage-insensitive Ca^{2+} channels in the plasma membrane (see references 30, 44, 46, and 52 for reviews). ET-1 does not directly activate voltage-gated Ca^{2+} channels, even though it can increase the probability of channel opening in response to other depolarizing stimuli (14, 30, 35, 44). Although voltage-insensitive Ca^{2+} channels are widely expressed in nonexcitable cells, they are not well characterized and relatively little is known about their ability to regulate gene expression in response to cell activation by agonists. Induction of c-*fos* is among the earliest genomic responses to ET-1, and previous experiments by Pribnow et al. (37) with fibroblasts (which express mostly voltage-insensitive Ca^{2+} channels) showed that chelation of extracellular Ca^{2+} inhibits transcription of the c-*fos* immediate-early gene by ET-1. These results suggest an important function for voltage-insensitive Ca^{2+} channels in c-*fos* induction by ET-1. Although mechanisms whereby voltage-gated Ca^{2+} channels induce c-*fos* have been extensively investigated (10), almost nothing is known about the potential role of voltage-insensitive Ca^{2+} channels and downstream effectors in c-*fos* regulation by ET-1 or other regulatory peptides.

In this study, we investigated the hypothesis that voltage-insensitive Ca^{2+} channels evoke nuclear signaling cascades that link ET-1 receptors in the plasma membrane to the c-*fos* promoter. These experiments employed glomerular mesangial cells, an important microvascular cell type in which ET-1 activates voltage-insensitive Ca^{2+} channels. We report that voltage-insensitive Ca^{2+} channels propagate ET-1 signals to the c-*fos* promoter. Activation of the c-*fos* promoter by ET-1 apparently involves Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) and multiple, interdependent *cis* elements including the serum response element (SRE), the FAP *cis* element, and to a lesser extent the Ca^{2+}/cyclic AMP (cAMP) *cis* element (Ca/CRE). These results point to an important function for voltage-insensitive Ca^{2+} channels and CaMKs in ligand-activated networks of gene expression in nonexcitable cells.

MATERIALS AND METHODS

Reagents. Antihemagglutinin (anti-HA) monoclonal antibodies (12CA5) were obtained from Boehringer Mannheim (Indianapolis, Ind.). Anti-CaMKII and anti-CaMKIV monoclonal antibodies were obtained from Transduction Laboratories (Lexington, Ky.). Peroxidase-labeled goat anti mouse immunoglobulin (heavy and light chains) was obtained from Kirkegaard & Perry (Gaithersburg, Md.). ET-1 was purchased from American Peptide Company (Sunnyvale, Calif.). Nifedipine, SKF-96365, and KN-93 were from Calbiochem (La Jolla, Calif.). Fetal bovine serum (FBS) was from HyClone (Logan, Utah).

Mesangial cell culture. Mesangial cell strains from male Sprague-Dawley rats were isolated and characterized as previously reported (45). Cells were maintained in RPMI 1640 medium supplemented with 17% FBS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 5 μg each of insulin and transferrin per

* Corresponding author. Mailing address: Department of Medicine, Division of Nephrology, Biomedical Research Building, Rm. 427, Case Western Reserve University, 2065 Adelbert Rd., Cleveland, OH 44106. Phone: (216) 368-1251. Fax: (216) 368-1249. Electronic mail address: mss5@po.cwru.edu.

ml, and 5 ng of selenite per ml at 37°C in a 5% CO_2 incubator. Cells in passages 5 to 30 were employed in this study. All experiments were performed in quiescent mesangial cells (approximately 80% confluence) incubated in Dulbecco modified Eagle medium (DMEM)–0.5% FBS for 24 h. Viability of mesangial cells was confirmed by incubation with 5(6)-carboxyfluorescein diacetate and monitoring retention of the cleaved, fluorescent product carboxyfluorescein by epifluorescence microscopy to assess membrane integrity as previously described (45).

Plasmids. The following plasmids were used: pCaMKII wt and pCaMKII 290 (31), pRSVCaMKIV wt and pRSVCaMKIV 313 (53), pCGN (54), pSRELUC (40), and p-356wt/fosCAT and point mutant (pm)/fosCATs (4, 12, 13, 15). To construct the p-356wt/fos and pm/fosLUC reporter plasmids, the *Hin*dIII-*Xba*I fragment of p-356wt/fosCAT or specific p-356pm/fosCATs, containing genomic DNA sequences −356 to +109 of the murine c-*fos* promoter, were subcloned into the *Hin*dIII-*Xba*I sites of the pBK-RSV (Promega, Madison, Wis.) multiple cloning site. The c-*fos* promoter fragments were then excised as *Sac*I-*Xba*I fragments and directionally subcloned into the *Sac*I-*Nhe*I sites of pGL3LUC (Promega). The resulting constructs, which were verified by restriction mapping, expressed the luciferase gene under transcriptional control of the wild-type or point-mutated −356 to +109 sequences of the c-*fos* promoter. To construct the CaMKII wt expression vector, a 1.4-kb *Xba*I-*Bam*HI fragment was made from a 35-cycle PCR with rat CaMKII cDNA (31) as the template, a 5′ oligodeoxynucleotide primer (GCTTCTAGAATGGCTACCATCACCTGC), and 3′ oligodeoxynucleotide primer (CGGGATCCATCAATGGGGCAGGACGGAG). For CaMKII 290, a 1.0-kb *Xba*I-*Bam*HI fragment was made from a 35-cycle PCR with rat CaMKII cDNA as the template, the upstream primer as described above, and a downstream primer (CGGGATCCATCACAGGCAGTCCACGG TC) that terminated the coding sequence at Leu-313. The full-length and truncated CaMKII cDNA fragments were then subcloned in frame into the unique *Xba*I-*Bam*HI sites of pCGN (54). The orientation and sequence of each plasmid were verified. The resultant plasmids, pCMVCaMKII wt and pCMVCaMKII 290, expressed the wild-type and constitutively active truncated CaMKII proteins under transcriptional control of the cytomegalovirus (CMV) promoter/enhancer, respectively.

Determination of intracellular free $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ in mesangial cell monolayers grown on Aclar coverslips was determined by dual-wavelength spectrofluorometry using the Ca^{2+}-sensitive dye fura-2 (Molecular Probes, Eugene, Oreg.) exactly as previously reported (47). Calibration of the fluorescent signal was made with ionomycin followed by EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and $[Ca^{2+}]_i$ was calculated by the formula of Grynkiewicz et al. (16) assuming the K_d of the fura-2–Ca^{2+} interaction to be 224 nM. All agonists were tested for autofluorescence, and after lengthy incubations leakage of fura-2 was assessed by addition of 100 μM Mn^{2+} followed by rapid chelation with 4 mM diethylenetriaminepentaacetic acid.

Mesangial cell transfection and reporter gene assays. We transiently transfected mesangial cells using the calcium phosphate method as previously reported (17, 49). Briefly, cells in six-well plates (35 mm; 2×10^5 cells per well) were transfected with 1 μg of the wt or pm fosLUC reporter, 0.1 μg of pRSVβGal internal control, and the indicated expression vectors (usually 2 μg) and carrier DNA (pUC19) to a total of 6 μg of DNA per well. Total promoter strength in control transfections was held equivalent by inclusion of expression plasmids lacking a cDNA insert. After incubating with precipitates for 16 to 18 h at 37°C, cells were washed three times with DMEM and incubated with DMEM–0.5% FBS for an additional 24 h before cell lysis (RLB buffer; Promega). Where indicated ET-1 or FBS was added for 16 h before lysis. Luciferase activity was measured as previously described (17) by adding 20 μl of cell lysate to a buffer containing 20 mM Tricine (pH 7.8), 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 0.3 mM coenzyme A, 0.5 mM luciferin, and 0.53 mM ATP and measuring relative light units in a Berthold Lumat Luminometer (Wallac, Gaithersburg, Md.) for two 10-s intervals. Transfection efficiency was determined with a pRSVβGal construct that directed β-galactosidase expression from the viral long terminal repeat. β-Galactosidase activity was measured by using the Galacto-Light protocol as described by the manufacturer (Tropix, Bedford, Mass.). All reporter gene assays were in the linear range.

Immunoblot assessment of CaMK protein levels. Quiescent mesangial cells or mesangial cells transiently transfected with pCMVCaMKII wt and pCMVCaMKII 290 in 100-mm dishes were washed once in Dulbecco phosphate-buffered saline and scraped into 1-ml sample lysis buffer (0.5 M Tris-HCl [pH 6.8] [2.5 ml], 10% [wt/vol] sodium dodecyl sulfate [SDS] [4.0 ml], glycerol [2 ml], 0.1% bromophenol blue [0.5 ml], β-mercaptoethanol [0.5 ml], distilled water to 10 ml). The lysate was vortexed and boiled for 5 min, aliquots were resolved on SDS–8 to 16% polyacrylamide gradient gels, and proteins were transferred to 0.2-μm-pore-size nitrocellulose filters. Transferred proteins were stained with Ponceau S, and the filter was blocked in blocking buffer (1.0% bovine serum albumin [BSA], 10 mM Tris [pH 7.5], 100 mM NaCl, 0.1% Tween 20) with shaking overnight at 4°C. To detect native CaMKs in untransfected mesangial cells, blots were incubated with monoclonal antibodies at 250 μg/ml in blocking buffer for 1 h at 22°C. To detect the HA epitope-tagged CaMKII wt and CaMKII 290 in transfected cells, blots were incubated with the monoclonal anti-HA antibody as described above. After extensive washing, the appropriate peroxidase-labeled secondary antibodies in blocking buffer (1:10,000) were added, and the proteins were detected by chemiluminescence (ECL; Amersham, Arlington Heights, Ill.). Typical exposure times were 30 to 60 s.

Semiquantitative RT-PCR analysis of c-*fos* mRNA. Preparations of total mesangial cell RNA and reverse transcription PCR (RT-PCR) were carried out as described previously (48) with modifications to permit the semiquantitative analysis of mRNA levels. Optimal oligonucleotide primer pairs of 21 nucleotides (40 to 60% GC) were designed (OLIGO; NBI, Plymouth, Minn.) on the basis of the sequence of the rat c-*fos* gene (6) to yield a predicted product of 308 bp spanning the third and fourth exons. The upstream rat c-*fos* primer was 5′CCGAAGGG AAAGGAATAAGAT; the downstream rat c-*fos* primer was 5′TGAGAAGAG GCAGGGTGAAGG. GAPDH primer pairs yielding a product of 452 bp were from Clontech (Palo Alto, Calif.). Total RNA (0.5 μg) was reverse transcribed in a reaction mixture containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 1 mM each deoxynucleoside triphosphate, 1 U of RNase inhibitor per μl, 1 μM downstream c-*fos* or GAPDH primer, and 2.5 U of avian myeloblastosis virus reverse transcriptase per μl at 42°C for 25 min and 99°C for 5 min. A 20-μl aliquot of the RT reaction mixture was then used for PCR in a reaction mixture containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Samples were denatured for 2 min at 95°C, and PCRs were conducted for 30 cycles (1 min at 95°C, 1 min at 60°C) followed by final extension for 7 min at 72°C. Concentrated PCR products were resolved on a 2.0% agarose gel stained with ethidium bromide and were quantified by scanning densitometry using NIH Image (version 1.55); the amount of c-*fos* mRNA was normalized for the amount of GAPDH in each lane. Under these conditions the amounts of c-*fos* and GAPDH PCR products were linear with respect to the amount of input RNA and the number of cycles (see Fig. 1).

Data analysis. Statistical significance was calculated by a paired *t* test in which repeated experiments run at different times were compared with their own controls. Data presented (three to five independent experiments) are means ± standard errors of the means normalized to the intraexperimental control value. Statistical analysis was performed with InStat for the Macintosh (version 2.0; GraphPAD software).

RESULTS

Voltage-insensitive Ca^{2+} channels control c-*fos* mRNA and promoter induction by ET-1. As a model to study voltage-insensitive Ca^{2+} channels and ET-1 nuclear signaling, we used rat glomerular mesangial cells, in which ET-1 stimulates c-*fos* induction and mitogenesis by binding to an ET_A receptor subtype (47). In these cells ET-1 rapidly increases intracellular free $[Ca^{2+}]_i$ by stimulating Ins(1,4,5)P_3-dependent release of intracellular Ca^{2+} stores and by activating voltage-insensitive Ca^{2+} channels (46, 51). Under certain conditions voltage-gated Ca^{2+} channels can also contribute (22), but most of the $[Ca^{2+}]_i$ waveform results from voltage-insensitive Ca^{2+} channels (46). Thus, mesangial cells are a useful model to investigate whether voltage-insensitive Ca^{2+} channels contribute to c-*fos* induction by ET-1.

As a first step we asked whether influx of extracellular Ca^{2+} was required for c-*fos* mRNA induction by ET-1. An RT-PCR assay was established to measure steady-state c-*fos* mRNA levels. As shown in Fig. 1A, oligodeoxynucleotide PCR primers spanned the third and fourth exons of the rat c-*fos* gene to allow detection of products amplified from potential contamination with genomic DNA. Under the conditions described, the assay was linear and demonstrated the expected rapid and transient pattern of c-*fos* mRNA induction by ET-1 (Fig. 1A, lanes 2 to 5). Omission of the RT reaction further confirmed that the products derived from amplification of mRNA transcripts (Fig. 1A, lane 5), and the amplicons were also verified by restriction mapping (data not shown). Endogenously expressed GAPDH mRNA was coamplified to control for sample-to-sample variation in RT or PCR and for recovery of RNA.

The effect of voltage-independent Ca^{2+} entry on c-*fos* mRNA induction was tested by addition of nonsaturating concentrations of the Ca^{2+} ionophore ionomycin. Ionomycin stimulated a 4.8-fold increase in c-*fos* mRNA at 30 min, similar to the 5.4-fold induction of c-*fos* mRNA by ET-1 (Fig. 1B). c-*fos* mRNA induction by both ionomycin and ET-1 was transient

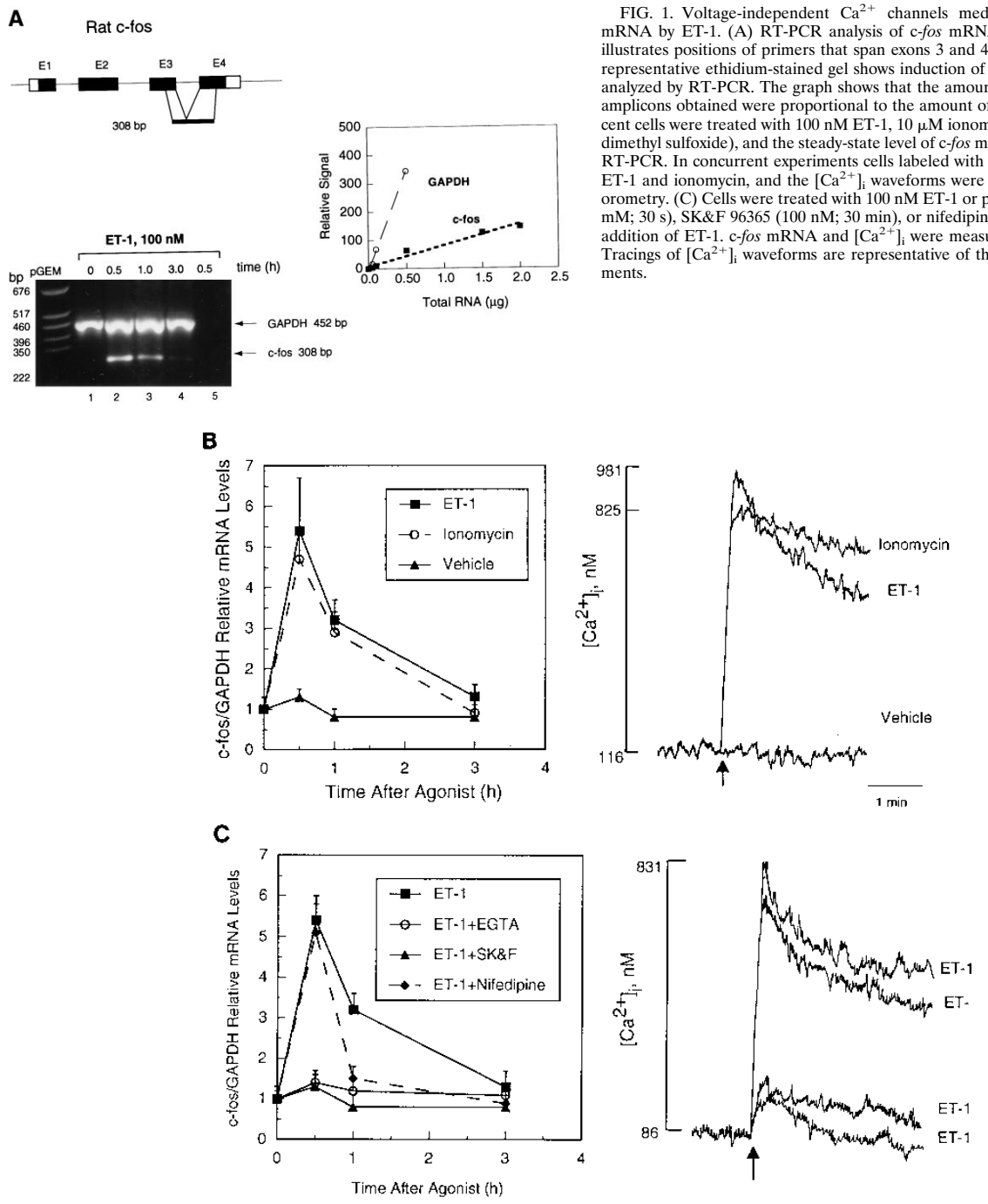

FIG. 1. Voltage-independent Ca^{2+} channels mediate induction of c-*fos* mRNA by ET-1. (A) RT-PCR analysis of c-*fos* mRNA levels. The schematic illustrates positions of primers that span exons 3 and 4 of the rat c-*fos* gene. A representative ethidium-stained gel shows induction of c-*fos* mRNA by ET-1 as analyzed by RT-PCR. The graph shows that the amounts of GAPDH and c-*fos* amplicons obtained were proportional to the amount of input RNA. (B) Quiescent cells were treated with 100 nM ET-1, 10 μM ionomycin, or vehicle (H_2O or dimethyl sulfoxide), and the steady-state level of c-*fos* mRNA was determined by RT-PCR. In concurrent experiments cells labeled with fura-2 were treated with ET-1 and ionomycin, and the $[Ca^{2+}]_i$ waveforms were measured by spectrofluorometry. (C) Cells were treated with 100 nM ET-1 or pretreated with EGTA (3 mM; 30 s), SK&F 96365 (100 nM; 30 min), or nifedipine (10 μM, 3 min) before addition of ET-1. c-*fos* mRNA and $[Ca^{2+}]_i$ were measured as described above. Tracings of $[Ca^{2+}]_i$ waveforms are representative of three independent experiments.

and declined to near basal levels by 3 h. Using mesangial cells loaded with the Ca^{2+}-sensitive fluorescent indicator fura-2, we confirmed that ionomycin at 10 μM and ET-1 at 100 nM produced similar maxima of $[Ca^{2+}]_i$, although, as expected, the $[Ca^{2+}]_i$ waveform was protracted in cells treated with ionomycin (Fig. 1B). These experiments demonstrated that Ca^{2+} influx in mesangial cells mimics c-*fos* induction by ET-1.

Several experiments demonstrated that Ca^{2+} influx was required for induction of c-*fos* mRNA by ET-1. Preincubation (30 s) with EGTA to chelate extracellular Ca^{2+} blocked c-*fos* mRNA induction by ET-1 (Fig. 1C). EGTA prevented ET-1-stimulated Ca^{2+} influx but not release of Ca^{2+} from the intracellular stores (Fig. 1C) (46), suggesting that intracellular Ca^{2+} release is insufficient to induce c-*fos* mRNA in response to ET-1. SK&F 96365 is a structurally distinct antagonist of voltage-independent Ca^{2+} channels (32). Preincubation with SK&F 96365 inhibited ET-1-stimulated c-*fos* mRNA induction (Fig. 1C) and attenuated the $[Ca^{2+}]_i$ waveform evoked by ET-1. Finally, a dihydropyridine that blocks voltage-gated Ca^{2+} entry, nifedipine, failed to block ET-1-stimulated c-*fos* mRNA or $[Ca^{2+}]_i$ (Fig. 1C). These results are consistent with a role for voltage-insensitive Ca^{2+} channels in mediating c-*fos* mRNA induction by ET-1.

We (17, 49) and others (37) have previously shown that ET-1

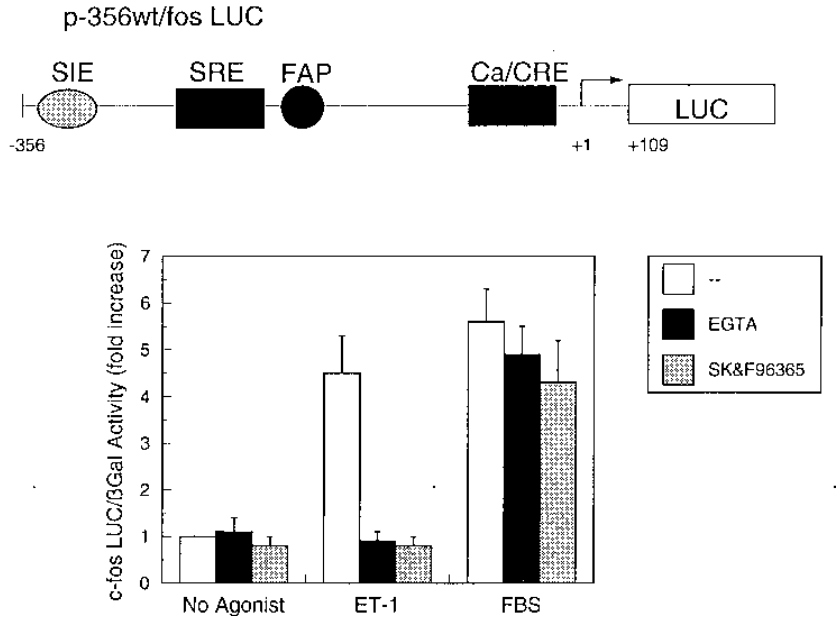

FIG. 2. Ca^{2+} influx is required for stimulation of the c-*fos* promoter by ET-1. Cells were transiently transfected with the p-356wt/fosLUC reporter plasmid (diagrammed at the top), rendered quiescent, and treated with 100 nM ET-1 or 10% FBS. Where indicated, cells were pretreated with EGTA (3 mM; 30 s) or SK&F 96365 (100 mM; 30 min) before agonist addition. Luciferase activity was measured as an index of c-*fos* promoter activity as described in Materials and Methods.

activates the c-*fos* promoter in mesangial cells. To test whether voltage-insensitive Ca^{2+} channels mediate c-*fos* promoter activation by ET-1, we transiently transfected mesangial cells with a plasmid containing a luciferase reporter gene under transcriptional control of a −356 to +109 genomic DNA fragment of the c-*fos* promoter. This c-*fos* promoter fragment, which responds to ET-1 (49), contains the major *cis* elements for ligand-induced c-*fos* transcription in vivo and in vitro (13, 38) (Fig. 2). ET-1 stimulated a 4.8-fold increase in c-*fos* promoter activity that was blocked by SK&F 96365 and EGTA (Fig. 2). SK&F 96365 and EGTA did not significantly alter c-*fos* promoter activity stimulated by FBS, suggesting that these compounds do not nonspecifically inhibit the c-*fos* promoter. Taken together, these results demonstrate that voltage-insensitive Ca^{2+} channels mediate activation of the c-*fos* promoter by ET-1 and that the *cis* elements responsive to Ca^{2+} influx reside between −356 and +109.

CaMKs are downstream effectors of ET-1 nuclear signaling to the c-*fos* promoter. Ca^{2+} influx activates diverse downstream effectors including CaMKs. Both CaMKII and CaMKIV have been implicated in transcriptional regulation of the c-*fos* gene (10, 31, 53); thus, we asked whether CaMKs contribute to activation of the c-*fos* promoter by ET-1. Mesangial cells expressed abundant amounts of a 54-kDa CaMKII, but CaMKIV was undetectable (Fig. 3A). Preincubation with a selective CaMK inhibitor, KN-93 (29), blocked activation of the c-*fos* promoter by ET-1 but did not inhibit activation by FBS (Fig. 3B). Inhibition by KN-93 was dose dependent. Although KN-93 might antagonize Ca^{2+} signaling by mechanisms other than CaMK blockade, these results suggest that CaMK contributes to activation of the c-*fos* promoter by ET-1.

To further support a role for CaMK, we asked whether overexpression of CaMKII would potentiate activation of the c-*fos* promoter by ET-1. Plasmid vectors were constructed expressing wild-type (pCMVCaMKII wt) and constitutively activated mutant (pCMVCaMKII 290) CaMKII under transcriptional control of the CMV promoter (Fig. 4). Western blotting (immunoblotting) of the epitope-tagged proteins demonstrated expression of CaMKII of the expected molecular weights (Fig. 4). These vectors were then cotransfected with the c-*fos* reporter construct to assess the effects of CaMK on activation of the c-*fos* promoter by ET-1. Wild-type CaMKII alone did not significantly stimulate the c-*fos* promoter, but mutant CaMKII 290 stimulated an 8.6-fold increase, indicating that CaMKII activates the c-*fos* promoter in mesangial cells (Fig. 5A). Moreover, wild-type CaMKII potentiated stimulation of c-*fos* transcription by ET-1 (Fig. 5B). ET-1 did not further increase c-*fos* promoter activity in cells transfected with CaMKII 290, presumably because of the robust stimulation of c-*fos* in these cells (data not shown). Taken together, these results suggest that CaMKII contributes to activation of the c-*fos* promoter by ET-1.

CaMKII requires multiple *cis* elements to activate the c-*fos* promoter in mesangial cells. To better understand the nuclear targets of ET-1 and CaMKII signaling, we identified *cis* elements of the c-*fos* promoter that respond to ET-1 and CaMKII in mesangial cells. We previously showed that activation of the c-*fos* promoter by ET-1 requires both the SRE and Ca/CRE *cis* elements (17, 49). CaMKII 290 did not stimulate transcription from a c-*fos* promoter construct containing an inactivating point mutation in the CArG sequence (i.e., SRF binding site) of the SRE (pm 12; Fig. 6), demonstrating that the SRE is required for the response to CaMKII. Stimulation of the c-*fos* promoter by an activated mutant of CaMKIV, CaMKIV 313, was inhibited but not completely blocked by pm12 (Fig. 6). It is important to note that transfection efficiencies in cells that received different reporter plasmids were not always equivalent; thus, a direct comparison of signals from fosLUC reporter plasmids in different panels cannot be made. To determine whether the c-*fos* SRE is sufficient for the response to CaMKII, we transfected mesangial cells with a minimal promoter construct containing only a single copy of the SRE upstream from a minimal c-*fos* promoter. Expression of CaMKII 290 or CaMKIV 313 did not increase transcription from this construct

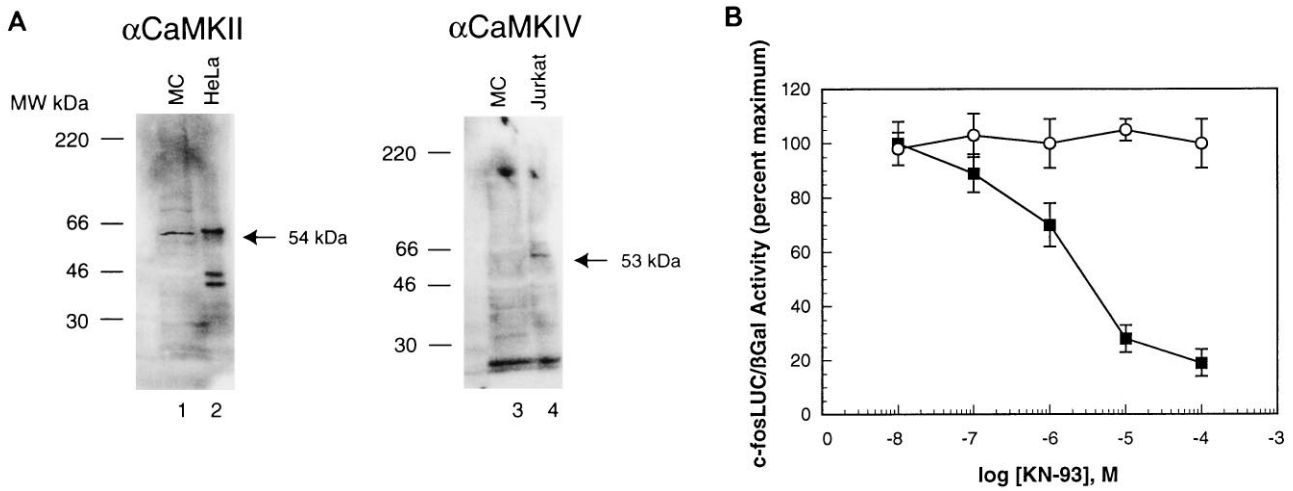

FIG. 3. A pharmacological CaMK antagonist inhibits activation of the c-*fos* promoter by ET-1. (A) Analysis of CaMK isotypes in mesangial cells (MC). Cells were lysed, and proteins (40 μg) separated by electrophoresis were transferred to nitrocellulose. Blots were probed with specific monoclonal antibodies against rat CaMKII and CaMKIV, and bands were visualized by chemiluminescence. Control lysates were from HeLa and Jurkat cells for CaMKII and CaMKIV, respectively. (B) Cells transiently transfected with the p-356wt/fosLUC reporter plasmid were pretreated for 20 min with increasing doses of KN-93, a pharmacological CaMK antagonist, before addition of 100 nM ET-1 (■) or 10% FBS (○) (mean ± standard error of the mean; $n = 3$ in duplicate).

(SRE; Fig. 6). However, treatment with FBS demonstrated that the SRE luciferase construct was responsive to signals. Collectively, these results demonstrate that the c-*fos* SRE is required but not sufficient for c-*fos* promoter activation by CaMKII. Thus, other *cis* elements must cooperate with the SRE to confer responsiveness to CaMKII.

Because the Ca/CRE at −65 responds to Ca^{2+} in neuronal cells (41) but not in T cells (26), we asked whether the Ca/CRE cooperates with the SRE to mediate c-*fos* promoter activation by CaMKII in mesangial cells. Inactivating point mutations in the Ca/CRE (C-to-G conversions, pm3) that block binding of cAMP response element binding proteins (CREB) (4) inhibited approximately 40% of the c-*fos* promoter activity stimulated by CaMKII 290 and CaMKIV 313 (Fig. 6). Inactivating point mutations in the Ca/CRE did not inhibit FBS-stimulated c-*fos* promoter activity (Fig. 6). Collectively these results suggest that the Ca/CRE makes a modest contribution to activation of the c-*fos* promoter by CaMKII and that other *cis* elements must cooperate with the SRE for full induction by CaMKII.

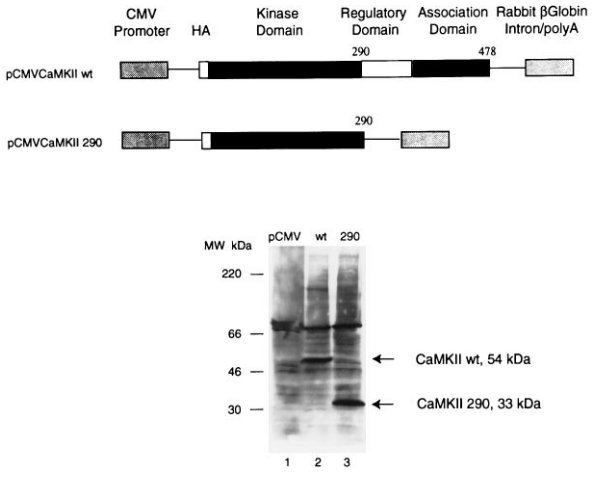

FIG. 4. Expression of epitope-tagged CaMKII wt and CaMKII 290. Cells were transiently transfected with plasmid vectors expressing N-terminal HA epitope-tagged CaMKII wt and a truncated, constitutively active mutant (CaMKII 290) lacking the regulatory and association domains encoded by amino acids 290 to 478. Control cells were transfected with the pCMV expression vector alone. Expression of the properly processed proteins was confirmed by immunoblotting of whole-cell lysates using an anti-HA monoclonal antibody.

The FAP *cis* element contributes to ET-1- and CaMK-stimulated c-*fos* transcription. Recent work by Lee and Gilman (26) with T cells demonstrates that Ca^{2+} signaling targets the FAP site, a CRE/AP-1-like site adjacent to the SRE. We therefore asked if ET-1, CaMKII, or both utilize the FAP site in mesangial cells to activate the c-*fos* promoter. Inactivating point mutations in the c-*fos* FAP (pm 9 [4]) inhibited ET-1- and CaMKII 290-stimulated c-*fos* promoter activity (Fig. 7). In contrast, FAP point mutations did not significantly affect activation of the c-*fos* promoter by CaMKIV 313 or FBS (Fig. 7). Inactivating point mutations in the *sis*-inducible element (pm 6 [4]), which is regulated by the JAK/STAT pathway, did not inhibit activation of the c-*fos* promoter by ET-1, CaMKII 290, and CaMKIV 313 but did inhibit FBS-induced activation (Fig. 7). Thus, the FAP, but not the *sis*-inducible element, apparently cooperates with the SRE to regulate c-*fos* promoter activation by ET-1 and CaMKII.

DISCUSSION

Activation of voltage-insensitive Ca^{2+} channels and influx of extracellular Ca^{2+} is one of the earliest postreceptor events in transmembrane signaling by ET-1. In this study we asked whether voltage-insensitive Ca^{2+} influx and Ca^{2+}-activated downstream effectors contribute to regulation of gene expression by ET-1. Our results show that Ca^{2+} influx through voltage-insensitive Ca^{2+} channels is essential for induction of c-*fos* mRNA and activation of the c-*fos* promoter by ET-1. CaMKII is apparently a downstream effector in pathways leading mainly to the c-*fos* SRE, FAP, and to a lesser extent the Ca/CRE at −65.

ET-1-stimulated Ca^{2+} influx is necessary for c-*fos* induction. Several experiments demonstrated that voltage-insensitive Ca^{2+} influx is required for c-*fos* mRNA induction by ET-1.

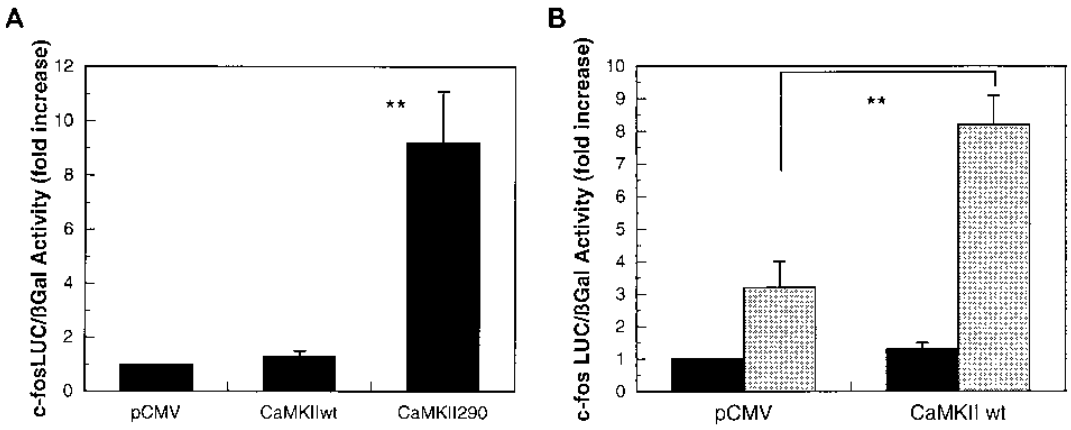

FIG. 5. CaMKII potentiates activation of the c-*fos* promoter by ET-1. (A) Mesangial cells were transiently transfected with the p-356wt/fosLUC reporter plasmid and with wild-type CaMKII or a truncated CaMKII 290 mutant. (B) Overexpression of CaMKII wt potentiates activation of the c-*fos* promoter by ET-1. Cells transfected with p-356wt/fosLUC and the plasmid expressing CaMKII wt (4 μg) or the vector control (pCMV) were treated with 100 nM ET-1 before lysis and measurement of luciferase activity (shaded bars). Solid bars, no addition. **, $P < 0.01$.

The Ca^{2+} ionophore ionomycin mimicked the kinetics and magnitude of c-*fos* mRNA induction by ET-1. Chelation of extracellular Ca^{2+} by EGTA, which prevented influx of extracellular Ca^{2+} but not intracellular release of Ca^{2+}, inhibited ET-1-stimulated c-*fos* mRNA induction. An antagonist of voltage-insensitive Ca^{2+} channels, SK&F 96365 (32), also blocked Ca^{2+} influx and c-*fos* mRNA induction by ET-1, providing further evidence for the importance of Ca^{2+} influx in the ET-1 c-*fos* signaling pathway. As expected (46), a dihydropyridine antagonist of voltage-gated Ca^{2+} channels, nifedipine, did not block ET-1-stimulated Ca^{2+} influx or c-*fos* mRNA induction.

We (17, 49) and others (37) have previously shown that ET-1 activates the c-*fos* promoter. Our present results demonstrate that Ca^{2+} influx mediates activation of the c-*fos* promoter in cells treated with ET-1. Transcription of a luciferase reporter gene by a −356 to +109 fragment of the c-*fos* promoter was blocked by chelation of extracellular Ca^{2+} with EGTA or by pretreatment with SK&F 96365. Using nuclear run-on assays, Pribnow et al. (37) previously showed that an increase in $[Ca^{2+}]_i$ is required for ET-1-stimulated c-*fos* transcription. Our present results confirm this finding and further demonstrate that the *cis* elements responsive to voltage-insensitive Ca^{2+}

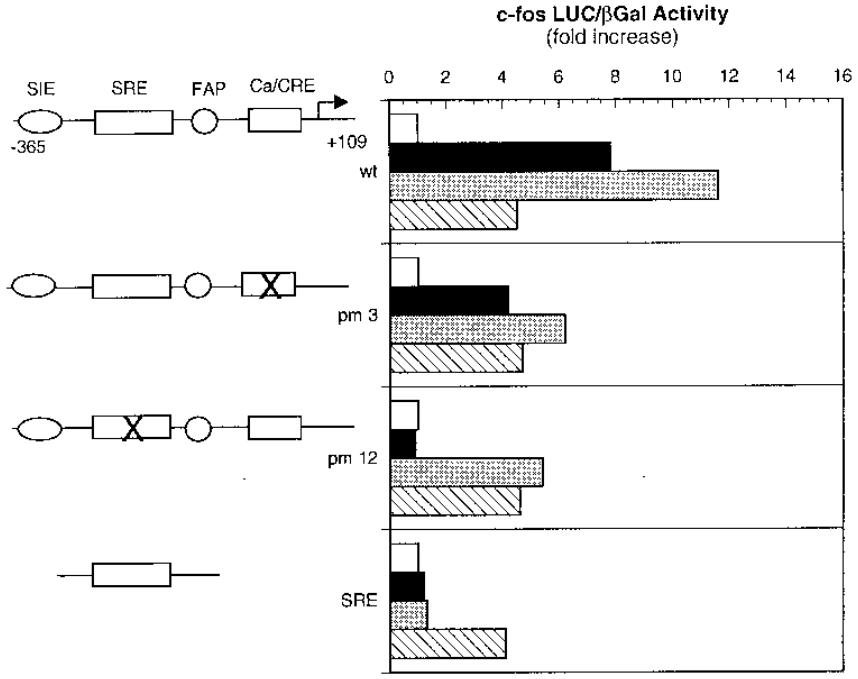

FIG. 6. The c-*fos* SRE is necessary but not sufficient for activation of the c-*fos* promoter by CaMKII 290. Cells were transfected with c-*fos* promoter LUC constructs with inactivating point mutations (pm) as indicated schematically at left. ×, point mutations in the Ca/CRE (pm3) and SRE (pm12). The SRELUC construct (bottom) contained a single-copy SRE upstream of a minimal c-*fos* promoter. Serum-starved cells were transfected with pCMV (no addition) (open bars), CaMKII 290 (solid bars), or CaMKIV 313 (shaded bars) or treated with FBS (hatched bars). The data are means from three independent experiments; error bars are omitted for clarity but were never more than 16% of the mean.

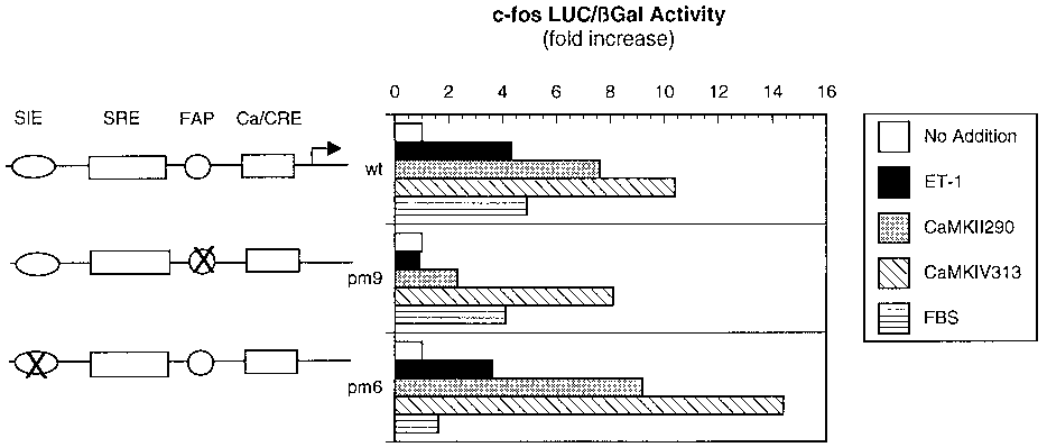

FIG. 7. The c-*fos* FAP *cis* element is required for c-*fos* promoter activation by ET-1 and CaMKII. Serum-starved cells transfected with the c-fosLUC promoter constructs indicated schematically at left (wt, pm9, and pm6) were cotransfected with plasmids expressing CaMKII 290 or CaMKIV 313. Alternatively, the cells were treated with 100 nM ET-1 or 10% FBS. Inactivating mutations (pm9) in the FAP *cis* element prevented activation of the c-*fos* promoter by ET-1 and CaMKII 290.

influx lie within DNA bp −356 to +109 of the c-*fos* promoter. The same c-*fos* genomic DNA sequences are regulated by voltage-gated Ca^{2+} channels in PC12 cells and neurons (1, 10, 33), suggesting that similar Ca^{2+}-based mechanisms might regulate gene expression following activation of voltage-insensitive and voltage-gated Ca^{2+} influx. These results argue for an important function of voltage-insensitive Ca^{2+} influx to regulate gene expression in nonexcitable cells.

CaMKs propagate ET-1 signals to the c-*fos* SRE and Ca/CRE. Having established a role for voltage-insensitive Ca^{2+} influx, we next attempted to identify Ca^{2+}-dependent effectors of c-*fos* promoter activation by ET-1. One of several mechanisms by which Ca^{2+} regulates c-*fos* gene expression is through activation of CaMKs (10, 31, 33, 41, 53). We found that mesangial cells expressed a 54-kDa CaMKII protein. A pharmacological antagonist of CaMKs, KN-93 (29), prevented activation of the c-*fos* promoter by ET-1. These results support a role for CaMKII in activation of the c-*fos* promoter by ET-1; however, KN-93 inhibits multiple isotypes of CaMKs, and it is possible that mesangial cells express CaMKs other than CaMKII that also contribute. In addition, overexpression of wild-type CaMKII potentiated the effects of ET-1, providing further evidence that CaMKII participates in signaling cascades that link ET-1 receptors to the c-*fos* promoter.

To further understand how Ca^{2+}-dependent signals regulate the c-*fos* promoter, we sought to identify the *cis* elements responsive to CaMKII. We have previously shown that activation of the c-*fos* promoter by ET-1 requires both the SRE and Ca/CRE (17, 49). Our present results suggest that CaMKII-mediated activation of the c-*fos* promoter requires the SRE, FAP, and to a lesser extent the Ca/CRE. Inactivating point mutations in the Ca/CRE only partly inhibited activation of the c-*fos* promoter by CaMKII in mesangial cells. Although the ability of Ca^{2+} influx and CaMKs to phosphorylate and activate CREB at the Ca/CRE is well established (31, 41, 53), regulation of the Ca/CRE and CREB by Ca^{2+} is cell type specific and does not appear to operate in T cells (26) or HeLa cells (8). Similarly, the Ca/CRE appears to be only partially responsible for activation of the c-*fos* promoter by CaMKII in mesangial cells. In addition, there are multiple sequences in the c-*fos* promoter that respond to cAMP (4, 9), and it is possible that these sequences are also responsive to Ca^{2+} and/or CaMKII.

We also found that CaMKII contributes to activation of the SRE by ET-1. Previous work demonstrates that the c-*fos* SRE contributes to c-*fos* transcription stimulated by NMDA type glutamate receptors (1); although not required, activation of voltage-gated Ca^{2+} channels in PC12 cells also stimulates SRE-dependent transcription that requires SRF but not ternary complex factors (33). Our present results suggest that the SRE is required but not sufficient for activation of the c-*fos* promoter by Ca^{2+} influx and CaMKII. Point mutations in the SRE (pm12) that inhibit SRF binding completely abolish stimulation of c-*fos* transcription by CaMKII. These results are consistent with a role for ET-1-stimulated Ca^{2+} influx and CaMKII in SRE activation. However, a single SRE upstream of a minimal promoter was not sufficient for activation by CaMKII 290, and other point mutant constructs with an intact SRE were similarly unresponsive to CaMKII 290 (i.e., pm3). Collectively, these results suggest that *cis* elements in addition to the SRE and Ca/CRE must be required for full activation of the c-*fos* promoter by CaMKs.

An additional *cis* element required for full activation of the c-*fos* promoter by ET-1 and CaMKII was the FAP site. The c-*fos* FAP site was a potential candidate since Ca^{2+} signaling targets this *cis* element in T cells (26). Indeed, inactivating mutations in the FAP prevented upregulation of the c-*fos* promoter by ET-1 and CaMKII 290. Interestingly, promoter activation by CaMKIV 313 was only slightly inhibited, suggesting that different isoforms of CaMKs interact differently with distinct *cis* elements in the c-*fos* promoter. Point mutations in the *cis*-inducible element had no effect on c-*fos* promoter activation by ET-1 or CaMKII 290. The specific *trans*-acting factors involved in Ca^{2+}-FAP signaling in T cells or mesangial cells have not been identified; however, our work suggests that CaMKII is probably involved in this pathway.

This ET-1-stimulated, Ca^{2+}-based pathway to the SRE is different in several important respects from the voltage-gated Ca^{2+} channel/SRE pathway in PC12 cells. For example, the voltage-gated Ca^{2+} channel/SRE pathway is independent of Ras (33), whereas we have previously shown that ET-1-stimulated SRE activity depends on Ras (17). Activation of the SRE by ET-1 (17, 49) and other G-protein-coupled receptor ligands (i.e., lysophosphatidic acid [18–20]) occurs independently of p62 ternary complex factors such as Elk-1 or Sap-1. However, unlike other G-protein-coupled receptor ligands, ET-1 utilizes Ras in this pathway (17–20). Differences in the requirement for Ras in this pathway might reflect differences in signals con-

trolled by the lysophosphatidic acid and ET-1 receptors or cell-type-specific differences in signaling. We also believe that a GTPase cascade (see references 5, 23, and 36 for reviews) is required for full activation of the SRE by ET-1 in mesangial cells. For example, we find that dominant negative mutants of RhoA also block ET-1-stimulated SRE activity (43a), a finding similar to the results of Hill et al. (20) in which dominant negative RhoA blocked activation of serum response factor by G-protein-coupled receptors. Although ET-1 might need to evoke a GTPase cascade for full activation of the SRE, this hypothesis remains to be tested.

In summary, our results favor a model in which Ca^{2+} influx contributes to a complex, interdependent network of signals whereby ET-1 regulates the c-*fos* promoter. One pathway of this network apparently involves a previously characterized mechanism requiring activation of the c-*fos* Ca/CRE by CaMKII, although effectors in addition to CaMKII might also contribute to this pathway in mesangial cells. Another pathway involves the SRE and effectors such as c-Src and Ras (17, 49), which are both activated by Ca^{2+} influx (39, 50, 58, 59). It is now apparent that CaMKII also contributes to the SRE pathway. Finally, ET-1 also activates the FAP *cis* element by mechanisms that apparently involve CaMKII. Further experiments are required to elucidate the mechanisms by which ET-1/CaMKII signals are relayed to the FAP site.

ACKNOWLEDGMENTS

We gratefully thank Michael G. Gilman, G. Stanley McKnight, Richard A. Maurer, and Kenneth Walsh for providing plasmids. We also thank William Herman for excellent technical assistance.

This work was supported by a grant from the National Institutes of Health DK-46939 and by grant-in-aid 93-1514 from the American Heart Association.

REFERENCES

1. **Bading, H., D. D. Ginty, and M. E. Greenberg.** 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science **260:**181–186.
2. **Baynash, A. G., K. Hosoda, A. Giaid, J. A. Richardson, N. Emoto, R. E. Hammer, and M. Yanagisawa.** 1994. Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell **79:**1277–1285.
3. **Benigni, A., C. Zoja, D. Corna, S. Orisio, L. Longaretti, T. Bertani, and G. Remuzzi.** 1993. A specific endothelin subtype A receptor antagonist protects against functional and structural injury in a rat model of renal disease progression. Kidney Int. **44:**440–444.
4. **Berkowitz, L. A., K. T. Riabowol, and M. Z. Gilman.** 1989. Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-*fos* promoter. Mol. Cell. Biol. **9:**4272–4281.
5. **Chant, J., and L. Stowers.** 1995. GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. Cell **81:**1–4.
6. **Curran, T., M. B. Gordon, K. L. Rubino, and L. C. Sambucetti.** 1987. Isolation and characterization of the c-fos(rat) cDNA and analysis of post-translational modification in vitro. Oncogene **2:**79–84.
7. **Douglas, S. A., C. Louden, L. M. Vickery-Clark, B. L. Storer, T. Hart, G. Z. Feuerstein, J. D. Elliott, and E. H. Ohlstein.** 1994. A role for endogenous endothelin-1 in neointimal formation after rat carotid artery balloon angioplasty. Circ. Res. **75:**190–197.
8. **Fisch, T. M., R. Prywes, and R. G. Roeder.** 1987. c-*fos* sequences necessary for basal expression and induction by epidermal growth factor, 12-*O*-tetradecanoyl phorbol-13-acetate, and the calcium ionophore. Mol. Cell. Biol. **7:**3490–3502.
9. **Fisch, T. M., R. Prywes, M. C. Simon, and R. G. Roeder.** 1989. Multiple sequence elements in the c-fos promoter mediate induction by cAMP. Genes Dev. **3:**198–211.
10. **Ghosh, A., and M. E. Greenberg.** 1995. Calcium signaling in neurons: molecular mechansisms and cellular consequences. Science **268:**239–247.
11. **Giad, A., M. Yanagisawa, D. Langleben, R. P. Michel, R. Levy, H. Shennib, S. Kimura, T. Masaki, W. P. Duguid, and D. J. Stewart.** 1993. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. N. Engl. J. Med. **328:**1732–1739.
12. **Gilman, M. Z.** 1988. The c-fos serum response element responds to protein kinase C-dependent and -independent signals but not to cyclic AMP. Genes Dev. **2:**394–402.
13. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6:**4305–4316.
14. **Goto, K., Y. Kasuya, N. Matsuki, Y. Takuwa, H. Kurihara, T. Ishikawa, S. Kiumura, M. Yanagisawa, and T. Masaki.** 1989. Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca2+ channel in vascular smooth muscle. Proc. Natl. Acad. Sci. USA **86:**3915–3918.
15. **Graham, R., and M. Z. Gilman.** 1991. Distinct protein targets for signals acting at the c-fos serum response element. Science **251:**189–192.
16. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
17. **Herman, W. H., and M. S. Simonson.** 1995. Nuclear signaling by endothelin-1: a Ras pathway for activation of the c-fos serum response element. J. Biol. Chem. **270:**11654–11661.
18. **Hill, C. S., and R. Treisman.** 1995. Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. EMBO J. **14:**5037–5047.
19. **Hill, C. S., J. Wynne, and R. Treisman.** 1994. Serum-regulated transcription by serum response factor (SRF): a novel role for the DNA binding domain. EMBO J. **13:**5421–5432.
20. **Hill, S. H., J. Wynne, and R. Treisman.** 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell **81:**1159–1170.
21. **Hosoda, K., R. E. Hammer, J. A. Richardson, A. G. Baynash, J. C. Cheung, A. Giaid, and M. Yanagisawa.** 1994. Targeted and natural (Piebald-Lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell **79:**1267–1276.
22. **Huang, S., M. S. Simonson, and M. J. Dunn.** 1993. Manidipine inhibits endothelin-1-induced [Ca2+]i signaling, but potentiates endothelin's effect of c-fos and c-jun induction in vascular smooth muscle and glomerular mesangial cells. Am. Heart J. **125:**589–597.
23. **Khosravi-Far, R., P. A. Solski, G. J. Clark, M. S. Kinch, and C. J. Der.** 1995. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol. Cell. Biol. **15:**6443–6453.
24. **Kurihara, Y., H. Kurihara, H. Oda, K. Maemura, R. Nagai, T. Ishikawa, and Y. Yazaki.** 1995. Aortic arch malformations and ventricular septal defect in mice deficient in endothelin-1. J. Clin. Invest. **96:**293–300.
25. **Kurihara, Y., H. Kurihara, H. Suzuki, T. Kodama, K. Maemura, R. Nagai, H. Oda, T. Kuwaki, W.-H. Cao, N. Kamada, K. Jishage, Y. Ouchi, S. Azuma, Y. Toyoda, T. Ishikawa, M. Kumada, and Y. Yazaki.** 1994. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. Nature (London) **368:**703–710.
26. **Lee, G., and M. Gilman.** 1994. Dual modes of control of c-*fos* mRNA induction by intracellular calcium in T cells. Mol. Cell. Biol. **14:**4579–4587.
27. **Lerman, A., B. S. Edwards, J. W. Hallett, D. M. Heublein, S. M. Sandberg, and J. C. Burnett.** 1991. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. N. Engl. J. Med. **325:**997–1001.
28. **Levin, E. R.** 1995. Endothelins. N. Engl. J. Med. **333:**356–363.
29. **Mamiya, N., J. R. Goldenring, Y. Tsunoda, I. M. Modlin, K. Yasui, N. Usuda, T. Ishikawa, A. Natsume, and H. Hidaka.** 1993. Inhbition of acid secretion in gastric parietal cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-93. Biochem. Biophys. Res. Commun. **195:**608–615.
30. **Masaki, T.** 1993. Endothelins: homeostatic and compensatory actions in the circulatory and endocrine systems. Endocr. Rev. **14:**256–268.
31. **Matthews, R. P., C. R. Guthrie, L. M. Wailes, X. Zhao, A. R. Means, and G. S. McKnight.** 1994. Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. Mol. Cell. Biol. **14:**6107–6116.
32. **Merritt, J. E., W. P. Armstrong, C. D. Benham, T. J. Hallam, R. Jacob, A. Jaxa-Chamiec, B. K. Leigh, S. A. McCarthy, K. E. Moores, and T. J. Rink.** 1990. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem. J. **271:**515–522.
33. **Miranti, C. K., D. D. Ginty, G. Huang, T. Chatila, and M. E. Greenberg.** 1995. Calcium activates serum response factor-dependent transcription by a Ras- and Elk-1-independent mechanism that involves a Ca^{2+}/calmodulin-dependent kinase. Mol. Cell. Biol. **15:**3672–3684.
34. **Muldoon, L. L., D. Pribnow, K. D. Roland, and B. E. Magun.** 1990. Endothelin-1 stimulates DNA synthesis and anchorage-independent growth of rat-1 fibroblasts through a protein kinase C-dependent mechanism. Cell Regul. **1:**379–390.
35. **Naitoh, T., T. Toyooka, and T. Sugimoto.** 1990. An endogenous Ca2+ channel agonist, endothelin-1, does not directly activate partially purified dihydropyridine-sensitive Ca2+ channel from cardiac muscle in a reconstituted system. Biochem. Biophys. Res. Commun. **171:**1205–1210.
36. **Olson, M. F., A. Ashworth, and A. Hall.** 1995. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science **269:**1270–1272.
37. **Pribnow, D., L. L. Muldoon, M. Fajardo, L. Theodor, L. S. Chen, and B. E. Magun.** 1992. Endothelin induces transcription of *fos*/*jun* family genes: a

prominent role for calcium ion. Mol. Endocrinol. **6:**1003–1012.
38. **Robertson, L. M., T. K. Kerppola, M. Vendrell, C. Bocchiaro, J. I. Morgan, and T. Curran.** 1995. Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. Neuron **14:**241–252.
39. **Rosen, L. B., D. D. Ginty, M. J. Weber, and M. E. Greenberg.** 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron **12:**1207–1221.
40. **Santoro, I. M., and K. Walsh.** 1991. Natural and synthetic DNA elements with the CArG motif differ in expression and protein-binding properties. Mol. Cell. Biol. **11:**6296–6305.
41. **Sheng, M., G. McFadden, and M. E. Greenberg.** 1990. Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron **4:**571–582.
42. **Simonson, M. S.** 1993. Endothelins: multifunctional renal peptides. Physiol. Rev. **73:**375–411.
43. **Simonson, M. S.** 1994. Endothelin peptides and compensatory growth of renal cells. Curr. Opin. Nephrol. Hypertens. **3:**73–85.
43a. **Simonson, M. S., et al.** Unpublished data.
44. **Simonson, M. S., and M. J. Dunn.** 1990. Cellular signaling by peptides of the endothelin gene family. FASEB J. **4:**2989–3000.
45. **Simonson, M. S., and M. J. Dunn.** 1990. Eicosanoid biochemistry in cultured glomerular mesangial cells. Methods Enzymol. **187:**544–553.
46. **Simonson, M. S., and M. J. Dunn.** 1991. Ca2+ signaling by distinct endothelin peptides in glomerular mesangial cells. Exp. Cell Res. **192:**148–156.
47. **Simonson, M. S., and W. H. Herman.** 1993. Protein kinase C and protein tyrosine kinase activity contribute to mitogenic signaling by endothelin-1: cross-talk between G protein-coupled receptors and $p60^{c\text{-}src}$. J. Biol. Chem. **268:**9347–9357.
48. **Simonson, M. S., and A. Rooney.** 1994. Characterization of endothelin receptors in mesangial cells: evidence for two functionally distinct endothelin binding sites. Mol. Pharmacol. **46:**41–50.
49. **Simonson, M. S., Y. Wang, and W. H. Herman.** 1996. Nuclear signaling by endothelin-1 requires Src protein tyrosine kinases. J. Biol. Chem. **271:**77–82.
50. **Simonson, M. S., Y. Wang, and W. H. Herman.** 1996. Ca2+ channels mediate protein tyrosine kinase activation by endothelin-1. Am. J. Physiol. **270:**F790–F797.
51. **Simonson, M. S., S. Wann, P. Mene', G. Dubyak, M. Kester, Y. Nakazato, J. R. Sedor, and M. J. Dunn.** 1989. Endothelin stimulates phospholipase C, Na+/H+ exchange, c-fos expression, and mitogenesis in rat mesangial cells. J. Clin. Invest. **83:**708–712.
52. **Simpson, A. W., and C. C. Ashley.** 1989. Endothelin evoked Ca2+ transients and oscillations in A10 vascular smooth muscle cells. Biochem. Biophys. Res. Commun. **163:**1223–1229.
53. **Sun, P., H. Enslen, P. S. Myung, and R. A. Maurer.** 1994. Differential activation of CREB by Ca2+/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. **8:**2527–2539.
54. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2: independent activation domains induce Oct-2 phosphorylation. Cell **60:**375–386.
55. **Yamashita, J.-I., M. Ogawa, H. Egami, S. Matsuo, H. Kiyohara, K. Inada, S.-I. Yamashita, and S. Fujita.** 1992. Abundant expression of immunoreactive endothelin 1 in mammary phyllodes tumor: possible paracrine role of endothelin-1 in the growth of stromal cells in phyllodes tumor. Cancer Res. **52:**4046–4049.
56. **Yanagisawa, M.** 1994. The endothelin system: a new target for therapeutic intervention. Circulation **89:**1320–1322.
57. **Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tombe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki.** 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature (London) **332:**411–415.
58. **Zhao, Y., M. Sudol, H. Hanafusa, and J. Krueger.** 1992. Increased tyrosine kinase activity of c-Src during calcium-induced keratinocyte differentiation. Proc. Natl. Acad. Sci. USA **89:**8298–8302.
59. **Zhao, Y., H. Uyttendaele, J. Krueger, M. Sudol, and H. Hanafusa.** 1993. Inactivation of c-Yes tyrosine kinase by elevation of intracellular Ca^{2+} levels. Mol. Cell. Biol. **13:**7507–7514.