Collaborative Roles for c-Jun N-terminal Kinase, c-Jun, Serum Response Factor, and Sp1 in Calcium-regulated Myocardial Gene Expression

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Electrical stimulation of contractions (pacing) of primary neonatal rat ventricular myocytes increases intracellular calcium and activates a hypertrophic growth program that includes expression of the cardiac-specific gene, atrial natriuretic factor (ANF). To investigate the mechanism whereby pacing increases ANF, pacing was tested for its ability to regulate mitogen-activated protein kinase family members, ANF promoter activity, and the trans-activation domain of the transcription factor, Sp1. Pacing and the calcium channel agonist BAYK 8644 activated c-Jun N-terminal kinase (JNK) but not extracellular signal-regulated kinase. Pacing stimulated ANF-promoter activity approximately 10-fold. Furthermore, transfection with an expression vector for c-Jun, a substrate for JNK, also activated the ANF promoter, and the combination of pacing and c-Jun was synergistic, with consistent with roles for JNK and c-Jun in calcium-activated ANF expression. Proximal serum response factor and Sp1 binding sites were required for the effects of pacing or c-Jun on the ANF promoter. Pacing and c-Jun activated a GAL4-Sp1 fusion protein by 3- and 12-fold, respectively, whereas the two stimuli together activated GAL4-Sp1 synergistically, similar to their effect on the ANF promoter. Transfection with an expression vector for c-Fos inhibited the effects of c-Jun, suggesting that c-Jun acts independently of AP-1. These results demonstrate an interaction between c-Jun and Sp1 and are consistent with a novel mechanism of calcium-mediated transactivation involving the collaborative actions of JNK, c-Jun, serum response factor, and Sp1.

Increased cytoplasmic calcium has been demonstrated to up-regulate gene transcription in a variety of excitable cell types (1–6). Following depolarization-induced calcium entry, Ca\(^{2+}\)/calmodulin kinase activation leads to the phosphorylation of CAMP response element-binding protein (7, 8), C/EBP (9), ATF-1 (10), and serum response factor (SRF)\(^1\) (11), leading to enhanced transcription. Calcium influx has also been shown to activate the tyrosine kinase, PYK2 (12), as well as the small GTPase, p21 Ras, and extracellular signal-regulated kinase (ERK) (13, 14), a MAPK family member that has been widely implicated in controlling gene expression associated with mitogenesis. An additional member of the MAPK family, c-Jun N-terminal kinase (JNK) is also calcium-activated in some cell types (15–18), which further implicates the potential participation of the proto-oncogene product c-Jun in pathways involving calcium-stimulated gene expression. Although c-Jun is usually thought to confer transcriptional enhancement through AP-1 elements (19, 20), c-Jun has also been demonstrated to augment transcription from constructs containing putative binding sites for SRF; the mechanism of this activation does not appear to require the binding of Jun to SRF or the binding of Jun to the serum response element (SRE) (21). Thus, it is conceivable that calcium activation of JNK could lead to enhanced transcription of genes containing AP-1 or SRE regulatory elements.

Cardiac myocytes exhibit transient increases in cytoplasmic calcium that serve as the driving force behind contraction (22, 23). Although the role of calcium in mediating cardiac myocyte contraction is well characterized, the potential participation of calcium in regulating cardiac gene expression has received less attention. Interestingly, cultured neonatal ventricular myocytes exhibit hypertrophic cellular growth that is dependent upon the contractile activity of the cells; cell size is increased in spontaneously contracting cell cultures (24, 25) and by the electrical pacing of contractions (26–28). Furthermore, atrial natriuretic factor (ANF), a cardiac-specific gene that serves as a marker of the hypertrophic phenotype (29, 30), is strongly up-regulated by pacing of contractions (26). Although the cis-elements in the ANF gene responsible for induction in response to pacing have not been elucidated, promoter mapping studies have shown that a promoter-proximal SRE and an Sp1-like element in the ANF 5'-flanking sequence are important for ANF induction in response to other growth promoters, such as the \(\alpha_1\)-adrenergic receptor agonist phenylephrine (31, 32). It is possible that even though pacing and \(\alpha_1\)-adrenergic receptor activation represent quite different modes of stimuli, they may nonetheless converge on common signaling pathways to confer ANF induction.

The present study was undertaken to evaluate the signaling mechanisms by which pacing leads to ANF promoter activation in cardiac myocytes. The findings support the view that induction by pacing involves the participation of members of the JNK, c-Jun, serum response factor, and Sp1 pathways, converging on an ANF promoter-proximal serum response element and an Sp1-like element. To our knowledge, this is the first...
report that relates increases in intracellular calcium with transcriptional induction via c-Jun and Sp1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Neonatal rat ventricular myocytes were dissociated as described previously (31, 33). In brief, 1–4-day-old rat hearts were dissected, and the apical one-third of the ventricle was removed, minced, and subjected to repeated rounds of trypsinization. For transfection, the dissociated cells were resuspended in minimal medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12; Life Technologies, Inc.) containing 1 mg/ml bovine serum albumin and BSA, electroporated in a Bio-Rad (Hercules) gene pulser (600 or 700 V, 25 microfarad, 100 ohm, 0.2-cm gap cuvette), and plated onto fibronectin-coated wells in the presence of 10% fetal bovine serum. DNA concentrations used in the transfections were 10 μg/well for reporter genes based on the ANF 5′-flanking sequence (described below) or 5 μg/well for the GAL4-sensitive reporter, pG5E1bluc; 3 μg/well CMV-β-gal (CLONTECH), a cytomegalovirus-driven expression vector for the β-galactosidase, was also routinely included. Phenylphosphate (10 μM) and propranolol (1 μM) were also present in the plating medium of transfected myocytes that improved plating efficiency of the cells. After 18–20 h, the cells were extensively rinsed and refed with minimal medium (without serum, phenylphosphate, or propranolol). Cells were then subjected to the electrical pacing of contractions (3 Hz) for 24–72 h utilizing the apparatus previously described (36, 39). After the experimental treatments, the cells were harvested and assayed for luciferase and β-galactosidase activity (35, 36).

ERK and JNK Assays—Myocytes plated at a density of 2 million cells/35-mm well were switched to serum-free medium for 24 h before treatment. Agonists were prepared in serum-free medium and added for the times indicated. Treatments were stopped by washing in cold phosphate-buffered saline, and the cells were scraped in 1 ml of buffer C (10 mM Tris/HCl, 5 mM EDTA, 50 mM NaF, 50 mM NaCl, 1% Triton X-100, 0.1% fatty acid-free bovine serum albumin, 20 μg/ml aprotinin, and 2 mM Na2VO4, pH 7.4 (37). Lysates were immunoprecipitated (for each sample, lysates from two wells were pooled) via either anti-ERK1 or anti-JNK-1 antibodies (Santa Cruz) and protein A-coupled Sepharose (Pharmacia Biotech Inc.) in phosphate-buffered saline. The ERK-1 antibody-Sepharose pellets were washed once in buffer C and then washed two times in Trius-buffered saline containing 1 mM Na2VO4 and 1 mM phenylmethylsulfonyl fluoride. Pellets were incubated in 30 μl of kinase buffer (30 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM MgCl2, 20 mM ATP, 6 μCi of [γ-32P]ATP) with 2 μg of myelin basic protein as a substrate for 20 min at 30 °C. JNK1 antibody-Sepharose pellets were washed twice in buffer C, washed twice in buffer D (50 mM Tris/HCl, 0.1 mM EDTA, 0.5 mM Na2VO4, and 0.1% (v/v) 2-mercaptoethanol, pH 8.0), and incubated for 20 min at 30 °C in 30 μl of kinase buffer (20 mM HEPES, pH 7.4, 20 mM MgCl2, 20 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na2VO4, 20 μM ATP, 10 μCi of [γ-32P]ATP) with 1 μg of GST-c-Jun (1–79) as a substrate (37). GST-c-Jun (1–79) was expressed in Escherichia coli through use of a pGEX-2T-c-Jun (1–79) plasmid (supplied by Dr. Michael Karin, University of California, San Diego) and isolated by standard techniques. Kinase reactions were stopped by boiling 4 μl of each sample for 5 min, the Sepharose pellets were centrifuged at 14,000 rpm, and the supernatants were run out on either 12% (ERK) or 15% (JNK) polyacrylamide gels. Incorporation of 32P into the bands corresponding to myelin basic protein or c-Jun (1–79) was quantified by Phosphor Imager (Molecular Dynamics) analysis. For sequential immunoprecipitations, the cell lysates were subjected to immunoprecipitation with the anti-JNK1 antibody followed by the anti-ERK1 antibody after which the pellets were subjected to the individual in vitro kinase reactions exactly as described above.

Plasmid Constructs—Preparation of the truncation mutants of chimeric rat ANF promoter/luciferase reporter constructs ANF-3003GL, ANF-638GL, ANF-122GL, ANF-109GL, and ANF-65GL has been previously reported (31). ANF-638GL/C114GL and ANF-638GL/C69GL were prepared with either pG2L or pGL3 (Promega) as the reporter plasmid, using previously published protocols (31). The ANF-65/3X SREGL construct was prepared beginning with the following oligonucleotide pair, which spans the ANF 5′-flanking sequence from positions –134 to –99: (+) 5′-ccggcttccggtgactgattcataaaaaagggcatctctctg3′ and (−) 5′-ctagcagagattctctttttttaaagttatcagttccggcaagc-3′. The uppercase nucleotides are native ANF sequences, and the lowercase nucleotides were added to provide PsPlI and NHeI ends for cloning purposes. Following phosphorylation and hybridization, the ANF –134/–99 oligonucleotide pairs were ligated to form a 3X concanomer, cloned into pBlueScript (Stratagene, La Jolla CA), and then subcloned into the multiple cloning site in ANF-65GL to create ANF-65/3X SREGL. This construct possesses three copies of ANF –134/–99 in alternating orientations driving the minimal ANF promoter, i.e. ANF-65/3X SREGL. The structures of all plasmid constructs were verified by dideoxy sequencing.

The plasmids pG5E1bluc (GAL4-sensitive luciferase reporter construct) and GAL4-Sp1 were obtained from Drs. Roger J. Davis and Michael R. Green (University of Massachusetts) and Dr. John Y.-J. Shyy (University of California, San Diego). GAL4-DBD, which contains only the DNA binding domain of GAL4, was obtained from Dr. Michael Karin (University of California, San Diego). The Rous sarcoma virus promoter-driven expression plasmids pRSV-c-Jun and pSV-fos were obtained from Dr. Kathleen McGuire (San Diego State University) and Dr. Michael Karin.

RESULTS

Agents that stimulate hypertrophic growth and ANF expression in cultured neonatal ventricular myocytes often also activate ERK (38, 39), a MAPK family member implicated in transcriptional up-regulation through SRF-dependent pathways (40, 41). To determine if the electrical pacing of contraction activates ERK, myocytes were paced, the endogenous ERK was immunoprecipitated, and in vitro kinase reactions were performed utilizing myelin basic protein as a substrate. Pacing did not appear to activate ERK at any time ranging from 5 to 60 min (Fig. 1A), whereas the phorbol ester phorbol 12,13-dibutyrate (PDBu) strongly activated ERK activity, serving as a positive control. In contrast, pacing was found to activate the related MAPK family member JNK (Fig. 1B). Maximum JNK activation was observed within 60 min of the initiation of pacing (Fig. 1C), and the overall time course was similar to the activation of JNK displayed by endothelin-stimulated cardiac myocytes (37). Over a total of six experiments, pacing at 3 Hz for 60 min activated JNK an average of 2.3 ± 0.6-fold (mean ± S.E.). The selective activation of JNK by treatments that raise intracellular calcium was also demonstrated through use of a sequential immunoprecipitation procedure, with JNK and ERK immunoprecipitated from the same cell lysates. Like pacing, BAYK 8644 or KCl + BAYK 8644 increase calcium influx and intracellular calcium concentration in cardiac myocytes (34), and these treatments activated JNK with no effect on ERK (Fig. 2). These results are consistent with the hypothesis that intracellular calcium may serve as a positive regulator of JNK activity in cardiac myocytes.

Because pacing is a known activator of ANF transcription (26), ANF promoter mapping studies were undertaken to assess how pace-induced JNK activation could lead to increased ANF expression. Cultured neonatal ventricular myocytes were transfected with a construct containing 3003 base pairs of the rat ANF 5′-flanking sequence (FS) ligated to the firefly luciferase (ANF-3003GL). Pacing at 3 Hz elicited an approximately linear increase in luciferase expression over a 72-h time course (Fig. 3A), which is consistent with the previously reported effects of pacing on ANF peptide and mRNA levels (26). To identify regions within the 5′-FS of ANF that confer pace inducibility, myocytes were transfected with a series of ANF promoter truncations, paced for 48 h, and tested for the production of luciferase activity. ANF-122GL, which contains just 122 base pairs of the ANF 5′-FS, was induced by pacing to the same degree as ANF-3003GL; truncations utilizing less of the ANF 5′-FS, however, were less sensitive to pacing, suggesting that responsiveness to pacing is conferred by sequences within the 122 base pairs of the ANF 5′-FS, which is consistent with the previously reported effects of pacing on ANF peptide and mRNA levels (26). To identify regions within the 5′-FS of ANF that confer pace inducibility, myocytes were transfected with a series of ANF promoter truncations, paced for 48 h, and tested for the production of luciferase activity. ANF-122GL, which contains just 122 base pairs of the ANF 5′-FS, was induced by pacing to the same degree as ANF-3003GL; truncations utilizing less of the ANF 5′-FS, however, were less sensitive to pacing, suggesting that responsiveness to pacing is conferred by sequences within the first 122 base pairs of the ANF promoter (Fig. 3B).

Previous studies have shown that a promoter-proximal SRE and an Sp1-like site, located at −114 and −70, respectively, in the ANF 5′-FS, are critical for ANF induction by α1-adrnergic
agonists (31, 32). To evaluate the roles of SRE/Sp1 and Sp1/SRF on promoter activity in the context of 638 base pairs of the ANF 5′-FS, reporter constructs possessing clustered point mutations (described in Fig. 4A) were tested. Pace inducibility was considerably decreased when either ANF-638/C114GL or ANF-655/3X SREGL, a construct comprised of a concatamer of three SRE/C114s ligated to an ANF minimal promoter (ANF-65Luc), was used as the test construct (Fig. 4B). This induction was inhibited by the calcium channel blocker nifedipine (data not shown), a compound that blocks both electrically stimulated calcium transients and pacing induction of ANF mRNA (26). These results suggest that SRE/C114 may confer pace inducibility to a minimal ANF promoter, confirming a linkage between increased intracellular calcium and SRF-enhanced transcription.

Because inducibility of the ANF promoter by pacing appeared to involve an SRE and an Sp1 element and because pacing activated JNK, studies were undertaken to establish a connection between the JNK-c-Jun pathway and SRE/Sp1 and Sp1/SRF. A well characterized function of JNK is the phosphorylation of c-Jun in a manner that leads to the activation of Jun as a transcription factor (19). Consistent with the involvement of the JNK-c-Jun pathway in pace-induced ANF expression, overexpression of c-Jun activated gene expression...
Effects of pacing on ANF promoter activation. Myocytes were transfected with constructs possessing the 5′-flanking sequence of the rat ANF gene ligated to firefly luciferase, plated for 24 h in serum and phenylephrine containing medium, and then switched to minimal medium prior to the initiation of pacing (3 Hz). A, time course of reporter expression from ANF-3003GL. ○, unpaced; □, paced. B, effects of pacing on reporter expression from ANF-3003GL as well as various truncated ANF/luciferase constructs. Pacing was for 48 h. For both A and B, data values represent the means ± S.E. for n = 3 cultures and are representative of two or three separate experiments.

from ANF-638GL (Fig. 4C) in a manner similar to pacing. This result was consistent with findings using the 5′-flanking sequence of the human ANF gene in rat cardiac myocytes (42). Moreover, inducibility by c-Jun was greatly decreased when cells were transfected with ANF-638/C114GL or ANF-638/C69GL (Fig. 4C), which is consistent with a collaborative arrangement between c-Jun and these two elements in the ANF gene. Also, c-Jun overexpression strongly activated reporter production from ANF-65/3X SREGL (Fig. 4C), further implicating a functional interaction between SRF and c-Jun.

Because c-Jun cotransfection enhanced ANF promoter activity, it is possible that increasing JNK via pacing of contractions might have a further, perhaps synergistic effect with c-Jun, as the ectopically expressed c-Jun would be a potential substrate for JNK. Consistent with this idea, the combination of pacing and c-Jun resulted in a 76-fold induction of ANF promoter activity, compared with about 16- and 26-fold induction by pacing or c-Jun alone, respectively (Fig. 4D). This result not only emphasizes the potential importance of the JNK-c-Jun pathway in regulating ANF expression but also suggests the possibility that pace activation of JNK might amplify ANF expression via other pathways that increase c-Jun expression in cardiac myocytes.

Because the mutation analyses suggested that Sp1 or a closely related protein might participate in pacing- and c-Jun-inducibility of ANF, it was of interest to evaluate whether either of these treatments could augment Sp1-enhanced transcription. Accordingly, an Sp1-based trans-activation assay was employed that utilized a plasmid (pG5E1bluc) encoding luciferase ligated to a 5× concatenator of a GAL4-binding cis-element and plasmids encoding either the DNA binding domain of GAL4 alone (GAL4-DBD) or a plasmid (GAL4-Sp1) encoding a chimeric protein comprised of the “A” trans-activation domain of Sp1 ligated to the DNA binding domain of GAL4 (43). c-Jun had no effect on luciferase expression from pG5E1bluc plus GAL4-DBD (Fig. 5A); this is consistent with the fact that there are no AP1 elements in the GAL4 binding sites (44) or backbone sequences of pG5E1bluc and the lack of a functional trans-activation domain in GAL4-DBD. Cotransfection of GAL4-Sp1 with pG5E1bluc resulted in increased luciferase expression, suggesting that the trans-activation domain of Sp1 may be slightly activated by endogenous factors in the control cardiac myocytes. Cotransfection with c-Jun, however, led to a further marked increase in luciferase expression over the level obtained with just the pG5E1bluc and GAL4-Sp1 constructs (Fig. 5A), suggesting that there may be functional interaction between c-Jun and trans-activation domain A of Sp1. For six separate experiments, including the experiments illustrated in Fig. 5, 5 µg/well c-Jun increased luciferase expression from myocytes cotransfected with the pG5E1bluc and GAL4-Sp1 constructs by an average of 12-fold (a range of 6-30-fold).

The GAL4-Sp1 system was also used to assess whether the supra-additive effects of pacing and c-Jun on ANF reporter activity could potentially be mediated by Sp1. In the experiment shown (Fig. 5B), pacing alone stimulated Sp1-enhanced transcription by about 2.6-fold, and c-Jun overexpression led to an approximate 6-fold enhancement; the combined treatment of c-Jun plus pacing resulted in an approximate 30-fold increase in Sp1-enhanced transcription, a synergistic profile similar to the effect of these treatments on gene expression from ANF638-luc (see above). Unlike c-Jun, c-Fos did not increase Sp1-enhanced transcription when tested at a relatively low concentration (0.1 µg/well in the transfection mixture) or at an input concentration identical to that used in the above experiments for c-Jun (5 µg/well) (Fig. 5C).

To directly compare the effects of c-Jun on the ANF promoter and Sp1, the dose-response relationships for c-Jun on luciferase expression from these reporter systems were determined utilizing myocytes that were paced to contract. For both reporters, increasing the input concentration of c-Jun increased luciferase expression in an almost linear fashion, with slight increases obtained at the very lowest concentration and little saturation of the effect, even at inputs of 10 µg of c-Jun construct/well (Fig. 6A). Because c-Jun could potentially be increasing gene expression in this system via heterodimerization with c-Fos to form AP-1 complexes, additional experiments were performed that utilized an intermediate concentration of c-Jun (5 µg/well) and increasing amounts of c-Fos. The lowest concentration of c-Fos tested (0.1 µg/well) had no effect on expression from the ANF-638 luc reporter and a slight (approximately 30%) stimulatory effect on the GAL4-Sp1 reporter (Fig. 6B). At higher doses, c-Fos was inhibitory for both systems, particularly when the input concentration was equimolar with the cotransfected c-Jun expression plasmid (5 µg/well). These results seem inconsistent with the hypothesis that c-Jun stimulates expression from ANF638-luc and from the Sp1 trans-activation sys-

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2 Transfection with 5 µg/well pRSV-c-jun consistently activated β-galactosidase expression from CMV-β-gal an average of 2-fold, an effect that has previously been noted by others (21). Therefore, results for experiments utilizing c-Jun or the combination of pacing plus c-Jun as stimuli were not normalized to β-galactosidase expression. Separate experiments in our laboratory have established that transfection efficiencies do not routinely vary by more than 20%.
tem via AP-1 complexes featuring c-Fos; instead, the stimulatory effects of c-Jun may occur via homodimer formation or dimerization with an as yet undetermined partner in the cardiac myocytes.

**DISCUSSION**

In the present study a model system featuring the electrical pacing of contractions was used to explore the relationship between contractile calcium transients and gene expression in cultured cardiac myocytes. The results suggest a mechanism for calcium-regulated gene expression that involves the activation of the MAPK family member JNK, the proto-oncogene product c-Jun, and the transcriptional regulators SRF and Sp1. Pacing was found to activate JNK, but not ERK, as did other treatments known to elicit calcium influx into cardiac myocytes, including the calcium channel activator BAYK 8644 and the combination of BAYK 8644 and the depolarizing agent KCl. Pacing of contractions was found to activate gene expression from ANF promoter/luciferase reporter constructs, and this induction was sensitive to truncations of the promoter that removed a proximal SRF binding site (SRE/−114) and to cluster mutations at the proximal SRE/−114 and Sp1 binding sites in the ANF promoter. Although not investigated in the present study, others have observed that electrical stimulation of contractions increases the expression of c-Jun and Jun-B in this cell type. The cotransfection of an expression plasmid for c-Jun, which is a substrate for JNK, elicited gene expression from the ANF promoter in a manner similar to pacing; c-Jun-induced gene expression was also inhibited by mutation of the SRF and Sp1 binding sites. Furthermore, both pacing and c-Jun expression activated luciferase expression in cells that were transfected with constructs designed to test for activation of the Sp1 trans-activation domain, and the combination of pacing and c-Jun, together, exhibited a synergism on Sp1-mediated transcription that was similar to their synergistic effect on the ANF promoter. To our knowledge, this is the first report to suggest a link between calcium transients and Sp1 activation and a potential interaction between Sp1 and c-Jun.

There have been several previous studies suggesting a role for JNK in cardiac growth responses and ANF expression. α1-Adrenergic agonists and endothelin activate both JNK as well as ERK in cultured neonatal ventricular myocytes (37, 38). Constitutively active MEKK1, an upstream regulator of the JNK pathway, has also been found to increase cell size and to increase expression from the promoters for the ANF, β-myosin heavy chain, and skeletal α-actin genes (45). Interestingly, mechanical stretch of cultured neonatal ventricular myocytes also elicits the development of the hypertrophic phenotype and ANF expression and is associated with the activation of JNK to a level consistent with the activation of JNK elicited by pacing (46).

The role of ERK activation in the generation of the hypertrophic response is not clearly understood, because results obtained from different laboratories regarding ERK activation

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**FIG. 4. Comparison of pace- and c-Jun-induced gene expression from the rat ANF 5′-flanking sequence.** A, the diagram of the ANF 5′-flanking sequence luciferase reporter constructs. The location and sequences corresponding to the SRE and Sp1 binding sites are shown, as are the cluster mutations denoted 638/114 and 638/69 that were prepared in the context of native ANF −638 to +65 base pair sequence. B, effects of pacing on luciferase expression from the native ANF 5′-flanking sequence (denoted 638), the cluster mutation constructs 638/114 and 638/69, and from the concatameric construct ANF-65/3X SREGL (denoted 3XSRE). C, effects of c-Jun on reporter expression from the native ANF 5′-flanking sequence, the cluster mutation constructs 638/114 and 638/69, and the concatameric construct ANF-65/3X SREGL. D, effects of the combination of pacing and c-Jun on the ANF 638 reporter construct. For B–D, data for the ANF 638 and mutant constructs were normalized to the maximal response obtained with ANF 638; the data obtained for ANF-65/3X SREGL were normalized to the maximal responses obtained for ANF-65/3X SREGL. Cells were harvested after 72 h in minimal medium. Where indicated, pacing was at 3 Hz and pRSV-c-jun was cotransfected at 5 μg/well. Each bar represents the mean ± S.E., for n = 3.
and gene expression in cardiac myocytes are difficult to completely reconcile. For example, down-regulation of both the ERK1 and ERK2 isoform expression in neonatal ventricular myocytes via antisense oligonucleotides inhibits \( \alpha_1 \)-adrenergic-induced increases in cell size and ANF expression (47), suggesting a crucial role for ERK in the development of the hypertrophic response. The muscarinic receptor agonist, carbachol, and the purinergic agent, ATP, also activate ERK1 and ERK2 in a manner similar to \( \alpha_1 \)-adrenergic stimulation. However, neither carbachol nor ATP increase cell size or ANF expression (48). In fact, ATP has been shown to inhibit adrenergic stimulation of hypertrophy (49). Additionally, ERK may differentially regulate ANF expression and the cytoskeleton organization changes that characterize the hypertrophic phenotype (50, 51). Hypertrophic stimuli including \( \alpha_1 \)-adrenergic stimulation and mechanical stretch may activate both MAPK pathways, whereas pacing of contractions may be unique in selectively activating JNK. Although the mechanism of calcium activation of JNK in cardiac myocytes has not yet been elucidated, it has been reported that both JNK and p38 may be activated by constitutively active calcium/calmodulin-dependent kinase IV in PC12 cells (18). Thus, calcium/calmodulin-dependent kinases may potentially provide a link between calcium transients and activation of MAPK family members. It is also quite likely that pacing activates additional pathways that are important for ANF expression in addition to JNK. Although the mechanism of calcium activation of JNK in cardiac myocytes has not yet been elucidated, it has been reported that both JNK and p38 may be activated by constitutively active calcium/calmodulin-dependent kinase IV in PC12 cells (18). Thus, calcium/calmodulin-dependent kinases may potentially provide a link between calcium transients and activation of MAPK family members. It is also quite likely that pacing activates additional pathways that are important for ANF expression in addition to JNK. In this regard, pacing activation of ANF expression has recently been found to be sensitive to genestein, a general inhibitor of tyrosine kinase activity.

The results obtained using cluster mutations in the ANF 5′-FS are consistent with previous reports investigating hormone-inducibility of the rat ANF promoter and the promoters of other cardiac genes. For ANF, the promoter-proximal SRE (31) and Sp1-like site (32) have been implicated in \( \alpha_1 \)-adrenergic-stimulated gene expression. Skeletal \( \alpha_1 \)-actin represents another gene that is up-regulated in neonatal ventricular myocytes undergoing hypertrophic growth and mutations in

\[ P. M. McDonough and N. Mellon, unpublished data. \]
putative SRE and Sp1 sites in the chicken skeletal α-actin promoter reduce TGFβ- and α1-adrenergic-stimulated gene expression (52, 53). The finding that similar cis-elements regulate gene expression in response to such different stimuli (e.g. α1-adrenergic agonists, transforming growth factor β, and pacing) suggests that the combinatorial action of SRF and Sp1-like proteins may be a fundamental theme for the induction of cardiac genes involved in the hypertrophic growth program.

Although classically thought of as a transcription factor associated with “housekeeping” or constitutively expressed genes, recent reports suggest that Sp1 can participate in tissue-specific or hormonally inducible gene expression and can cooperate with other transcription factors (43, 53–57). The increase in Sp1-mediated transcriptional activation by c-Jun reported in this study is similar to previously reported effects of the bovine papillomavirus enhancer protein E2 and the retinoblastoma gene product, Rb, which are known to activate Sp1-dependent transcription (58–63). c-Jun has also been reported to activate transcription from GAL4 chimeric constructs containing the trans-activation domain of the Ets-like transcription factor ERM (64) or the trans-activation domain of the androgen receptor (65). Thus, in the examples cited above and in the present investigation, GAL4-Sp1 permitted enhanced trans-activation of transcription via higher order protein-protein interactions. Similarly, c-Jun activated transcription from transcription factors in the absence of traditional AP-1 binding sites. This suggests the possibility that a similar interaction between c-Jun and Sp1 may occur at the endogenous ANF promoter, representing a novel pathway for the regulation of gene expression via contractile calcium transients.

Another potential participant in the functional interaction between c-Jun and Sp1 is p300, a molecule related to CREB-binding protein (CBP), which interacts with the trans-activation domain of c-Jun (66). However, there are no published reports describing interactions between Sp1 and p300. Additionally, there is the possibility that Sp1-related proteins such as Sp2 and Sp3 (67, 68) compete for interaction with the rat ANF promoter. Consistent with this idea, Ardati and Nemer (32) demonstrated the cardiac nuclear extract binding activity to DNA probes encoding the putative ANF Sp1 binding site, but only a portion of this binding activity was shifted by antibodies directed against Sp1; the remainder of this binding activity might correspond to additional Sp1 family members.

Given the striking effects of c-Jun on gene expression from the ANF promoter and Sp1 trans-activation system, it is relevant to consider the possible involvement of potential AP-1 complexes in c-Jun-mediated cardiac gene expression. Because c-Fos represents a dimerization partner for c-Jun to form the AP-1 complex, over-expression of c-Fos alone would be expected to increase AP-1-dependent gene expression (via potential interactions with endogenous c-Jun), and overexpression of both c-Fos and c-Jun together would be expected to yield an even greater response. Indeed, results consistent with these predictions have been observed in many cell types transfected with AP-1 binding site-containing reporter constructs (69–71), including cardiac myocytes (72, 73). The results of the present study, though, argue against the involvement of traditional c-Fos-c-Jun complexes in the regulation of expression from the rat ANF promoter and the Sp1 protein. For the ANF promoter, transfection of c-Fos in the presence of c-Jun inhibited the effect of c-Jun. Likewise, for the GAL4-Sp1 system, expression of c-Fos alone had no effect on Sp1-mediated transcription and was inhibitory when cotransfected with c-Jun at equimolar amounts. Similar effects of c-Fos on gene expression from the human ANF promoter have been observed by others, and the inhibitory effect has been localized to the C-terminal region of the c-Fos molecule (42, 73). In contrast to the results with c-Fos, JunB cotransfection has been shown to potentiate the effect of c-Jun on the human ANF promoter (73). Notably, JunB mRNA expression is up-regulated in neonatal ventricular myocytes subjected to pacing3 and ATP (74) and also in cardiac tissue isolated from rats exposed to isoproterenol infusion (75); all of these treatments increase intracellular calcium levels in cardiac myocytes. Thus, it is conceivable that either c-Jun-c-Jun homodimers or c-Jun-JunB heterodimers are responsible for the effects of c-Jun on the rat ANF promoter and Sp1 trans-activation domain observed in the present study. Another possibility to be considered is the potential involvement of c-Jun-JunD heterodimers. Whereas JunD is not an effective substrate for JNK due to the lack of a docking site for JNK, it has recently been demonstrated that c-Jun can recruit JunD to JNK allowing subsequent phosphorylation of JunD (76); whether such a mechanism occurs in cardiac myocytes is yet to be determined.

In summary, the results of the present study suggest that increased intracellular calcium resulting from electrical pacing of contractions leads to increased JNK activity. The combination of increased JNK activity and up-regulation of c-Jun and related proteins may activate gene transcription via interactions between c-Jun, SRF, and the trans-activation domain of Sp1. The effects of other hypertrophic stimuli, which increase c-Jun levels in cardiac myocytes might additionally be amplified by the calcium sensitivity of the JNK system. Calcium activation of JNK, with subsequent phosphorylation of c-Jun and activation of Sp1-mediated transcription, might be relevant to muscle, neuronal, and neuro-secretory cells, which also undergo oscillations in intracellular calcium concentration as part of their cellular functions.

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