Electrical Stimulation of Neonatal Cardiac Myocytes Activates the NFAT3 and GATA4 Pathways and Up-regulates the Adenylosuccinate Synthetase 1 Gene*

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Electrical stimulation of cultured cardiomyocytes serves as an experimentally convenient and physiologically relevant in vitro model of cardiac hypertrophy. Electrical pacing triggers a signaling cascade that results in the activation of the muscle-specific Adss1 gene and the repression of the nonmuscle Adss2 isoform. Activation of the Adss1 gene involves the calcineurin-mediated dephosphorylation of NFAT3, allowing its translocation to the nucleus, where it can directly participate in Adss1 gene activation. Mutational studies show that an NFAT binding site located in the Adss1 5′-flanking region is essential for this activation. Electrical pacing also results in the increased synthesis of GATA4, another critical cardiac transcription factor required for Adss1 gene expression. MEF2C also produces transactivation of the Adss1 gene reporter in control and paced cardiac myocytes. Using the Adss1 gene as a model, these studies are the first to demonstrate that electrical pacing activates the calcineurin/NFAT3 and GATA4 pathways as a means of regulating cardiac gene expression.

Cardiac hypertrophy is an adaptive process that allows the heart to maintain or increase cardiac output in response to increased workload (1, 2). Physiological hypertrophy refers to the enlargement of the heart that results from repeated endurance exercise. This form of cardiac hypertrophy is both beneficial and reversible. In contrast, prolonged hypertrophy, usually secondary to other pathology, leads to irreversible cardiomyopathy and heart failure. In recent years, a number of transgenic mouse models of cardiomyopathy have been constructed that mimic critical aspects of human heart disease (1–3). A number of these features can also be duplicated using an in vitro model of cardiac hypertrophy employs primary cultures of neonatal rat cardiomyocytes. This valuable model system has allowed the biochemical and molecular characteristics of cardiac hypertrophy to be studied under experimentally controlled conditions (2, 4–6). Neonatal cardiac myocytes display features of the hypertrophic response after stimulation with α-adrenergic agonists (7–9), endothelin-1 (10–12), transforming growth factor-β (13), insulin-like growth factor (14), angiotensin II (3), and other hormonal stimuli (15, 16). Stimulated cardiac myocytes undergo increases in cell size and in myofibrillar abundance with extensive parallel sarcomere alignment (4, 8, 17). Total RNA content per myocyte increases without a concomitant increase in DNA content, reflecting the absence of cell division. The hypertrophic response is characterized by the activation of immediate early gene expression followed by the transcriptional activation of certain embryonic genes such as skeletal α-actin, β-myosin heavy chain (18, 19), and atrial natriuretic factor (ANF) (20, 21). The reemergence of β-myosin heavy chain in cardiac ventricles is viewed as the canonical adaptive genetic response in cardiac hypertrophy (22).

It is also possible to elicit hypertrophy by the use of pulsatile electrical stimulation to pace the contractions of neonatal rat cardiac myocytes (23). In response to this treatment, the myocytes display dramatic increases in cellular size and myofibrillar organization and a 5–10-fold increase in the expression of several genes encoding sarcomeric proteins and mitochondrial enzymes (23, 24). Induction of the Anf promoter by electrical pacing involves the participation of c-Jun, c-Jun N-terminal kinase, serum response factor, and Sp1 acting on the Anf promoter-proximal serum response element and an Sp1-like element (25). In electrically paced cardiac myocytes, the hypertrophic response is characterized by a pronounced increase in mitochondrial content, including the activation of nuclear genes encoding specific mitochondrial proteins (26–28). The activation of these genes is preceded by an increase in the abundance of mRNA encoding the transcription factors c-Fos, c-Jun, Jun-B, and nuclear respiratory factor-1 (NRF-1). These results indicate that a battery of genes encoding mitochondrial components are activated in response to electrical pacing and that the transcription factors NRF-1 and AP-1 are involved in this process. Thus, electrically induced pacing of neonatal rat cardiomyocytes is a well-documented in vitro model of cardiac hypertrophy (23–29).

Recent studies have shown that a variety of physiological and pharmacological compounds illicit hypertrophic responses

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† The abbreviations used are: ANF, atrial natriuretic factor; Adss1 and -2, adenylosuccinate synthetase 1 and 2 genes, respectively; CAT, chloroamphenicol acetyltransferase; CMV, cytomegalovirus; NFAT, nuclear factor of activated T cells; NRF-1, nuclear respiratory factor-1; AP-1, activating protein-1; GFP, green fluorescent protein.
Regulation of Adss1 Gene Expression in Cardiac Myocytes

through calcium-mediated signaling pathways involving the activation of calcineurin. This cytoplasmic phosphatase acts to dephosphorylate the phosphorylated form of nuclear factor of activated T cells (NFAT), thereby allowing the dephosphorylated NFAT to enter the nucleus and activate genes. We wished to determine if electrical pacing also activated the calcineurin/NFAT pathway as a means of activating cardiac gene expression. As a model for these studies, we chose Adss1, the gene encoding the muscle-specific isoform of adenylosuccinate synthetase. This enzyme is believed to play a role in cardiac energy metabolism via the purine nucleotide cycle (30–32). Considerable information is available concerning the combinatorial mechanisms that regulate the cardiac specific expression of the Adss1 gene. This gene is activated early during cardiac development (by embryonic day 9.0 in the mouse) and up-regulated during the neonatal period to achieve very high levels of expression in the adult heart (33). Within 1.9 kb of DNA immediately upstream of the Adss1 transcription start site are genetic elements that function as a cardiac specific enhancer that confers proper developmental activation and neonatal enhancement in the cardiac lineage. The Adss1 cardiac enhancer confers copy number-dependent expression in transgenic mouse hearts (33). The enhancer region contains binding sites for NKX2.5, GATA4, MEF2C, E12, HAND1, and HAND2 that are essential for high level expression of a reporter gene in the hearts of transgenic mice and in transfected neonatal rat cardiomyocytes. Each site is capable of sequence-specific interactions with proteins present in neonatal rat primary cardiac myocyte nuclear extracts. Thus, the cardiac regulatory elements of the murine Adss1 gene are well characterized and when driving the expression of a reporter gene serve as a sensitive and reliable reporter of cardiac specific gene expression (33). We show here that the expression of the Adss1 gene is considerably enhanced in cardiomyocytes when electrical pacing is used to increase myocyte contraction rates. This induction of Adss1 gene expression is mediated by NFAT dephosphorylation as a consequence of calcineurin activation. Adss1 induction acts through an NFAT binding site associated with the Adss1 gene. Electrical pacing also results in the increased synthesis of GATA4, another critical cardiac transcription factor required for Adss1 gene expression. Our studies are the first to identify the calcineurin/NFAT and GATA4 pathway as a link between electrically paced myocyte contraction and changes in cardiac gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Electrical Stimulation—Neonatal (1–3-day-old) rat cardiac myocytes were isolated and plated in six-well dishes (for RNA isolation and transient transfection) or 12-well dishes (with coverslips for fluorescent microscopy) in Dulbecco’s modified Eagle’s medium supplemented with 10% Hyclone calf serum at a plating density of 5 x 10⁵ cells/21 cm or 4 x 10⁴ cells/16 cm as described previously (26). After 24 h, the serum-containing medium was removed, and the cells were washed and subsequently maintained for 24–72 h in Dulbecco’s modified Eagle’s medium, in the absence of serum containing 1% bovine serum albumin (fraction V). The medium was exchanged with fresh serum-free medium at 2 days. The four wells containing myocytes were electrically paced for 72 h using the method of Brevet et al. (34) as modified by McDonough and Glembozki (23). The cardiac myocytes in the other six-well dishes were maintained under the same conditions as the stimulated cells but in the absence of electrical stimulation. During the experimental period, the medium bathing the control and paced cells was changed after 48 h to fresh Dulbecco’s modified Eagle’s medium plus 1% bovine serum albumin. In some experiments, cyclosporin A (1 μM) was added to both the control and electrically paced cells at the initiation of electrical stimulation.

RNA Isolation and Northern Blot Analysis—RNA was isolated with Stat60 (UL-traspec RNA isolation system, Biotex Laboratories, Houston, TX) at the indicated times from control or electrically paced cells. For Northern analysis, 20 μg of total RNA was fractionated by electro-phoresis on 1% agarose gels containing 0.6% formaldehyde at 50 V for 3 h. Fractionated RNA was transferred onto a Duralon-UV nylon membrane (Stratagene, La Jolla, CA) by capillary action for 24 h. After blotting, the RNA was cross-linked to the filter by UV irradiation. Following prehybridization (50% formamide, 25 mM potassium phosphate, 5× SSC, 5× Denhardt’s solution, and 100 μg/ml salmon sperm DNA) at 42°C, the blot was hybridized in the prehybridization solution plus 1% dextran sulfate and the labeled DNA fragment. After washing, the blots were visualized by autoradiography following storage at –70°C for various times. To probe for additional transcripts, the blots were stripped in boiling water for 5 min. The prehybridization, hybridization, and washing procedures were repeated as described above.

Preparation of RNA Probes for Northern Blot Analysis—A hybridization probe for Adss1 mRNA was prepared from the Sty–BamHI genomic fragment spanning exon 1 of the Adss1 gene (32) using the random primer labeling kit from Promega. The complete cDNA of Adss2 (31) was also prepared using random primer labeling. Gata4 and Hand1 cDNA probes were excised from pCGGATA4 (35) and pCMVHand1 (33) and labeled as described.

Preparation of Plasmids for Transfection—The reporter constructs, 1.9Adss1/CAT and 0.6Adss1/CAT (33), as well as the expression constructs, pCGGATA4 (35), pCMVMFE2C (36), pCMV E12 (36), and pCMVMHand1 (33), have been previously described. The basic helix-loop-helix transcription factors, NKx2.5, MEF2, and GATA site-directed mutants were generated by a polymerase chain reaction megaprimer procedure (33) as described previously. The putative NFAT binding site within 1.9Adss1/CAT was disrupted using a polymerase chain reaction-based mutagenesis procedure (37). The primers were designed to replace the NFAT consensus sequence (TGGAAAAC) in the Adss1 promoter with a KpnI cutting site as CCATGG. The primers that were used for polymerase chain reaction-based mutagenesis of the NFAT site are as follows (mutated nucleotides are capitalized): P1, 5’-CCATGGTgattgatcctctgctcctgtgtccct-3’; P2, 5’-aagagacctggggcttgattgtct-3’.

Plasmids used to express NFAT-GFP and ΔNFAT-GFP were constructed in pEGFP-1 (CLONTECH) as described (37). The expression construct pCMV-NFAT and pCMV-ΔNFAT were prepared by deletion of the GFP coding region of NFAT-GFP and ΔNFAT-GFP expression vectors, respectively (37). Both plasmids were digested by AgeI and NotI to release the GFP fragment. The correct size fragments of CMV-ΔNFAT and CMV-NFAT were isolated and purified from the gel, blunted, and self-ligated.

Transient Transfection—Primary cardiac myocytes were cultured, transfected with plasmid vectors, and assayed for CAT activity and GATA4 expression, as described previously (27). Cells in six-well dishes were transfected with 0.5 μg of total DNA, including the promoter-reporter plasmid and pCMVlacZ, was held constant as empty vector and/or pCMV-NFAT or pCMV-ΔNFAT was varied. For NFAT localization studies, the cardiac myocytes were transfected with 0.5 μg of pNFAT-GFP. After 72 h of electrical stimulation, GFP localization was determined using fluorescence microscopy (see below).

Microscopic Techniques—Changes in cardiac myocyte size and sarcomeric morphology were determined as described previously (38) in control and electrically paced cardiac myocytes in the absence and presence of 2 mM Ca²⁺. Cells were stained with BODIPY FL phallacidin (Molecular Probes, Invitrogen, OR). The fluorescent images were viewed and photographed as described below.

For cells expressing the NFAT-GFP fusion protein, cardiac myocytes were cultured on laminin-coated (10 μg) glass coverslips inserted into the multwell dishes. After 24 h, cells were transfected with 2 μg of NFAT-GFP construct, and 6 h later they were washed twice with phosphate-buffered saline. Half of the cells were electrically paced for 72 h, and half were maintained under the same conditions in the absence of electrical pacing. After 72 h, all cells were washed with 1× phosphate-buffered saline, and the cells on the coverslips were viewed using an Olympus BX 60 fluorescence microscope equipped with dark field optics with a green filter and photographed using a SPOT digital camera (Diagnostics).

Statistics—The significance of the experimental differences reported here was determined using Student’s t test for paired and unpaired variants. Data are presented as the mean ± S.E.
The results are expressed as the mean ± S.E. of three independent determinations. The expression of the 1.9 Adss1 gene was determined by Northern blot hybridization using Adss1 and Adss2 DNA probes, respectively. The results are representative of at least three independent experiments. B, 1.9Adss1/CAT was cotransfected into control and electrically paced cardiomyocytes. Duplicate cultures were transfected with 0.6Adss1/CAT to serve as a negative control. The expression of the 1.9Adss1/CAT construct was expressed as a 6-fold increase over that of the 0.6Adss1/CAT, where the asterisk represents p < 0.01. The results are expressed as the mean ± S.E. of three independent determinations.

**RESULTS**

The Adss1 Gene Is Activated in Response to Electrically Induced Pacing—Neonatal rat cardiac myocytes were cultured for periods of up to 72 h in the absence and presence of electrically induced pacing. To determine if pacing results in increased Adss1 gene expression, we isolated total RNA from cardiomyocyte cultures at various times following the initiation of electrical stimulation. Adss1 mRNA levels were assessed by Northern blot hybridization using a murine Adss1 DNA probe (32). Adss1 mRNA was readily detected in control and paced cultures throughout the 72-h time course of the experiment (Fig. 1A). At 24 h, the Adss1 mRNA levels increased significantly in the electrically paced cultures only, reached a maximum at 48 h, and remained high at 72 h. These results indicate that Adss1 gene expression is significantly up-regulated in response to electrical pacing.

We wished to determine if the nonmuscle Adss2 gene (31) is expressed in rat primary cardiomyocytes and if Adss2 also responds to electrical stimulation. Adss2 mRNA levels were determined by blot hybridization analysis of the RNA isolated at various times from control and electrically paced cardiomyocytes. Adss2 mRNA was present in neonatal cardiac myocytes, and its abundance abruptly declined beginning 48 h following the initiation of electrical stimulation (Fig. 1A). Thus, electrically induced pacing results in reduced expression of the Adss2 gene (nonmuscle) and the enhanced expression of the Adss1 gene (muscle-specific) in neonatal cardiomyocytes.

Previous studies have shown that the promoter and immediate 1.9 kilobase pairs of 5’-flanking sequence of the murine Adss1 gene contain all of the cis-regulatory elements required to achieve proper activation and expression in the cardiac lineage (33). To determine if this regulatory region also contains genetic recognition signals that respond to electrically induced pacing, we measured the expression of a 1.9Adss1/CAT reporter construct following transfection in control and electrically paced rat neonatal cardiomyocytes. Expression of the 1.9Adss1/CAT reporter construct was induced approximately 7-fold in response to pacing (Fig. 1B). These results indicate that the Adss1 promoter and 1.9 kilobase pairs of 5’-flanking sequence contain regulatory sequence information that responds to electrically induced pacing.

The Increase in Adss1 Gene Expression following Electrically Induced Pacing Is Blocked by Cyclosporin A—We have previously shown that electrically induced pacing of rat neonatal cardiac myocytes results in a significant increase in intracellular calcium (26). Calcium can influence cardiac gene expression and the development of cardiac hypertrophy by a Ca2+/calcmodulin-mediated activation of calcineurin, an intracellular phosphatase (3, 39). Calcineurin activation and the cellular consequences of this activation, i.e. hypertrophy, are prevented by cyclosporin A. To determine if pacing regulates Adss1 gene expression through a calcineurin-dependent pathway, we examined Adss1 gene expression in control and electrically paced cardiomyocytes in the presence and absence of cyclosporin A. Adss1 gene expression was measured by Northern blot analysis. Cyclosporin A blocked the induction of Adss1 mRNA that normally occurs following 48 h of electrical pacing (Fig. 2A). As an internal control, we also examined the abundance of β-myosin heavy chain mRNA in control and paced cultures in the presence and absence of cyclosporin A. Here also, we found that the presence of cyclosporin A blocked the induction of β-myosin heavy chain mRNA. In contrast to the effect of cyclosporin A to block induction of Adss1 and β-myosin heavy chain mRNAs, the presence of cyclosporin A did not prevent the induction of c-fos, which encodes one of the immediate early proteins of the AP-1 complex. Based on these findings and the published effects of cyclosporin A, it is likely that the effects of electrical stimulation on Adss1 and β-myosin heavy chain gene expres-
sion are mediated through a calcineurin pathway, whereas the induction of c-fos is not.

Gene transfection experiments were conducted to determine if the effect of cyclosporin A on Adss1 mRNA levels was mediated through transcriptional inhibition. For this purpose, the 1.9Adss1/CAT construct was introduced into control and electrically paced cardiomyocytes in the absence or presence of cyclosporin A. The level of CAT activity in cell extracts was determined 48 h following transfection. The presence of cyclosporin A had no effect on reporter gene expression in the nonstimulated cultures (Fig. 2B). However, the presence of cyclosporin A completely blocked the activation of the 1.9Adss1/CAT reporter construct in the electrically paced cultures. These results suggest that cyclosporin A blocks the pacing-induced transcriptional activation of the Adss1 gene.

We also examined the effect of cyclosporin A on the hypertrophic changes in the cardiac myocytes paced by electrical stimulation. After 72 h, control and paced cardiomyocytes, without and with cyclosporin A, were fixed and examined following labeling with the actin stain, BODIPY FL phallacidin. Paced myocytes in the absence of cyclosporin A were larger than control cells, and actin fiber staining demonstrated a high degree of actin fiber organization (Fig. 3A). However, these changes were blocked by the presence of cyclosporin A in the stimulated cultures (Fig. 3B). Cyclosporin A had no effect on control cultures (Fig. 3, C and D). These results suggest that the cardiac hypertrophy and intracellular organization that accompanies electrical pacing are mediated in part through a signaling pathway involving calcineurin.

A Member of the NFAT Family of Transcription Factors Is Involved in the Activation of the Adss1 Gene following Electrically Induced Pacing—NFAT transcription factors translocate from the cytoplasm to the nucleus following dephosphorylation by calcineurin, a Ca\(^{2+}\)/calmodulin activated phosphatase (40–42). Cyclosporin A is known to prevent the activation of calcineurin by binding to cyclophilins and competing for the Ca\(^{2+}\)/calmodulin binding site (42). Thus, the fact that cyclosporin A blocks the induction of the Adss1 gene following electrical pacing of cardiomyocytes suggests that this induction may be mediated through the action of NFAT. To initially evaluate this possibility, we examined the 5'-flanking region of the Adss1 gene for the presence of an NFAT consensus sequence (5/AGA/TTGGAAANN(A/T/C)). The search identified a potential NFAT binding site situated approximately 556 base pairs upstream of the transcription initiation site. To test the importance of the NFAT consensus sequence, we prepared a mutationally altered 1.9Adss1/CAT reporter construct in which the NFAT binding site was destroyed by site-directed mutagenesis. The wild type 1.9Adss1 promoter construct and the 1.9Adss1 promoter mutant with a destroyed NFAT binding site were transfected into control and paced cardiomyocytes. After 72 h, the transfected cells were harvested, and extracts were tested for CAT reporter activity. The results indicate that mutation of the NFAT consensus sequence blocked induction of the reporter gene that accompanies electrically induced pacing (Fig. 4). However, the absence of the NFAT consensus sequence did not affect the level of expression in the control nonstimulated cells. These results suggest that the 5'-flanking region is required for enhanced Adss1 gene expression in the response to electrical pacing, but it is not required for basal expression.

The importance of the NFAT consensus sequence in the response of Adss1 gene expression to electrical pacing suggests that NFAT is part of the signaling pathway that controls pacing-induced activation of the Adss1 gene. To test this hypothesis, we cotransfected the 1.9Adss1/CAT reporter gene into nonpaced cardiomyocytes along with several different concentrations of expression constructs encoding either wild type NFAT or a constitutively active mutant NFAT (∆NFAT). The
ΔNFAT mutant is known to localize preferentially to the nucleus and in this way mimic the action of dephosphorylated NFAT (37). Cotransfection of wild type NFAT did not activate the 1.9Adss1/CAT reporter construct, whereas cotransfection of the ΔNFAT mutant did activate the Adss1 promoter in a dose-dependent manner (Fig. 5). These results provide strong evidence that NFAT proteins are involved in the pacing-induced activation of the Adss1 gene.

Based on the results presented above, we propose that electrical pacing results in the dephosphorylation of NFAT and its translocation to the nucleus. To test this hypothesis, we transfected an expression construct encoding an NFAT-GFP fusion protein into control and electrically pacer cells of rat neonatal cardiac myocytes. After 72 h, cells were washed with phosphate-buffered saline and viewed by fluorescence microscopy to determine the cellular localization of green fluorescence. In control cultures, green fluorescence was cytoplasmic and was excluded from nuclei (Fig. 5, inset). However, in electrically paced cells, the green fluorescence became concentrated in myocyte nuclei in 68% of transfected cells. In control cardiac myocytes, the GFP fluorescence in the transfected cells was only found in the cytosol. These results indicate that the electrically induced pacing results in the translocation of cytoplasmic NFAT into the nucleus.

**GATA4 Transcription Factor Is Induced as a Result of Electrical Pacing and Is Essential for Pacing-induced Adss-1 Gene Activation**—GATA4 and HAND1 are known to play significant roles in cardiac development and gene expression. For this reason, we wished to evaluate the potential role of these transcription factors in pacing-induced activation of the Adss1 gene in neonatal cardiomyocytes. RNA was isolated from control and electrically paced cultures and analyzed for Gata4 and Hand1 mRNA by Northern blot hybridization. The levels of Gata4 mRNA began to increase approximately 1 h after the initiation of electrical stimulation and remained high for 12 h of pacing (Fig. 6A). By 24 h, Gata4 mRNA levels returned to nonpaced levels. In contrast, the abundance of Hand1 mRNA was less than that of GATA4 mRNA, and Hand1 mRNA levels did not change with the duration of pacing (Fig. 6A). These results suggest that GATA4 may play a role in the pacing-induced activation of gene expression in cardiomyocytes.

The effect of GATA4 on Adss1 gene expression was examined in a series of cotransfection assays using control and electrically paced cardiomyocytes. In the first series of experiments, the 1.9Adss1/CAT reporter construct was introduced into control and paced cardiomyocytes in the absence or presence of the pGATA4 expression construct. The 1.9Adss1/CAT reporter construct was transactivated by GATA4 in both control and paced cardiomyocytes (Fig. 6B). In each case, the presence of the GATA4 expression vector resulted in a 3–4-fold increase in Adss1 reporter gene expression. These results show that GATA4 plays a rate-limiting role in expression of the 1.9Adss1/CAT reporter constructs in control and electrically paced cardiomyocytes.

The importance of GATA4 in Adss1 gene expression in cardiomyocytes was further examined by mutational analysis of GATA factor binding sites located in the Adss1 cardiac regulatory region. To this end, four of the five GATA sites in the Adss1 cardiac regulatory region were destroyed by site-directed mutagenesis (33), and the expression of the resulting mutant 1.9Adss1/CAT reporter construct was examined in control and electrically paced cardiomyocytes. Mutant reporter gene expression was drastically reduced in control cultures and was not significantly increased following electrical stimulation (Fig. 6B). These results reinforce the findings presented above and reported previously (33) that GATA factors play a critical role in the regulation of Adss1 gene expression in the heart.

Recent studies have suggested a functional interaction between GATA4 and NFAT3 (3, 43). To determine if GATA4 and NFAT3 interact synergistically in the activation of the Adss1 gene in cardiomyocytes, we compared the ability of these factors alone and in combination to activate the 1.9Adss1/CAT reporter construct following transfection into control and electrically paced neonatal cardiomyocytes. In control cells, GATA or ΔNFAT alone resulted in a 3-fold increase in 1.9Adss1/CAT reporter gene expression, respectively, while the two together achieved a 7-fold increase in expression of the reporter gene (Fig. 6B). These results show that GATA4 is able to activate Adss1 gene expression alone and in combination with NFAT3. However, the effects of GATA4 and NFAT3 are additive rather than synergistic.

In paced cardiac myocytes, cotransfection of the 1.9Adss1/CAT reporter construct with GATA4 alone activated reporter gene expression by 3-fold, while cotransfection with ΔNFAT alone had no effect. Similar to the results with GATA4 alone, ΔNFAT in combination with GATA4 also resulted in a 3-fold induction. These results suggest that overexpression of ΔNFAT has no effect on Adss1 gene expression in electrically paced cells. Since NFAT is dephosphorylated and activated as a result of pacing, it is likely that nuclear binding of NFAT is
The Role of MEF2C in the Control of Adss1 Gene Expression in Cardiomyocytes—We have recently shown that MEF2 proteins are critical for the proper expression of the Adss1 gene during cardiac development (33). The effect of MEF2C on Adss1 gene expression in cardiac myocytes was examined in a series of co-transfection assays using control and electrically paced cells. In the first series of experiments, the 1.9 Adss1/CAT reporter construct was introduced into control and paced cardiomyocytes in the absence or presence of the MEF2C expression construct. MEF2C transactivated 1.9 Adss1/CAT in both cultures of cardiomyocytes (Fig. 7). In each case, the presence of the MEF2C expression vector resulted in an approximately 2-fold increase in Adss1 reporter gene expression. These results show that MEF2C is capable of enhancing the expression of the 1.9 Adss1/CAT reporter construct in both control and paced cells.

The importance of MEF2 factors in Adss1 gene expression in cardiomyocytes was further examined by mutational alteration of a MEF2 factor-binding site located in the Adss1 cardiac regulatory region (33). The expression of the resulting MEF2 mutant 1.9 Adss1/CAT reporter construct was examined in control and paced cardiomyocytes. Reporter gene expression was drastically reduced in control cultures and was not significantly increased following electrical pacing (Fig. 7). These results reinforce the findings, presented above, that MEF2 factors play a critical role in the regulation of Adss1 gene expression in cardiomyocytes.

Recent studies have suggested a functional interaction between MEF2 and NFAT proteins in striated muscle (33). To determine if MEF2C and NFAT3 interact synergistically in the activation of the Adss1 gene in cardiomyocytes, we compared the ability of these factors alone and in combination to transactivate the 1.9 Adss1/CAT reporter construct following transfection into control and paced cardiomyocytes. In control cells, MEF2C or ΔNFAT alone resulted in a 2- and 3-fold increase in Adss1 reporter gene expression, respectively, while the two together achieved a 7-fold increase in expression of the reporter gene (Fig. 7). These results show that MEF2C is able to activate Adss1 gene expression alone and in combination with NFAT3. However, the results suggest that the effects of MEF2C and ΔNFAT are additive rather than synergistic. When MEF2C or ΔNFAT was introduced into electrically paced cardiomyocytes, there was already saturated in the transfected and electrically paced myocytes.

The Role of MEF2C in the Control of Adss1 Gene Expression in Cardiomyocytes—We have recently shown that MEF2 proteins are critical for the proper expression of the Adss1 gene during cardiac development (33). The effect of MEF2C on Adss1 gene expression in cardiac myocytes was examined in a series of co-transfection assays using control and electrically paced cells. In the first series of experiments, the 1.9 Adss1/CAT reporter construct was introduced into control and paced cardiomyocytes in the absence or presence of the MEF2C expression construct. MEF2C transactivated 1.9 Adss1/CAT in both cultures of cardiomyocytes (Fig. 7). In each case, the presence of the MEF2C expression vector resulted in an approximately 2-fold increase in Adss1 reporter gene expression. These results show that MEF2C is capable of enhancing the expression of the 1.9 Adss1/CAT reporter construct in both control and paced cells.

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FIG. 6. Gata4 is induced by electrical pacing and is important for activation of Adss1. A, RNA was isolated from control (C) and paced (P) cardiomyocytes at the times indicated, and the abundance of Gata4 and Hand1 mRNA was determined by Northern blot hybridization analysis. The results are representative of at least three separate experiments. B, control and paced cells were transfected with either the wild type 1.9Adss1/CAT reporter construct or a mutant construct lacking four GATA sites (mGATA4). In other experiments, the wild type 1.9Adss1/CAT was cotransfected with a GATA4 and/or ΔNFAT expression construct. Duplicate cultures were transfected with 0.6Adss1/CAT to serve as a negative control. After 72 h, the level of CAT activity in cell extracts was determined, and the expression from the 1.9Adss1/CAT construct was expressed as a -fold increase over that seen with the 0.6Adss1/CAT construct. The results are expressed as the mean ± S.E. of three independent determinations.

FIG. 7. MEF2C plays an important role in the induction of the Adss1 gene following electrical pacing. Control and paced cardiomyocytes were transfected with either the wild type 1.9Adss1/CAT reporter construct or a mutant construct lacking the single MEF2 site. In other experiments, the wild type 1.9Adss1/CAT was cotransfected with a MEF2C and/or ΔNFAT expression construct. Duplicate cultures were transfected with 0.6Adss1/CAT to serve as a negative control. After 72 h, the level of CAT activity in cell extracts was determined, and the expression from the 1.9Adss1/CAT construct was expressed as a -fold increase over that seen with the 0.6Adss1/CAT construct. The results are expressed as the mean ± S.E. of three independent determinations.

cultures, MEF2C resulted in an approximately 2-fold induction, whereas ΔNFAT had no stimulatory effect. Together, the two had only a 2-fold effect, reflecting the lack of additional gene activation by ΔNFAT in the paced cultures. Thus, the effects of MEF2C and ΔNFAT are additive rather than synergistic in control and paced myocytes.

DISCUSSION

Pulsatile electrical stimulation serves as a contractile stimulus for cultured cardiomyocytes that provides an experimentally convenient method for inducing cardiac hypertrophy. The first direct evidence that cardiomyocytes are able to “sense” the contractile stimulation in the absence of neuronal or hormonal
factors came from studies by McDonough et al. (23, 24), who demonstrated that the regulation of ANF secretion is primarily dependent on contractile calcium transients and calmodulin kinase, independent of protein kinase C. Furthermore, contractile stimulation of cardiac myocytes in serum-free cultures results in cardiac growth and maturation in vitro (26, 27). More recently, both Xia et al. (27, 28) and McDonough et al. (25) have reported that contractile pacing regulates transcription in cardiac myocytes first by activating the expression of a series of early genes, c-fos, c-jun, and jun-b. These studies suggest that long term electrical stimulation of cardiac myocytes in vitro is a suitable experimental system to determine how external contractile stimuli are transduced into intracellular signals regulating cardiac gene expression through increased calcium transients and immediate early gene induction.

We have shown here that electrical pacing triggers a signaling cascade that results in the activation of the muscle-specific gene, Adss1, and repression of the nonmuscle gene, Adss2. The induction of Adss1 was blocked by the presence of cyclosporin A, suggesting that the activation of calcineurin, a cellular serine/threonine phosphatase is involved in the signaling cascade (3). Activated calcineurin is known to catalyze the dephosphorylation of cytoplasmic NFAT3, allowing the translocation of the latter into the nucleus, where it can directly participate in the activation of the Adss1 gene. We have shown that a constitutively activated NFAT3 is a potent activator of Adss1 gene expression and that an NFAT3 binding site in the Adss1 5’-flanking region is essential for this activation. These studies are the first to demonstrate that electrical pacing activates cardiac myocyte gene expression via the calcineurin/NFAT pathway (3).

Cyclosporin A did not inhibit the rapid induction of c-fos mRNA following electrical stimulation. This result suggests that calcineurin does not play a role in immediate early gene activation. Therefore, induction of immediate early genes by electrical pacing responds to hypertrophic signals different from the calcineurin-dependent pathway. These results indicate that multiple signaling pathways, acting in parallel, coordinately regulate cardiac specific genes in response to electrically induced pacing. In this model, calcium content increases in the cytosol, activating NFAT3 by calcineurin-dependent dephosphorylation. Simultaneously, non-calcineurin-dependent pathways regulate cardiac specific genes through immediate early gene induction. In this regard, previous studies have shown that AP-1 protein is essential to activate the cytochrome c gene by electrical pacing (28). Although the induction of AP-1 proteins is independent of calcineurin, AP-1 proteins may cooperate with NFAT proteins in the activation of target genes (see below). NFAT proteins are also known to cooperate with other transcription factors such as GATA4 (3) and MEF2 (37) in the activation of target genes.

GATA sites are located within the cardiac control region of the Adss1 gene and are essential for activation of the Adss1 gene during murine cardiac development (33). These sites are also important for Adss1 reporter gene expression following transfection into rat neonatal cardiac myocytes (33). Here we have shown that these GATA sites are essential for the pacing-induced activation of Adss1 reporter genes in neonatal cardiomyocytes. The importance of GATA4 in Adss1 gene activation is consistent with the finding that pacing-induced activation of the Gata4 gene occurs well in advance of Adss1 gene activation. Presumably, this timing allows for the synergistic activation of new GATA4 protein that is required to achieve transcriptional enhancement of Adss1. It is interesting to note that GATA4 can transactivate Adss1 expression constructs in control and in electrically paced cardiomyocytes. These findings suggest that GATA4 is rate-limiting in both control and electrically paced cardiomyocytes. Our findings concerning the role of GATA factors in gene regulation in cardiomyocytes are in good agreement with those of Molkentin et al. (3), who have recently reported that GATA4 is involved in regulating gene expression in angiotensin II- or phenylephrine-stimulated cardiomyocytes. Their studies provide evidence for a direct physical interaction between NFAT and GATA4 transcription factors and suggest that these transcription factors act together to activate gene expression in cardiomyocytes (3). Our results are the first to show that the Gata4 gene is activated in response to electrical pacing and that GATA4 plays a critical role in pacing-induced activation of cardiac specific genes.

MEF2 proteins are members of the MCM1, agamous, deficiens, SRF (MADS) family of transcription factors and are known to play a role in cardiac and skeletal muscle gene expression (37). We have recently provided transgenic data suggesting that MEF2 transcription factors are essential for proper Adss1 gene expression during cardiac development (33). We have shown here that the single MEF2 site in the 5’-flanking region of the Adss1 gene is also critical for pacing-induced activation of the Adss1 gene following electrical stimulation of neonatal cardiomyocytes. Furthermore, transfection studies shown here indicate that MEF2C can activate Adss1 reporter constructs in both control and electrically paced cardiomyocytes. These findings suggest a rate-limiting role for MEF2 proteins in the activation of Adss1 gene expression in cardiomyocytes. A role for MEF2 proteins in linking energy demand with changes in gene expression has also been reported for skeletal muscle. Chin et al. (37) have recently reported that MEF2 factors cooperate with NFAT to selectively activate slow fiber-type-specific genes following persistent motor neuron stimulation of skeletal muscle. Thus, it appears that in both skeletal and cardiac muscle MEF2 proteins participate with NFAT proteins in linking contractile activity with selective gene expression.

Electrical pacing triggers a signaling cascade that results in an orderly program of gene activation and repression. Here we have shown that the muscle-specific gene, Adss1, is activated, and the nonmuscle gene, Adss2, is repressed as a result of electrically induced increases in the contraction rate of cardiomyocytes. A similar isotype switch has been reported for the carnitine palmitoyltransferase genes, in which the muscle CPT1 gene is activated and the liver CPT1 gene is repressed in response to electrically induced pacing (26, 27). These changes represent a reprogramming of cardiomyocyte gene expression and are believed to reflect a commitment to a mature cardiac phenotype characterized by high levels of ADSS1 and muscle-specific CPT1. The activation of genes encoding the muscle-specific isoforms presumably reflects the genetic response of the cardiomyocyte to the increased energy demand that accompanies increased contraction rates. A reprogramming of gene expression also occurs in skeletal muscle in response to experimentally induced stimulation of contraction rates. Chin et al. (37) have recently shown that as a consequence of more frequent neuronal stimulation, slow fibers maintain higher levels of calcium, leading to calcineurin activation and selective up-regulation of slow fiber-type-specific gene promoters in skeletal muscle. Our studies along with those of Chin et al. (37) suggest that calcineurin/NFAT signaling is a common pathway in striated muscle for linking increased energy demand with changes in cellular gene expression. In each case, the isotype or fiber type switch is mediated through a calcium-regulated pathway involving calcineurin activation and NFAT dephosphorylation. A variety of transcription factors are involved in coupling contractile stimulation with cardiomyocyte gene expression.
The immediate early proteins of the AP-1 transcription factor complex (c-Fos, c-Jun, and Jun-B) and NRF-1 are elevated significantly in response to electrical stimulation (44, 45). We have shown previously that AP-1 proteins are induced very early following the commencement of electrically induced pacing and that AP-1 is required for the activation of the cytochrome c gene (28). NRF-1 is a transcription factor that is believed to play a critical role in regulating the expression of nuclear genes that encode mitochondrial proteins (46, 47). Recent studies by McDonough et al. (29) have shown that ATF6 plays a critical role in mitogen-activated protein kinase (p38MAPK), leading to ANF gene activation by electrical pacing. Here we have provided evidence that NFA3 and GATA4 and MEF2 proteins are involved in the pacing-induced stimulation of Adss1 gene expression. Thus, as a result of the research reported here, NFA1, GATA4, and MEF2 can now be added to a list of transcription factors that function to couple electrically induced pacing with changes in cardiac gene expression. A number of studies indicate that NFA proteins function in combination with a variety of other transcription factors. Previous studies of calcineurin-mediated transactivation of cytochrome c gene promoters in T cells provide evidence that AP-1 cooperates with NFA in both DNA binding and transactivation. Recent studies have shown that NFA cooperates with GATA factors in cardiomyocytes (3) and with MEF2 proteins in skeletal muscle (37). We have provided evidence that all of these factors are involved in pacing-induced changes in cardiomyocyte gene expression.

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