Extracellular Signal-regulated Protein Kinase Activation Is Required for the Anti-hypertrophic Effect of Atrial Natriuretic Factor in Neonatal Rat Ventricular Myocytes*

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Atrial natriuretic factor (ANF) inhibits proliferation in non-myocardial cells and is thought to be anti-hypertrophic in cardiomyocytes. We investigated the possibility that the anti-hypertrophic actions of ANF involved the mitogen-activated protein kinase signal transduction cascade. Cultured neonatal rat ventricular myocytes treated for 48 h with the α1-adrenergic agonist phenylephrine (PE) had an 80% increase in cross-sectional area (CSA). ANF alone had no effect but inhibited PE-induced increases in CSA by approximately 50%. The mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD098059 minimally inhibited PE-induced increases in CSA, but it completely abolished ANF-induced inhibition of PE-induced increases. ANF-induced extracellular signal-regulated protein kinase (ERK) nuclear translocation was also eliminated by PD098059. ANF treatment caused MEK phosphorylation and activation but failed to activate any of the Raf isoforms. ANF induced a rapid increase in ERK phosphorylation and in vitro kinase activity. PE also increased ERK activity, and the combined effect of ANF and PE appeared to be additive. ANF-induced ERK phosphorylation was eliminated by PD098059. ANF induced minimal phosphorylation of JNK or p38, indicating that its effect on ERK was specific. ANF-induced activation of ERK was mimicked by cGMP analogs, suggesting that ANF-induced ERK activation involves the guanylyl cyclase activity of the ANF receptor. These data suggest that there is an important linkage between cGMP signaling and the mitogen-activated protein kinase cascade and that selective ANF activation of ERK is required for the anti-hypertrophic action of ANF. Thus, ANF expression might function as the natural defense of the heart against maladaptive hypertrophy through its ability to activate ERK.

Atrial natriuretic factor (ANF)1 is a peptide hormone that is normally expressed only in the cardiac atria and has a well characterized endocrine role in blood volume regulation (1). The ANF gene is also inducible in the ventricle under pathological circumstances and after exposure to hypertrophic agents (2–4), including the α1-adrenergic agonist phenylephrine (PE) (5). Thus, significant increases in ANF mRNA are found in the mouse ventricle that is stressed by aortic banding (6), in genetic hypertension (7), and in viable ventricular myocardium following experimental infarction (8). Even though ventricular ANF expression has been considered to be a specific molecular marker of hypertrophy, its role in hypertrophy is unclear. ANF has been shown to have growth-inhibitory effects in both non-myocardial cells (9, 10) and cardiac myocytes (11). Furthermore, knockout mice lacking the ANF guanylate cyclase receptor appear to die suddenly due to hypertrophic cardiomyopathy. Thus, ventricular ANF may play a compensatory role that modifies the ventricular myocyte’s growth response to hypertrophic signals.

Diverse stimuli lead to cardiac hypertrophy, including mechanical loading (6), myocardial infarction (12), and the effects of growth factors such as IGF-I (2–5). Although the molecular mechanisms responsible for mediating the cardiac growth response are poorly understood, they probably involve the integration of multiple, potentially antagonistic, pathways. Many hypertrophic signals, including those initiated by PE (13), IGF-I (14), and afterload stress (15), appear to merge at the level of the mitogen-activated protein kinase (MAPK) cascade. Consequently, each of the MAPK family members, ERK (13, 16), JNK (17), and p38 (18), have been implicated in the hypertrophic response.

Until recently, ERK was thought to be a positive regulator of the hypertrophic phenotype (13, 16). It is now known that, although the ERK pathway plays a primary role in the proliferative response in skeletal muscle, it is in fact inhibitory to the myogenic response in L6A1 myoblasts (19). Similarly, in cardiomyocytes, inactivation of ERK does not inhibit the ability of PE to increase ANF promoter activity (a key molecular marker of cardiac hypertrophy) (20). Others (21) have demonstrated that transfected dominant-negative Rac1 GTPase, a member of the Rho family, fails to inhibit PE-induced ERK activation but disrupts PE-induced sarcomerogenesis and leucine incorporation. Indeed, a number of recent investigations have implicated other ERK-independent signal transduction pathways as being critical in mediating the hypertrophic action of PE, including...
the MEKK1/JNK (22), p38 (18), Rac1 (21), and calcineurin pathways (23). Thus, ERK does not appear to be involved in initiating or maintaining cardiac hypertrophy.

We initially hypothesized that the anti-hypertrophic effect of ANF resulted from interference with ERK activation. However, in preliminary experiments we found that, although ANF pre-treatment does inhibit subsequent growth factor-induced activation, ANF treatment alone causes rapid and robust ERK phosphorylation (24). On the basis of this evidence and work suggesting that ERK activation is not itself a hypertrophic stimulus, we hypothesized that the anti-hypertrophic action of ANF in the ventricular myocyte is mediated by activation of the ERK signaling cascade. Therefore, the present investigation was undertaken to determine if ERK signaling was involved in the anti-hypertrophic action of ANF and to better define the site of intersection between ANF signaling and the ERK cascade.

Most ANF actions are mediated through activation of its transmembrane guanylyl cyclase receptor, NPR-A (25). Receptor-generated cGMP binds to cGMP-dependent protein kinase (PKG), which is thought to mediate the principal biological functions of cGMP. However, very little is known about downstream PKG effects. cGMP-dependent nuclear localization of PKG causes transactivation of the Fos promoter, suggesting that the cGMP/PKG system plays a potentially important role in transcriptional regulation (26). Recently, Hood and Granger (28) reported that the stimulation of the ERK system by nitric oxide (NO) in endothelial cells is dependent on cGMP (27) and PKG activation. The ability of ANF, presumably through cGMP/PKG, to activate the ERK cascade in cardiac myocytes has not been previously studied.

Classical ERK activation involves a series of protein-protein interactions initiated by GTP loading of Ras that results in phosphorylation of the serine-threonine kinase Raf through recruitment to the cell membrane (29). Activated Ras in turn phosphorylates the MAPK/ERK kinase, MEK. MEK activation by Ras isoforms Raf-1 (30), B-Raf, and A-Raf has been well demonstrated, although A-Raf appears to activate selectively only MEK1 (31). Other phosphorylating proteins are also capable of activating MEK, such as the c-mos protooncogene (32) and MEK kinase 1 (33), a yeast STE11 homologue that is a more potent activator of JNK. MEK displays extremely high substrate selectivity toward ERK. An anchoring sequence on the cytoplasmic tail of MEK1 is required for interaction with the MEK kinase activity of the Raf isoforms (adapted from the method described by Bogoyevitch et al. (39)) following treatments, cells were lysed as described above in order to verify equal loading. Quantitation was done using the Molecular Image System (Bio-Rad).

In Vivo Kinase Assays—In all experiments, following specific treatments, myocytes plated on 10-cm dishes were washed twice in cold phosphate-buffered saline (PBS) and lysed on ice in buffer containing 10% sucrose, 1% Igepal CA-630 (a Nonidet P-40 equivalent from Calbiochem), 20 mM Tris-Cl (pH 8.0), 157 mM NaCl, 10 mM MgCl2, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 1 mM NaVO3, and 10 mM NaF. For Raf Western immunoblots, the lysis buffer contained 10 μg/ml Triton X-100, 20 mM n-octyl-β-D-glucopyranoside, 0.1% (w/v) fatty acid-free bovine serum albumin, 20 μg/ml aprotinin, and 2 mM Na3VO4. Lysates were centrifuged at 20,000 × g to remove nuclei, and total protein concentration was determined by the Bradford method (38). Samples containing 100 μg of protein were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to a Hybond-ECL membrane (Amersham Pharmacia Biotech) using a wet transfer apparatus (Bio-Rad). Membranes were incubated in a blocking buffer containing 5% (v/v) nonfat dry milk (Lucerne) in TBS with 0.1% Tween 20. Membranes were probed with phospho-p42/44 MAPK, phospho-SAPK/JNK, phospho-p38 MAPK, or phospho-MEK1/2 antibodies overnight at 4°C (all at 1:1000 dilution), washed twice in TBS-T, and then detected using a horseradish peroxidase-conjugated secondary antibody and ECL (Amersham Pharmacia Biotech). The membranes were stripped under reducing conditions in a solution containing 62.5 mM Tris-Cl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C. Following re-blocking, membranes were probed with the respective Raf-1 (1:1000), A-Raf (1:1000), B-Raf (1:1000), p42/44 MAPK (1:1000), SAPK/JNK (1:1000), p38 MAPK (1:5000), or MEK1/2 antibodies (1:1000) overnight at 4°C and detected as described above in order to verify equal loading. Quantitation was determined by the Bradford method (38). Samples containing 100 μg of protein were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to a Hybond-ECL membrane (Amersham Pharmacia Biotech) using a wet transfer apparatus (Bio-Rad). Membranes were incubated in a blocking buffer containing 5% (v/v) nonfat dry milk (Lucerne) in TBS with 0.1% Tween 20. Membranes were probed with phospho-p42/44 MAPK, phospho-SAPK/JNK, phospho-p38 MAPK, or phospho-MEK1/2 antibodies overnight at 4°C (all at 1:1000 dilution), washed twice in TBS-T, and then detected using a horseradish peroxidase-conjugated secondary antibody and ECL (Amersham Pharmacia Biotech). The membranes were stripped under reducing conditions in a solution containing 62.5 mM Tris-Cl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C. Following re-blocking, membranes were probed with the respective Raf-1 (1:1000), A-Raf (1:1000), B-Raf (1:1000), p42/44 MAPK (1:1000), SAPK/JNK (1:1000), p38 MAPK (1:5000), or MEK1/2 antibodies (1:1000) overnight at 4°C and detected as described above in order to verify equal loading. Quantitation was done using the Molecular Image System (Bio-Rad).

Anti-hypertrophic Action of ANF—In preliminary experiments, we found that ANF treatment alone inhibits subsequent growth factor-induced activation of ERK (24). However, the precise point at which ANF acts is unclear. In this study we have confirmed that ANF significantly inhibits the hypertrophic effects of PE. Furthermore, we have extended this observation by demonstrating that activation of the ERK cascade is required for the anti-hypertrophic action of ANF, and we have established that ANF-induced ERK activation through cGMP involves MEK but not Raf. The role of Raf in regulating the MEK/ERK cascade in cardiac myocytes remains to be determined.
Acetate, and 1 μCi of [γ-32P]ATP to assay activity of ERK. The reaction was continued for 15 min at 30 °C and then terminated by spotting on PS1 paper and washed 4 times in 75 mM phosphoric acid. Control reactions in which antibodies were incubated with buffer were done in parallel. Results were quantitated using scintillation counting. In addition, the activity of the ERK protein was assessed as described for Raf activity, except that full-length MEK was omitted from the reaction buffer.

For ERK activity assays, the ERK Western immunoblot lysis buffer was used. Samples containing 100 μg of total protein were immunoprecipitated in a total volume of 500 μl of lysis buffer with ERK-specific, agarose-coupled antibodies overnight at 4 °C. The ERK immunoprecipitate was washed three times in buffer containing 50 mM Tris-HCl (pH 7.5), 0.2% Igepal CA-630, 0.5 mM NaCl, 1 mM PMSF, and 5% sucrose (w/v). Kinase activity was determined by incubation of 10 μg of bovine MBP and 10 μCi of [γ-32P]ATP in 25 μl of assay buffer containing 80 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 0.4 mM PMSF, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Lysates were centrifuged at 2000 × g to remove nuclei, and total protein concentration was measured. Samples containing 100 μg of total protein were immunoprecipitated in a total of 500 μl of lysis buffer with specific SAPK/JNK antibodies bound to protein A-Sepharose overnight at 4 °C. The immunoprecipitated JNK was washed twice in buffer containing 50 mM LiCl, 100 mM Tris-HCl (pH 7.6), 1 mM DTT, and 0.1% Triton X-100 and then boiled for 3 min in assay buffer containing 20 μM MOPS (pH 7.2), 10 mM MgCl2, 2 mM EGTA, 1 mM DTT, and 0.1% Triton X-100. Kinase activity was determined by incubation with 3 μg of c-Jun substrate and 10 μCi of [γ-32P] ATP in 20 μl of assay buffer for 30 min at 30 °C. The reaction was terminated by the addition of 65 μl of Laemmli sample buffer, and MBP was resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. Results were quantitated by phosphorimaging.

For c-Jun kinase activity, following specific treatments, myocytes were washed twice in cold PBS, and whole-cell lysates were prepared in buffer containing 20 mM Hepes-ROH (pH 7.4), 2 mM EDTA, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, and 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 0.4 mM PMSF, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Lysates were centrifuged at 2000 × g to remove nuclei, and total protein concentration was measured. Samples containing 100 μg of total protein were immunoprecipitated in a total volume of 500 μl of lysis buffer with specific SAPK/JNK antibodies bound to protein A-Sepharose overnight at 4 °C. The immunoprecipitated JNK was washed twice in buffer containing 50 mM LiCl, 100 mM Tris-HCl (pH 7.6), 1 mM DTT, and 0.1% Triton X-100 and then boiled for 3 min in assay buffer containing 20 μM MOPS (pH 7.2), 10 mM MgCl2, 2 mM EGTA, 1 mM DTT, and 0.1% Triton X-100. Kinase activity was determined by incubation with 3 μg of c-Jun substrate and 10 μCi of [γ-32P] ATP in 20 μl of assay buffer for 30 min at 30 °C. The reaction was terminated by the addition of 65 μl of Laemmli sample buffer, and MBP was resolved by SDS-polyacrylamide gel electrophoresis. Results were quantitated using the Molecular Imager System (Bio-Rad).

Cell Staining—Prior to plating, chamber slides were pretreated with laminin (Life Technologies, Inc.) for at least 1 h. Following treatments, myocytes were washed twice in PBS, fixed in 3.7% formaldehyde/phosphate-buffered saline at room temperature for 10 min, washed once in PBS, and then permeabilized for 1 min in cold methanol, followed by another wash. After blocking with 0.2% bovine serum albumin in PBS for 30 min, fixed cells were incubated with ERK-specific antisera (1:50), washed three times in blocking buffer, followed by immunofluorescent staining with fluorescein-5-isothiocyanate-conjugated goat anti-rabbit IgG (1:500) (Molecular Probes, Eugene, OR). As an immunocytochemical control, the ERK primary antibody was replaced with primary antibodies pre-absorbed with synthetic ERK peptide (Santa Cruz Biotechnology) containing the antigenic sites for the antibody. The specificity of the antibody was also confirmed by Western immunoblotting which demonstrated protein bands only at the expected molecular weight of ERK 42/44. Cells were visualized with a Bio-Rad 1024ES laser scanning confocal system, equipped with a krypton-argon laser, attached to a Nikon TE300 inverted microscope. Images were acquired using Leershard acquisition software (Bio-Rad) and prepared for publication using Lasersharp post-processing software (Bio-Rad).

Transfections—For liposome-mediated transient transfections, myocytes plated on 60-mm dishes were exposed to a DNA-liposome complex according to the manufacturer’s protocol for Lipofectin (Life Technologies, Inc.) using 8 μg of pcDNA/β-galactosidase (CLONTECH, Palo Alto, CA). Cells were washed and incubated in serum-free media as described. Following treatments, cells were washed in PBS (pH 7.4), fixed for 5 min in a solution containing 74% PBS (pH 7.4), 4% paraformaldehyde, and 0.25% glutaraldehyde, washed again, and then stained for 48 h at 20 °C in a reaction solution containing 95.4% PBS (pH 7.4), 1× MgCl2, 1× potassium ferricyanide, 1× potassium ferrocyanide, and 0.1% X-gal. Cells were washed twice and covered with 30% glycerol. To assess the effect of various treatments on cardiomyocyte growth, we measured the CSA of transfected cells with light microscopy using a computer-assisted automatic edge detection and measurement software package (BioScan Optimas, BioScan, Inc., Edmonds, WA).

RESULTS

ANF Inhibition of PE-induced Myocyte Hypertrophy Is ERK-dependent (Fig. 1)—To confirm that ANF treatment inhibits PE-induced hypertrophy, we measured cross-sectional area (CSA) in primary cultures of neonatal rat ventricular myocytes (NRVMC). PE treatment increased NRVMC cross-sectional area by 80%. Treatment with a combination of PE and ANF reduced the PE-induced increase in cell size by approximately 50%. To determine that ANF-induced activation of ERK was necessary for its anti-hypertrophic actions, we assessed the ability of the MEK antagonist PD98059 to inhibit the inhibitory effect of ANF on PE-induced increases in CSA. PD98059 treatment alone had no effect on NRVMC CSA. PD98059 had a small inhibitory effect on the PE-induced increase in CSA but completely abolished ANF-induced inhibition of PE-induced increases in CSA, suggesting that the anti-hypertrophic action of ANF is ERK-dependent. This is consistent with previous findings that ANF is anti-proliferative in other cell types (9, 10) and inhibits thymidine uptake and leucine incorporation in cardiac myocytes (11).

ANF Causes ERK Translocation to the Nucleus—Although the subcellular localization of ERK is predominantly cytoplasmic, nuclear translocation of the phosphorylated protein is thought to be necessary for many ERK functions (40). We reasoned that if ERK was significantly involved in the anti-hypertrophic effect of ANF, then ANF treatment should result in ERK nuclear translocation. In serum-starved quiescent NRVMC, diffuse cytoplasmic localization (and absent nuclear staining) of ERK was observed by confocal imaging (Fig. 2A). In contrast, intense nuclear staining was seen 1 h after ANF treatment (Fig. 2B). Nuclear translocation was evident as early
as 15 min in nearly all cases and was significantly diminished at 2 h (data not shown). Furthermore, PD098059 inhibited ANF-induced nuclear translocation of ERK (Fig. 2C). Taken together, these data suggest that the anti-hypertrophic effect of ANF is ERK-dependent and that ANF-induced nuclear localization may be an essential component of this action.

ANF Activates Components of the ERK Cascade—To test more directly whether the anti-hypertrophic actions of ANF might occur through activation of the ERK signaling cascade, NRVMC were treated with ANF, and in vitro kinase activity assays were performed using immunoprecipitated Raf-1, MEK, and ERK. Treatment with TPA caused robust Raf-1 activation. However, ANF treatment had no effect on Raf-1 activation. In parallel experiments no effect was seen with other Raf isoforms, A-Raf and B-Raf (Fig. 3). ANF treatment resulted in a 3–4-fold increase in MEK activity (Fig. 4C). There was no difference between the ability of ANF to activate MEK1 or MEK2 (data not shown). This rapid increase in MEK activity was reflected in the ability of ANF to phosphorylate MEK as demonstrated by Western immunoblotting with anti-phospho-MEK antibody (Fig. 4A). These data suggest that the ANF signal intersects with the ERK cascade at the level of MEK and appears to be Raf-independent.

We next determined that ability of ANF to activate and phosphorylate ERK itself. ANF caused rapid ERK activation (Fig. 5, A and C). Furthermore, simultaneous treatment with both PE and ANF was clearly not inhibitory and, in fact, appeared to be additive (Fig. 5B). In additional experiments, pretreatment with the MEK antagonist PD98059, as expected, eliminated the ability of ANF to phosphorylate ERK, indicating that ANF-induced ERK phosphorylation is MEK-dependent (Fig. 4B). ANF stimulated the phosphorylation of ERK in NRVMC in a dose-dependent fashion (Fig. 6).

ANF-induced ERK Phosphorylation Is Specific—To determine if the effect of ANF on ERK was specific, we tested its ability to activate other members of the MAPK family. ANF did not activate JNK in vitro, despite evidence of significant JNK activation by the protein kinase C inhibitor RO-31–8220 in these cells (data not shown and Ref. 41). ANF stimulated the phosphorylation of ERK but had a minimal effect on phosphorylation of JNK or p38 (Fig. 7). ERK phosphorylation was significantly increased after 5 min and was maximal at 10 min. ERK2 (p42) appeared to be the predominant ERK isoform identified by Western immunoblot. However, both isoforms appeared to be phosphorylated following ANF treatment.

cGMP Mimics ANF-induced Activation of ERK—Most ANF actions are mediated through activation of its transmembrane guanylyl cyclase receptor, NPR-A (25), which is particularly abundant in the myocardium (42). To determine if ANF-stimulated ERK phosphorylation is mediated by cGMP, we used the cell-permeable derivative 8-bromo-cGMP. This cGMP analog caused a marked increase in ERK phosphorylation after 5- and 10-min treatments (Fig. 8), suggesting that ANF-induced ERK phosphorylation involves the guanylyl cyclase activity of the ANF receptor. Since most cGMP-mediated effects occur through activation of PKG, we attempted to inhibit the effect of 8-bromo-cGMP with the selective PKG antagonist KT5822 (43). However, KT5822 alone caused ERK phosphorylation, making it impossible to interpret the effect of KT5822 pretreatment on cGMP-induced ERK activity.
DISCUSSION

Changes in cardiomyocyte morphology in response to hypertrophic signals are reflected at the molecular level by the regulated induction of important sarcomeric proteins, including the \( \beta \) myosin heavy chain, myosin light chain-2V, and skeletal muscle \( \alpha \)-actin (44). Invariably, ventricular ANF mRNA is also rapidly expressed in this setting, although the physiological relevance of the production of a non-structural protein like ANF is unclear. In uncomplicated cardiac hypertrophy, circulating ANF levels are not increased (45), suggesting that local rather than systemic ANF actions might modify the hypertrophic response. In the present report we have confirmed that ANF has direct anti-hypertrophic effects in the ventricular cardiomyocyte, and we have extended this observation by establishing that ERK activation is required for this effect. Thus, the ability of ANF to activate ERK selectively and rapidly and to cause ERK nuclear translocation appears to be essential for the anti-hypertrophic action of ANF.

This study provides the first evidence of an important link between ANF and MAPK pathway in the heart and begins to define the molecular mechanisms involved in this interaction. Given that the classical pathway of ERK activation by growth factors involves the sequential activation of Ras, Raf, MEK, and ERK, there are a number of levels where ANF signaling

**Fig. 4.** ANF phosphorylates and activates MEK. A, serum-starved NRVMC were treated with 100 nM ANF for 10 min. Phosphorylation was determined by Western immunoblotting with specific phospho-MEK antibodies as described under “Experimental Procedures” using 100 \( \mu \)g of total protein. The blot was stripped and reprobed for total MEK to verify equal loading. B, myocytes were treated with ANF or IGF-I alone for 10 min or were pretreated with the MEK inhibitor PD98059 at the concentrations indicated for 30 min prior to a 10-min exposure to ANF. Western immunoblots were probed with specific phospho-ERK antibodies as described in A. C, in three separate identical experiments, endogenous MEK was immunoprecipitated, and kinase activity assays were performed as described under “Experimental Procedures” (data are means ± S.D.).

**Fig. 5.** ANF activates ERK. A, serum-starved NRVMC were treated with ANF (100 nM) for the indicated times. Endogenous p42/44 ERK was immunoprecipitated and assayed for kinase activity by measuring incorporation of \([\gamma-32P]ATP\) into MBP. B, quiescent cells were left untreated, treated with PE (1 \( \mu \)M), or PE and ANF (100 nM) for 10 min (for A and B relative kinase activities are provided above). C, ERK activation after 10 min of ANF (100 nM) treatment. Data points represent the means ± S.D. from three separate experiments and are expressed as fold stimulation relative to unstimulated controls.

**Fig. 6.** Effect of increasing ANF dose on ERK phosphorylation. Serum-starved NRVMC were treated for 10 min with the indicated concentration of ANF (C = unstimulated control). Phosphorylation was determined by Western immunoblotting of whole cell lysates with specific phospho-ERK. Data were adjusted for total ERK levels and are expressed as fold stimulation relative to unstimulated controls (means ± S.E. of data from three separate experiments).
Phosphorylation was determined as in Fig. 4. Serum-starved NRVMC were treated with ANF or 8Br-cGMP for the indicated periods (C = time 0-min unstimulated control). Phosphorylation was determined by Western immunoblotting of whole cell lysates with specific phospho-ERK (arrows indicate p42 and p44 isoforms), phospho-JNK (arrows indicate JNK1 and JNK2 isoforms), or phospho-p38 antibodies (A–C, respectively) as described under “Experimental Procedures” using 100 μg of total protein. Blots were stripped and reprobed for total ERK, JNK, or p38 to verify equal loading (less than 5% variation in all cases). Data are adjusted for total ERK, JNK, or p38 and expressed as fold stimulation relative to unstimulated controls (means ± S.E. of data from three separate experiments (D)).

Fig. 7. ANF treatment rapidly phosphorylates ERKs but not JNK or p38. Serum-starved NRVMC were treated with 100 nm ANF for the indicated periods (C = time 0-min unstimulated control). Phosphorylation was determined by Western immunoblotting of whole cell lysates with specific phospho-ERK (arrows indicate p42 and p44 isoforms), phospho-JNK (arrows indicate JNK1 and JNK2 isoforms), or phospho-p38 antibodies (A–C, respectively) as described under “Experimental Procedures” using 100 μg of total protein. Blots were stripped and reprobed for total ERK, JNK, or p38 to verify equal loading (less than 5% variation in all cases). Data are adjusted for total ERK, JNK, or p38 and expressed as fold stimulation relative to unstimulated controls (means ± S.E. of data from three separate experiments (D)).

Fig. 8. ANF phosphorylation of ERK is mimicked by cGMP. Serum-starved NRVMC were treated with ANF or 8Br-cGMP for the times indicated. Phosphorylation was determined as in Fig. 4A. Arrows indicate p42 and p44 ERK isoforms.

through cGMP and (presumably) PKG could activate the MAPK pathway. Our findings that ANF treatment resulted in the phosphorylation and activation of MEK in the absence of any effect on Raf isoforms suggests that the ANF signal converges with the ERK cascade at the level of MEK. Additional evidence supporting an effect of cGMP signaling on ERK activation has come from studies utilizing NO, a signaling molecule with many important cGMP-mediated physiological functions (46). Hood and co-workers (27) have demonstrated that the activation of vascular endothelial growth factor (VEGF) on ERK is also NO/cGMP-dependent and further suggest, unlike our results, that this effect is Raf-dependent as well. The reason for this difference is unclear. It is possible that cGMP effects are tissue-specific (endothelial cells versus cardiac myocytes). Alternatively, a nonspecific assay was utilized by the study cited. Raf phosphorylation of the catalytic domain may be presumed in that study because an in vitro kinase assay demonstrated that immunoprecipitated Raf is capable of phosphorylating the nonspecific substrate, syntide-2. In our study, phosphorylation of immunoprecipitated Raf from ANF-treated cells is coupled to activation of full-length ERK, thereby specifically implicating or excluding involvement of the MAPK pathway. Consistent with our findings, Suhasini et al. (47) have established that PKG plays no role in the activation of Raf. These investigators went on to demonstrate that PKG phosphorylates Raf Ser43, a residue adjacent to the Ras binding domain and that preincubation with cGMP inhibits ERK activation by EGF. Phosphorylation of Ser43 is thought to uncouple the Ras-Raf kinase interaction. Thus, the demonstration that PKG phosphorylates Ser43 provides an intriguing explanation for our previous observation that pretreatment with ANF inhibits ERK activation by IGF-I (24). Taken together, these data suggest a dual role for ANF signaling through cGMP in the ERK system; ANF is capable of rapidly activating MEK and ERK and is also able to prevent the action of subsequent growth factor activation of ERK through its ability to prevent the Ras-Raf interaction.

ERK activation was previously thought to be critical in the molecular pathways that initiate the hypertrophic response, since dominant-interfering MEK mutants (13) or ERK depletion using specific antisense oligonucleotides inhibited growth factor-stimulated ANF and MLC-2 expression and attenuated PE-stimulated increases in myocyte area and sarcomereogenesis (16). However, there is now a preponderance of evidence, including our own, that the initiation and maintenance of cardiac hypertrophy are ERK-independent processes. ERK inhibition does not prevent, and in fact enhances, skeletal muscle cell differentiation in IGF-I-treated cells (19). Furthermore, ERK inhibition fails to down-regulate biochemical markers of hypertrophic activity in PE-treated cardiac myocytes (20). Conversely, ATP and carbachol, molecules that are known to activate ERK, do not cause hypertrophy (20). Indeed, ATP has been shown to have anti-hypertrophic actions in PE-treated cardiomyocytes (48). Finally, our observations that (a) ERK inhibition with PD098059 minimally inhibits PE-induced increase in CSA, (b) ANF rapidly activates ERK but inhibits PE-induced increases in cell size, and (c) ERK inhibition with PD098059 blocks the anti-hypertrophic action of ANF support the conclusion that ERK activation does not promote PE-induced hypertrophy. The idea that cardiac ERK activation is anti-hypertrophic is supported by our own observations and other investigations in which constitutively activated MEK through ERK activation induces the c-Fos promoter in transient transfection assays but inhibits PE-induced activation of key hypertrophic markers (49). Furthermore, MEK inhibition in cardiac myocytes transfected with constitutively active MEKK1 appears to promote hypertrophy through JNK activation (50). These data suggest that modulation of MEK itself, the intersecting point of the ANF signal and the ERK cascade, may significantly modulate the hypertrophic phenotype.

Cardiac hypertrophy is the functional consequence of virtually all diseases of the heart. In acquired heart diseases, remodeling of the left ventricle occurs as the direct result of afterload stress. In these cases, hypertrophy initially plays a beneficial role by normalizing wall stress but subsequently becomes a significant independent risk factor for dying from cardiovascular disease (51). Cardiac hypertrophy can also result from more than 100 different disease-causing genetic mutations of the contractile apparatus (hypertrophic cardiomyopathy). Thus, hypertrophic signal transduction mechanisms
present an attractive therapeutic target for treating heart disease because hypertrophy appears to be the final common pathway for the pathogenesis of a vast array of acquired and inherited disorders. Angiotensin-converting enzyme inhibitors and calcium channel antagonists are thought to be effective because of the anti-hypertrophic action (52). In animal models of familial hypertrophic cardiomyopathy, cyclosporin and FK506 can prevent the hypertrophic action of calcineurin, a calcium-regulated phosphatase (53). Our results suggest that, in addition to inhibiting hypertrophic pathways, enhancing the activity of endogenous anti-hypertrophic factors may also be useful in the treatment of maladaptive hypertrophy. The development of such new therapeutic tools that mimic or modulate the anti-hypertrophic effects of ANF in the cardiomyocyte will require a complete understanding of ANF signal transduction pathways in the heart.

In summary, our data suggest that activation of the ERK cascade is required for the anti-hypertrophic action of ANF. We have presented evidence for a novel interaction between ANF and ERK signaling cascades in NRVMC. These data suggest that ANF treatment results in functional activation of the ERK system, including ERK phosphorylation and nuclear translocation. Furthermore, ERK activation by ANF appears to be cGMP-mediated and requires MEK phosphorylation but occurs independently of classical ERK activation through Raf. Therapeutic strategies aimed at enhancing cGMP-mediated mechanisms in the cardiomyocyte may be beneficial in hypertrophic diseases such as aortic stenosis and familial hypertrophic or dilated cardiomyopathy.

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