A Raf-induced, MEK-independent signaling pathway regulates atrial natriuretic factor gene expression in cardiac muscle cells

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Abstract The atrial natriuretic factor (ANF) gene is activated in cardiac myocytes by Ras and its effector Raf. However, MEK, the best-characterized Raf substrate, cannot efficiently activate ANF suggesting that Raf uses a MEK-independent pathway to activate ANF. By manipulating MEK and Raf activities so that they are equally effective at activating ERK, we now demonstrate that Raf activates at least two signaling pathways in cardiac myocytes that regulate the ANF promoter; the MEK → ERK pathway inhibits ANF gene expression while a Raf-induced, MEK-independent pathway activates expression. This mechanism may provide increased ability to regulate ANF expression in response to hypertrophic stimuli.

Key words: Atrial natriuretic factor; MEK-independent Raf signaling; Cardiac myocyte; MAP kinase

1. Introduction

During post-natal development, cardiac muscle cells grow by hypertrophy rather than proliferation. Along with numerous characteristic phenotypic changes, such as increased cell size and protein synthesis, certain genes, such as the atrial natriuretic factor (ANF), are expressed during hypertrophy and are therefore considered hypertrophic markers [1]. Many different stimuli lead to hypertrophy, and several different signal transduction pathways have been shown to regulate expression of the ANF gene primarily using rat neonatal ventricular myocytes as a model system. Treatment of these cells with various hypertrophic agonists leads to many of the characteristics associated with hypertrophy in vivo.

Mitogen-activated protein (MAP) kinases translate extracellular signals into changes in gene expression. These pathways regulate gene expression and affect cell growth, death and/or differentiation in many cell types [2,3]. The best characterized MAP kinase pathways leading to ERK, JNK and p38 activation have been implicated in the regulation of ANF expression and other hypertrophic phenotypes in a large number of studies over recent years [4–20]. These studies have mostly agreed on the stimulatory role of the 'stress-induced' MAP kinases, JNK and p38, in regulating ANF expression. However, there have been conflicting conclusions regarding the role of the ERK 1 and 2 MAP kinases that are activated by the prototypical Ras → Raf → MEK → ERK pathway in the regulation of this promoter. A catalytically inactive ERK mutant [7], antisense oligonucleotides that deplete ERK1 and ERK2 [20], or MKP1, a phosphatase that inactivates the ERKs (and other MAP kinases) [9,15], have all been shown to inhibit ANF expression in response to various hypertrophic stimuli. In addition it has been reported that active MEK, the enzyme immediately upstream of the ERKs, is sufficient to stimulate ANF expression [5]. These data are consistent with a stimulatory role for the ERK pathway on the ANF promoter. However, it has also been shown that ERK activation can be dissociated from ANF expression [16], and that activation of MEK, and thus ERK activity, can actually inhibit ANF gene expression [9]. Furthermore, the MEK inhibitor PD98059 was unable to prevent ANF expression [16] and has been shown to further stimulate ANF expression in some situations [10,21]. These data are consistent with no stimulatory role and in fact an inhibitory role for the ERK pathway on ANF expression. Thus, although we previously found that active Raf is sufficient to stimulate ANF expression [8], it is not clear whether Raf's known downstream target, MEK, is responsible for mediating Raf's ability to stimulate ANF gene expression.

To further investigate the mechanism through which Raf is able to induce ANF expression, we separately manipulated Raf and MEK activity in cardiac myocytes and monitored the effect on a bona fide target of the ERK pathway and the ANF promoter. These experiments indicate that Raf activates the ANF promoter through a signal transduction pathway that does not involve MEK and that increased MEK activity inhibits Raf-induced ANF gene expression. Thus, Raf utilizes at least one novel substrate to activate gene expression in cardiac myocytes while simultaneously activating the ERK pathway, which counteracts this induction.

2. Materials and methods

2.1. Cell culture and transfections

All experiments were performed by calcium phosphate-mediated transient transfection of primary neonatal rat ventricular myocytes. Myocytes were prepared, cultured and transfected as previously described [9,10,21] using the amounts of plasmid DNAs noted in the figure legends for each experiment. Where required, the total amount of plasmid in the transfection mix was kept constant by adding the relevant empty expression vectors. Luciferase and β-galactosidase assays were performed with reagents from Promega (Madison, WI, USA) or Tropix (Bedford, MA, USA) as described by the manufacturer. Transfection efficiencies were normalized by dividing the luciferase activity from each dish by the β-galactosidase activity from the same dish. Data in each experiment are presented as the mean ± S.D. of triplicates from a representative experiment. All experiments were performed at least three times with different preparations of plasmids and primary cells, producing qualitatively similar results.

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2.2. Plasmids

The activated MEK molecule (ΔN3/S218E/S222D MEK, also designated R4F) was provided by Natalie Ahn (University of Colorado, Boulder, CO, USA). The estrogen-regulated Raf-1 expression vector (pCEP4ΔRaf-1:ER) [8] was based on molecules provided by Martin McMahon (UCSF, San Francisco, CA, USA) and expresses the kinase domain of Raf-1 fused to the steroid-binding domain of the human estrogen receptor. The kinase activity of this molecule is dependent on the addition of exogenous estradiol to the culture medium [22,23].

The ERK2-MEK1 plasmid [24] was provided by Melanie Cobb (UT Southwestern Medical School, Dallas, TX, USA). BXB-Raf-MEK was constructed by replacing the ERK2 in the ERK2-MEK1 molecule with the kinase domain of Raf-1 (BXB-Raf). The ANF-luciferase plasmids contained regions of the ANF promoter (as specified in Section 3) driving a luciferase reporter gene derived from pGL3 (Promega, Madison, WI, USA). Gal4:Elk-1 (pFA-Elk-1, from Stratagene, La Jolla, CA, USA) contains a CMV-driven Gal4 DNA binding domain fused to the Elk-1 activation domain. Gal4:luciferase (pFR-Luc, from Stratagene) contains five Gal4 binding elements driving a luciferase reporter gene. For luciferase assays, ANF-luciferase or Gal4-luciferase reporters were co-transfected with a Rous sarcoma virus (RSV)-β-galactosidase reporter plasmid (provided by Michael Kapiloff, Vollum Institute, Portland, OR, USA) that was used to normalize transfection efficiencies.

3. Results

3.1. Active MEK does not mimic the effects of active Raf on ANF gene expression

To determine whether MEK can mimic Raf activation of the ANF promoter, we performed experiments with active forms of these molecules. A constitutively active form of MEK1 [25], referred to as R4F, contains a deletion of the N-terminal regulatory domain and has two negatively charged amino acids replacing serines on which Raf phosphorylates and activates MEK. ΔRaf-1:ER is an estradiol-inducible form of Raf-1 whose enzymatic activity can be controlled by titrating estradiol into the culture media [22,23]. We performed transient transfections in primary cardiac myocytes with these molecules to determine their ability to activate −3003ANF:luc, a luciferase reporter gene driven by base pairs −1 to −3003 of the ANF promoter. In parallel experiments, Gal4:Elk-1 was used to induce expression from Gal4:luc. Gene expression from this molecule measures activity through the Raf→MEK→ERK cascade. By separately titrating estradiol concentrations to induce ΔRaf-1:ER and introducing different amounts of active MEK into the cells, it is possible to regulate the amount of ERK activity so that equivalent amounts of active ERK are stimulated by either Raf or MEK.

A dose response was performed to determine whether equivalent signaling to ERK from Raf and MEK, as measured by Gal4:luc expression, has equivalent effects on −3003ANF:luc expression. In control experiments, treatment of cells with estradiol failed to activate reporter expression in the absence of ΔRaf-1:ER (data not shown). Fig. 1A shows that at their highest doses, R4F and ΔRaf-1:ER have similar abilities to activate ERK. However, Fig. 1B indicates that these doses of MEK cannot mimic that ability of Raf to activate −3003ANF:luc. In multiple experiments using different preparations of primary cells, the ability of ΔRaf-1:ER to activate −3003ANF:luc varied from 2.5-fold to 7-fold between experiments. In some experiments (such as that shown in Fig. 1B but not the experiment in Fig. 2A), we observed some activation of −3003ANF:luc by active MEK. However,

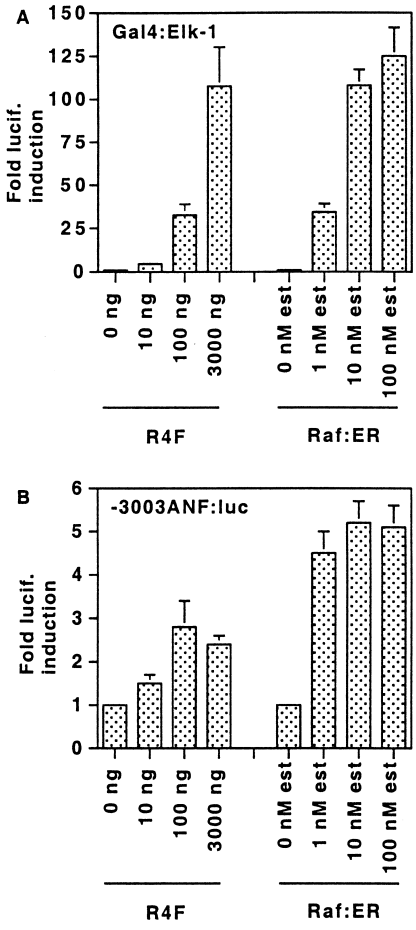


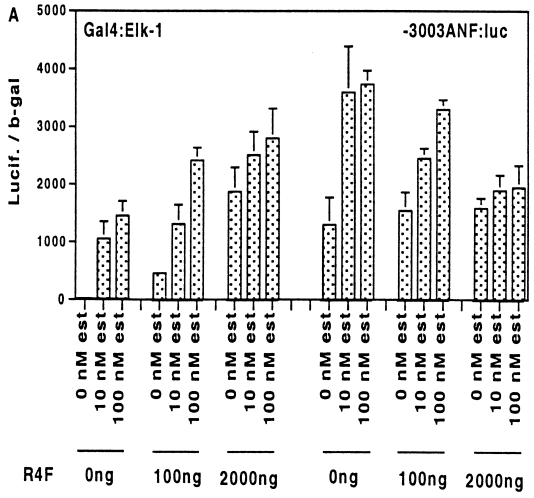
Fig. 1. MEK cannot mimic Raf activation of −3003ANF:luc. A: Empty expression plasmid plus or minus increasing amounts of R4F (as indicated and supplemented with empty expression plasmid to total 3 μg), or 3 μg ΔRaf-1:ER with increasing amounts of estradiol (as indicated, with the same total amount of ethanol vehicle in each instance), were transfected along with RSVβgal and the Gal4:Elk-1/Gal4:luc reporter system. B: Cells were transfected and treated the same as in (A) but with the −3003ANF:luc reporter.

for any particular preparation of cells, Raf was always more effective than MEK at activating −3003ANF:luc even when activation of Gal4:Elk-1, a bona fide target of the ERK pathway was equivalent. Thus, equal amounts of signaling to ERK by Raf and MEK have different effects on ANF gene expression with Raf being more efficient at inducing the ANF promoter.

3.2. Increased MEK activity inhibits Raf-dependent activation of −3003ANF:luc

We previously found that active MEK inhibits PE-induced expression of −3003ANF:luc [9]. To determine if increased MEK activity could also inhibit Raf-induced −3003ANF:luc expression, we performed experiments using ΔRaf-1:ER and R4F simultaneously. Dose response curves were performed using increasing concentrations of estradiol, at three different concentrations of R4F. Once again Gal4:Elk-1/Gal4:luc constructs were included in parallel to monitor the amount of ERK activity in each case. The results are shown in Fig. 2A.

With no added MEK, increasing concentrations of estradiol led to increasing activation of both Gal4:Elk-1 and



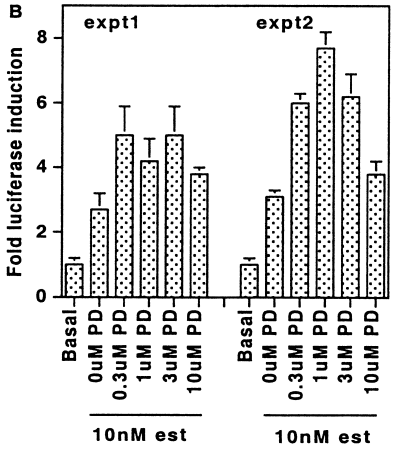


Fig. 2. MEK inhibits Raf-dependent activation of ANF:luc. A: Empty expression plasmid plus or minus increasing amounts of R4F (as indicated and supplemented with empty expression plasmid to total 2 μg), were transfected along with 1 μg ΔRaf-1:ER, RSVβgal, and with either Gal4:Elk-1/Gal4:luc or −3003ANF:luc. At each amount of R4F, three different concentrations of estradiol were added to the media (as indicated, with the same addition of ethanol vehicle in each instance). B: Cells were transfected with ΔRaf-1:ER and −3003ANF:luc and treated with either vehicle alone or 10 nM estradiol plus or minus increasing amounts of PD98059 (as indicated).

−3003ANF:luc as expected. However, at 0 nM estradiol, i.e. no ΔRaf-1:ER activity, increasing amounts of MEK led to a dose-responsive activation of Gal4:Elk-1, but no overall activation of −3003ANF:luc. Thus, as before, active MEK was able to induce Gal4:luc through Gal4:Elk-1 but was unable to induce the ANF promoter. Additionally, while increasing amounts of MEK potentiated the ability of Raf to activate Gal4:Elk-1, they led to a dose-dependent inhibition of Raf-induced −3003ANF:luc expression. These data indicate that MEK can inhibit Raf-dependent activation of −3003ANF:luc and further suggest that increased MEK activity and thus increased ERK activity is not responsible for Raf activation of −3003ANF:luc.

If MEK is in fact acting as an inhibitor of ANF expression, then blocking MEK activation should increase Raf-dependent activation of ANF. To test this hypothesis, we performed experiments with ΔRaf-1:ER and increasing amounts of the MEK inhibitor PD98059. Fig. 2B shows two separate experiments with PD98059. Experiment 1 showed a slight increase in Raf activation of ANF in the presence of PD98059. Experiment 2 showed a more pronounced increase in Raf activation of ANF at lower concentrations of PD98059, then a return to normal levels of Raf activation at higher concentrations. At the highest concentrations, the PD inhibitor was becoming insoluble explaining the variability between experiments. These data indicate that the MEK inhibitor does not prevent Raf-dependent activation of the promoter and even potentiates activation supporting the view that MEK and ERK activity is primarily a negative influence on the ANF promoter.

3.3. *Raf-dependent activation and MEK-dependent inhibition are achieved through separate DNA elements in the ANF promoter*

To test whether Raf activation and MEK inhibition of the

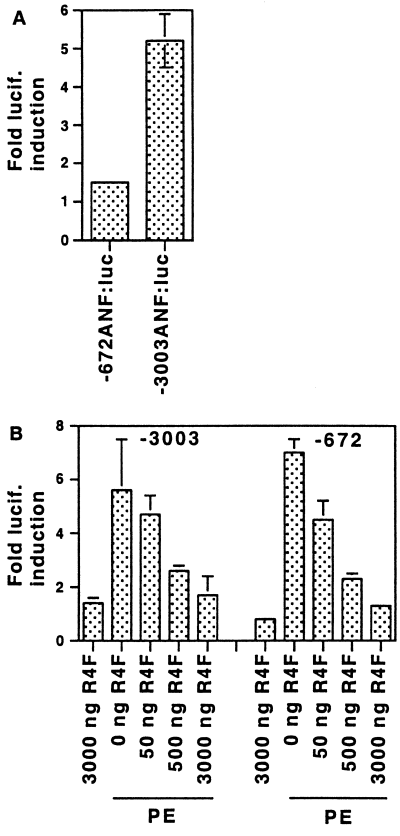


Fig. 3. Raf-dependent activation and MEK-dependent inhibition are achieved through separate DNA elements in the ANF promoter. A: Cells were transfected with ΔRaf-1:ER (treated with either 100 nM estradiol or the same amount of ethanol vehicle), RSVβgal, and either −3003ANF:luc or −672ANF:luc (as indicated). B: Empty expression plasmid plus or minus 100 μM phenylephrine (PE) or increasing amounts of R4F (as indicated and supplemented with empty expression plasmid to make the total 3 μg) were transfected with RSVβgal and either −3003ANF:luc or −672ANF:luc.

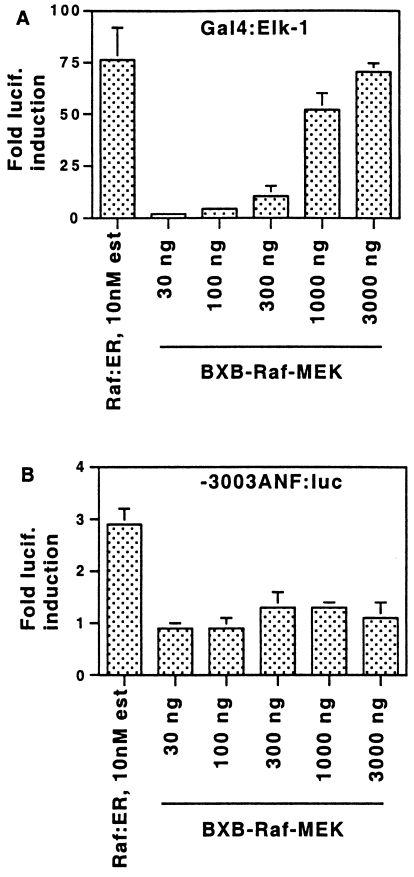


Fig. 4. A Raf molecule that preferentially activates MEK cannot activate −3003ANF:luc. A: Cells were transfected with either ΔRaf-1:ER or increasing amounts of BXB-Raf-MEK (as indicated and supplemented with empty expression plasmid to make the total 3 μg), RSVβgal, and Gal4:Elk-1/Gal4:luc. Cells transfected with ΔRaf-1:ER were treated with either 100 nM estradiol or the same amount of ethanol vehicle. B: Cells were treated as in (A) but with −3003ANF:luc reporter.

promoter are mediated through the same DNA elements, we tested the ability of Raf to activate −672ANF:luc, a luciferase reporter gene driven by base pairs −1 to −672 of the ANF promoter. Fig. 3A shows that Raf can activate −3003ANF:luc but not −672ANF:luc. This result indicates that DNA sequences required for Raf stimulation of the promoter lie between base pairs −672 and −3003 in the ANF promoter. To test if active MEK could still inhibit −672ANF:luc expression, we treated cells with 100 μM PE (an efficient inducer of both −3003ANF:luc and −672ANF:luc expression) and performed a dose response with MEK. The results are shown in Fig. 3B. As expected, MEK alone did not significantly activate either −3003ANF:luc or −672ANF:luc, while PE alone activated both −3003ANF:luc and −672ANF:luc to a similar extent. As previously demonstrated [9], increasing amounts of MEK led to inhibition of −3003ANF:luc expression. The same results were observed using −672ANF:luc indicating that DNA sequences that are required for MEK-dependent inhibition of gene expression, are located downstream of −672ANF. These results demonstrate that within the ANF promoter, DNA sequences that are required for Raf-dependent activation are separate from sequences that are sufficient for MEK-dependent inhibition.

3.4. A Raf molecule that preferentially signals to MEK cannot activate −3003ANF:luc

If Raf is indeed activating the ANF promoter via a MEK-independent signaling pathway, a Raf molecule that preferentially activates MEK rather than other substrates should be compromised in its ability to stimulate ANF but should still be able to efficiently activate ERK-induced transcription. To construct such a molecule we modified the ERK2-MEK1 fusion protein that was constructed by Robinson et al. [24] by inserting an active form of Raf-1 (BXB-Raf, which has a deletion of the N-terminal regulatory domain) in place of ERK2 in the ERK2-MEK1 fusion protein, thereby creating a BXB-Raf-MEK fusion protein. This molecule tethers an active Raf molecule to the wild-type MEK1 protein and thus creates a Raf molecule that preferentially activates MEK rather than other potential Raf substrates. We performed a dose response curve with this fusion molecule and asked if it was able to activate Gal4:Elk-1 and −3003ANF:luc.

Fig. 4A shows that increasing amounts of BXB-Raf-MEK generate a dose responsive increase in Gal4:Elk-1 activation such that 1000–3000 ng of BXB-Raf-MEK activates ERK to the same extent as estradiol-stimulated ΔRaf-1:ER. Fig. 4B shows that ΔRaf-1:ER activates −3003ANF:luc. However, increasing amounts of BXB-Raf-MEK do not lead to any significant activation of −3003ANF:luc. Thus although the Raf molecule that preferentially activates MEK and ΔRaf-1:ER (which is able to phosphorylate all potential Raf substrates) were equally effective at stimulating Gal4:Elk-1, only ΔRaf-1:ER was able to activate ANF. These data indicate that the ability of Raf to stimulate the ANF promoter is not due to its ability to activate MEK.

4. Discussion

In this study we showed that Raf activates the hypertrophic marker gene ANF in cardiac myocytes through a MEK-independent signal transduction pathway. This conclusion is based upon several lines of evidence. First, we separately manipulated Raf and MEK activities so that they were equally effective at inducing transcription from a bona fide target of the ERK pathway yet found that only Raf was effective at inducing ANF promoter-driven reporter gene expression (Fig. 1). Second, we constructed a Raf molecule that is preferentially able to activate only one substrate, MEK, and found that it is able to efficiently activate ERK targets but not the ANF promoter (Fig. 4). In addition, we showed (Fig. 2A) that increasing MEK activity could inhibit Raf-induced ANF expression, while inhibition of MEK further potentiates Raf-induced activation (Fig. 2B). This result suggested that not only does Raf activate the promoter via MEK-independent signals, the simultaneous activation of the ERK pathway by Raf actually inhibits ANF expression. Previously, we showed that the ERK pathway inhibits ANF expression in response to phenylephrine, activated G_q or active MEKK1 [9,10,21]. Thus ANF expression is regulated by competing positive and negative stimuli that are simultaneously induced by MAP kinase kinase kinases such as Raf or MEKK1.

These competing signals are regulated through separate DNA sequences in the promoter. We found that Raf could activate −3003ANF:luc, but not −672ANF:luc (Fig. 3). Therefore, Raf activation of ANF requires DNA sequences

upstream of −672ANF. However, MEK was able to inhibit PE activation of −672ANF:luc in a similar manner to that demonstrated with −3003ANF:luc. Although sequences between −672 and −3003 are required for Raf-dependent induction, we were unable to obtain Raf-induced expression when these sequences were fused behind a heterologous promoter (data not shown). Further deletion analysis indicates that no single deletion between −672 and −3003 results in loss of expression (data not shown). These data suggest that the mechanism of activation by the Raf-dependent signal is complex, perhaps requiring multiple DNA sequences and the native ANF promoter. It has been suggested that the ANF promoter might require formation of an enhancesome-like structure for proper activation [26,27]. Our data are consistent with the idea that the ultimate targets of the Raf-induced signaling pathway only induce transcription in such a context.

In some experiments, we observed modest activation of ANF by MEK (e.g. see Fig. 1B). Although this implies a stimulatory role for MEK (as suggested by Gillespie-Brown et al. [5]) Raf was always more effective than MEK at activating −3003ANF:luc even when activation of Gal4:Elk-1, a single, bona fide target of the ERK pathway was equivalent. One way to reconcile all the data from our group and others would be if there was a requirement for some ERK activity to allow ANF activation. In this case, highly efficient ERK inhibition might be incompatible with ANF activation via other signaling pathways. In some experiments (see Fig. 2B) inhibition of MEK→ERK signaling by PD98059 led to a dose responsive increase in Raf activation of ANF at lower concentrations, but ANF expression was diminished at higher concentrations of the inhibitor supporting this hypothesis. A complicating factor in the interpretation of such experiments is that the inhibitor was becoming insoluble at the higher concentrations. Such limitations make it difficult to conclusively test the hypothesis.

Although there have been several suggestions that Raf might have physiological substrates other than MEK [28–30], there are few examples where Raf is thought to alter gene expression via a MEK-independent pathway. The clearest example where this is the case comes from Rosner and colleagues [31,32]. These investigators showed that Raf could activate a protein kinase that is distinct from ERK1 or ERK2, that is able to phosphorylate and activate Elk-1 on the same sites as ERK1 and ERK2 [31]. The ANF promoter clearly cannot be activated solely by Elk-1 since we would expect that MEK, which is an efficient Elk-1 activator in our cells, would then be able to activate ANF. However, it is possible that the kinase that was discovered by Rosner and colleagues or a similar molecule is also able to activate other transcription factors in addition to Elk-1 that contribute to ANF activation. It will be possible to test this hypothesis when this kinase is further characterized.

In conclusion, our data suggest that the MEK/ERK pathway is not primarily responsible for Raf-dependent activation of ANF and in fact plays a negative role in ANF gene expression. Therefore, Raf activates at least two signaling pathways that compete against each other to regulate the ANF promoter. Similar competing pathways arise when active MEKK1 induces the ANF promoter [10]. Further complexity in the regulation of the ANF promoter is indicated by the fact that a low-affinity serum response element controls the activity of multiple transcription factors at other sites via a mechanism that is reminiscent of enhancesome-like activity [26]. Together, these data imply that ANF expression in hypertrophic heart cells has to be very tightly regulated. A possible explanation for this conclusion comes from a recent study by Silberbach and colleagues [33] who showed that ANF itself inhibits hypertrophy by directly activating the MEK→ERK pathway without activating Raf or other MAP kinase pathways such as JNK or p38. These findings in combination with our data imply that a delicate balance of ANF gene expression is required to maintain an optimal level of hypertrophy. Thus, as MAP kinase kinase kinases such as Raf or MEKK1 are activated by hypertrophic stimuli, this will lead to expression of ANF perhaps via JNK and p38-dependent signals [6,10,14] or the MEK-independent pathway that is shown here. Then, as ANF expression exceeds a threshold, a feedback loop will be initiated via the ANF receptor and cGMP-dependent signaling leading to MEK/ERK activation and subsequent inhibition of the hypertrophic phenotype [33]. In this scenario ANF-dependent activation of MEK should be effectively similar to our artificial stimulation of MEK and would therefore down-regulate ANF expression. This effect could be counteracted by a sustained hypertrophic stimulus that was still able to activate Raf or MEKK1 and the MEK-independent pathways that stimulate ANF expression. In this manner, the level of expression of ANF that is achieved through the complex interacting pathways that we have identified may serve as a 'rheostat' to regulate the extent and maintenance of the hypertrophic response.

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