Mitogen-activated Protein Kinases Mediate Changes in Gene Expression, but Not Cytoskeletal Organization Associated with Cardiac Muscle Cell Hypertrophy

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Abstract. Shortly after birth, cardiac myocytes lose the ability to divide, and, in adult animals, heart muscle grows by a process of cellular hypertrophy where each individual cell gets larger. We have previously shown that activated Ras protein can induce markers of the hypertrophic phenotype, including atrial natriuretic factor (ANF) expression and organization of contractile proteins, and that Ras is at least partially required for the hypertrophic effect of phenylephrine. In the present study, we examine the requirement for the mitogen-activated protein kinases (MAP kinases) in the hypertrophic response induced by phenylephrine. We find that phenylephrine treatment results in the activation of the MAP kinases and that this activity is required for transactivation of the *fos*, ANF, and MLH promoters. However, inhibition of MAP kinases does not prevent phenylephrine-induced organization of actin. These results suggest that the signal transduction pathways leading to different hypertrophic responses diverge upstream of the MAP kinases but possibly downstream of Ras.

THE molecular mechanisms which regulate cardiac muscle cell hypertrophy are unclear; however, it is becoming apparent that activation of various signaling molecules that are known to be involved in the growth and differentiation of other cell types is also important in the hypertrophic response. Culture systems using neonatal rat ventricular cardiomyocytes have been developed (Simpson, 1983) which respond to treatment with various agonists by displaying many features of the hypertrophic response (for review see Chien et al., 1991). Both heterotrimeric G protein–coupled agonists (e.g., phenylephrine [Lee et al., 1988], Endothelin-1 [Ito et al., 1991; Shubeita et al., 1990] and Angiotensin II [Sadoshima and Izumo, 1993*b*]), tyrosine kinase–coupled agonists (e.g., fibroblast growth factor [Parker et al., 1990]), phorbol esters (Dunnmon et al., 1990), and mechanical stretch (Komuro et al., 1991) have been shown to induce the hypertrophic phenotype. The hypertrophic response in neonatal ventricular myocytes is characterized by a series of phenotypic changes. These changes include (*a*) an increase in myocyte size; (*b*) increased protein synthesis; (*c*) activation of the expression of specific genes including embryonic markers like atrial natriuretic factor (ANF)[1] (Knowlton et al., 1991), contractile proteins like myosin light chain-2 (MLC-2) (Lee et al., 1988), and immediate early genes including c-*fos* (Izumo et al., 1988); and (*d*) increased organization of contractile proteins into sarcomeric units (Iwaki et al., 1990).

The mitogen-activated protein (MAP) kinases are ubiquitously expressed serine/threonine protein kinases which are activated in response to diverse stimuli (Boulton et al., 1991; Cobb et al., 1991; Thomas, 1992), and they are thought to be critical components in the signal transduction pathways which regulate cell growth and differentiation. MAP kinase activation occurs as a result of phosphorylation on threonine and tyrosine residues (Payne et al., 1991) by a MAP kinase kinase (MEK) (Ahn et al., 1992). MEKs are themselves activated by phosphorylation indicating the existence of a MEK kinase activity in the cell (Gomez and Cohen, 1991). A number of different kinases have been shown to function as MEK kinases including Raf-1 (Dent et al., 1992; Howe et al., 1992; Kyriakis et al., 1992), MEK Kinase (Lange-Carter et al., 1993), and Mos (Posada et al., 1993). It has been suggested that the MAP kinases may represent a convergence point where diverse signaling pathways are brought together (Lange-Carter et al., 1993).

Recent work suggests that treatments which result in

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1. *Abbreviations used in this paper*: ANF, atrial natriuretic factor; 2AP, 2-amino purine; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase kinase; MLC-2, myosin light chain-2; TPA, phorbol 12-myristate 13-acetate; 6TG, 6-thio guanine.

cardiac myocyte hypertrophy also induce MAP kinase activation (Bogoyevitch et al., 1993; Sadoshima and Izumo, 1993*a*; Yamazaki et al., 1993; Bogoyevitch et al., 1994). However, a correlation between MAP kinase activation and the hypertrophic response does not necessarily mean that MAP kinase activity is actually important for hypertrophy. We have attempted to address this question by studying the requirement for MAP kinase activity for two different phenotypes associated with cardiac myocyte hypertrophy, activation of gene expression and organization of actin into contractile units. In this report, we demonstrate that phenylephrine treatment causes MAP kinase activation in cardiac myocytes and that this activation is required for transcriptional activation but not for the organization of actin into contractile units. Thus the signal transduction pathway activated by phenylephrine separates above the MAP kinases leading to different hypertrophic phenotypes.

Materials and Methods

Cell Culture

Tissue culture media, phenylephrine, phorbol 12-myristate 13-acetate (TPA), 2-amino purine (2AP), and 6-thio guanine (6TG) were from Sigma Chem. Co. (St. Louis, MO), serum was supplied by GIBCO-BRL (Gaithersburg, MD). Neonatal rat ventricular myocytes were isolated by five 20-min rounds of collagenase/pancreatin digestion (95 U/ml collagenase, 0.6 mg/ml pancreatin, GIBCO-BRL) in a buffer containing 116 mM NaCl, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.5 mM $MgSO_4$, pH 7.35. The cells were pelleted by low speed centrifugation and suspended in plating media (4:1, DMEM:Media 199 containing 10% horse serum and 5% FBS). The isolated cells, a mixture of myocytes and non-myocytes (mostly fibroblasts) were plated onto an untreated tissue culture flask for two 45-min incubations. Non-myocytes adhered to the flask so that the remaining unattached cells were ~90% myocytes as determined by myosin staining, morphology, and beating. Non-myocyte cultures were passaged twice to remove any contaminating myocytes. For biochemical analysis, 10^6 cells were plated overnight onto 6-cm gelatin-coated dishes, for transfection experiments, 5×10^5 cells were plated onto 3.5-cm dishes while for immunofluorescence analysis, 5×10^5 cells were plated onto NUNC chamber slides (Naperville, IL) (culture area 45 mm × 21 mm). After plating for 16 h, the media was changed to serum-free media (4:1, DMEM:199) for 24–36 h before treatment as required.

MAP Kinase Activation

Cultured cells were treated with TPA (1 μM), serum (10% FBS), or phenylephrine (100 μM) as described for various times. The cells were washed in PBS and lysed in a buffer containing 50 mM TRIS pH 7.4, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na orthovanadate, 1% Triton X-100, 0.5% NP-40 plus 1 mM PMSF, 20 μg/ml leupeptin and 20 μg/ml pepstatin. For direct Western analysis of the apparent mobility of the kinases on SDS gels, the protein samples were boiled in Laemmli sample buffer and separated on 8% polyacrylamide gels. After transfer to nitrocellulose, the filters were blocked in 4% wt/vol non-fat milk (Carnation) in PBS, 0.1% wt/vol Tween 20 and probed with anti-Erk antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, SC-94) diluted 1:2,000 in the blocking solution. After washing, the filter was incubated with diluted (1:5,000) HRP-anti-rabbit antibody and detected by ECL as directed by the manufacturer (Amersham Corp., Arlington Heights, IL). For anti-phosphotyrosine immunoprecipitation, the protein extract was incubated with agarose-conjugated anti p-Tyr (Santa Cruz, SC-20A) for 4 h at 4°C, washed with cold lysis buffer, and analyzed by gel electrophoresis and Western blotting as before. For the IP kinase assay, the protein extract was incubated with anti-Erk antibody, and then with Protein A/G PLUS Agarose (Santa Cruz, SC-2003). After washing with lysis buffer, the pellets were washed in kinase buffer (20 mM TRIS, pH 8, 10 mM $MgCl_2$, 1 mM DTT) and suspended in kinase buffer plus 50 μM ATP supplemented with 10 μCi [^{32}P]ATP (ICN Biomedicals, Inc., Costa Mesa, CA) and 2 μg myelin basic protein (Sigma Chem. Co.). The kinase reaction was allowed to proceed for 10 min at room temperature and stopped by adding Laemmli sample buffer and boiling. The labeled protein was separated on gels, stained, and the myelin basic protein (MBP) was excised and counted by liquid scintillation counting or exposed to X-ray film.

Ribonuclease Mapping and Northern Analysis

For RNase mapping analysis, antisense riboprobes were generated for ANF and UlsnRNA. Labeling was achieved by including [α^{32}P]CTP (ICN Biomedicals, Inc.) in the reaction mix. All reagents for the ribonuclease mapping were from Promega Corp. (Madison, WI) and used as described by the manufacturer. For Northern analysis of MLC-2 and *fos* gene expression, RNA samples were separated on formaldehyde containing agarose gels, transferred to nitrocellulose, and probed with random-primed probes generated from isolated cDNA fragments using standard methods. Reagents for probe generation were from Stratagene (La Jolla, CA) and used as recommended by the manufacturer.

Transfection Analysis

All transfections were performed in triplicate in 3.5-cm dishes. Plasmids for transfection were purified by alkaline lysis followed by polyethylene glycol precipitation. Transfections were performed by calcium phosphate precipitation as previously described (Knowlton et al., 1991; Thorburn et al., 1993). The ANF-luciferase plasmid contains a 3-kb promoter fragment, the MLC-2 luciferase plasmid contains a 2.7-kb promoter fragment while the *fos*CAT plasmid contains a 750-bp fragment. For transfection experiments using purine inhibitors (see Fig. 3 *a*), 1 μg CMV βGal plasmid and 3 μg ANF-luciferase plasmid were used per dish. After transfection, the cells were treated with the inhibitors as required for one hour, and then phenylephrine was added to 100 μM to the relevant dishes.

For the experiments using the dominant inhibitor Erk1 mutant (see Figs. 3 *b* and 4), transfections were performed using 1 μg ANF-luciferase, MLC-2 luciferase or *fos*CAT; 0.7 μg CMVβGal or SV40βGal and 3 μg pCEP4 (Invitrogen) or pCEP4K71RErk1. pCEP4K71RErk1 was constructed and generously supplied by Dr. T. Geppert, Dallas, TX. *fos*CAT-transfected cells were harvested after 20 h, while ANF-luciferase and MLC-2 luciferase-transfected cells were harvested after 48 h and luciferase, CAT, and βGal activities determined. The luciferase or CAT values were divided by the βGal values to normalize for differences in the transfection efficiencies. All transfection data is presented as the mean ± SD from three dishes of cells per sample. The data shown in each figure is from a single representative experiment, similar results were obtained in at least three experiments using different preparations of cells and plasmids.

Immunofluorescence Analysis

Cells were cultured on plastic chamber slides (Nunc). For analysis of the effect of pharmacological inhibitors of MAP kinases on actin organization, cells were treated with 2AP (10 mM), and then phenylephrine (100 μM) as required. The cells were maintained for 48 h before fixation. Cells were washed with PBS, fixed with 3.6% formaldehyde in PBS, lysed with 0.3% Triton X-100 in PBS and blocked in 10% goat serum in PBS plus 0.1% Tween 20. Then stained with FITC-conjugated phalloidin (Sigma Chem. Co., 40 μg/ml in PBS, 0.5% NP-40, 2 mg/ml BSA), the cells were washed in PBS, 0.1% Tween 20 and mounted for fluorescence microscopy.

For analysis of the effect of expression of the dominant negative Erk1 on actin organization, cells on chamber slides were either transfected or microinjected with the dominant negative K71RErk1 expression plasmid plus an RSV-luciferase plasmid. Control transfections with the wild-type pCEP4Erk1 were also performed. 3 μg of the Erk1 expression vectors and 0.7 μg of the RSV-luciferase plasmid were used in each chamber slide. Transfections were performed as described above, phenylephrine was added after 24 h. Nuclear microinjection of expression plasmids was performed as previously described (Thorburn et al., 1993) using 0.4 μg/μl pCEP4K71 and 0.2 μg/μl RSV-luciferase. Cells were incubated for 1 h after injection to allow expression (Alberts et al., 1993), and then treated with phenylephrine for 48 h, fixed, and stained for luciferase and actin. To identify luciferase-expressing cells, a rabbit anti-luciferase antibody followed by Texas red anti-rabbit was used, and then actin was stained as described above with FITC-phalloidin. FITC and Texas red fluorescence was detected using a Zeiss Axiophot fluorescence microscope (40× or 63× objectives) and photographed with Kodak TMAX 400 film pushed to 800 ASA.

Results

The α-adrenergic agonist phenylephrine induces the hy-

pertrophic response in neonatal rat ventricular myocytes through a pathway which is partially dependent on Ras function (Thorburn et al., 1993). Since it is known that activated Ras can activate MAP kinases (Leevers and Marshall, 1992), we first examined whether these kinases were activated after phenylephrine treatment. Activation of MAP kinases Erk1 and Erk2 is associated with phosphorylation on threonine and tyrosine residues (Payne et al., 1991), and results in reduced mobility of the proteins in SDS-polyacrylamide gels. Fig. 1 *a* shows that both phenylephrine and the phorbol ester TPA, induce a rapid change in the mobility of both Erk1 and Erk2. Furthermore, phenylephrine treatment increases the phosphotyrosine content of both Erk1 and Erk2 (Fig. 1 *b*). To confirm that these kinases were activated by phenylephrine treatment, we immunoprecipitated Erks and performed an in vitro kinase assay using MBP as a substrate. Fig. 1 *c* confirms that the kinases are indeed activated by phenylephrine treatment and that this activation persists for at least twenty minutes before starting to diminish. Phenylephrine-induced activation of Erk1 and Erk2 is specific to the cardiac myocytes since non-myocytes purified from the same hearts did not show any MAP kinase activation in response to phenylephrine, although the kinases in these cells were activated by treatment with TPA (Fig. 1 *d*). Downregulation of protein kinase C (PKC) by chronic treatment with TPA prevents activation of these kinases by either TPA or phenylephrine but not by serum (Fig. 1 *e*) suggesting that activation of PKC by phenylephrine may be important in this response.

To determine whether the kinase activation observed was necessary for the hypertrophic response, we performed a series of experiments designed to examine the effects of inhibition of these kinases. The nucleotide analogues 2AP and 6TG have been shown to inhibit the MAP kinases and to prevent the differentiation of PC12 cells in response to nerve growth factor (Qiu and Green 1992; Volonte et al., 1989). Fig. 2 *a* demonstrates that pretreatment of myocytes with either 2AP or 6TG results in a significant inhibition of MBP kinase activity, indicating that the inhibitors work in cardiac myocytes as they do in PC12 cells (Qiu and Green, 1992). To determine whether this inhibition had any effect on the changes in the pattern of gene expression associated with hypertrophy, we performed experiments to measure the levels of specific mRNAs whose expression is known to be upregulated in phenylephrine-induced hypertrophy. Increased expression of the c-*fos*, ANF, and MLC-2 genes was monitored as examples of the various classes of genes (immediate early, embryonic, and contractile protein genes) whose expression is increased in hypertrophy. Fig. 2 *b* shows the result. Phenylephrine treatment for 24 h causes an increase in the amount of ANF, MLC-2, and c-*fos* mRNA which is inhibited if the cells are preincubated with 2AP or 6TG. No effect on the amount of U1 snRNA or ribosomal RNAs which were used as loading controls was observed.

To confirm this finding, and to determine whether the effect of the inhibitors was actually due to decreased promoter activity, a transient transfection experiment was performed in which the activity of an ANF promoter–luciferase plasmid was determined in the presence and absence of the inhibitors. Fig. 3 *a* shows that treatment of the cells with phenylephrine for 48 h results in a significant activation of the ANF promoter as previously shown (Knowlton et al., 1991). However, if the cells were also treated with either 2AP or 6TG, this transactivation was abolished. The inhibitors had no apparent effect on the basal level of ANF promoter activity (data not shown).

While the purine analogs inhibit the MAP kinases, they

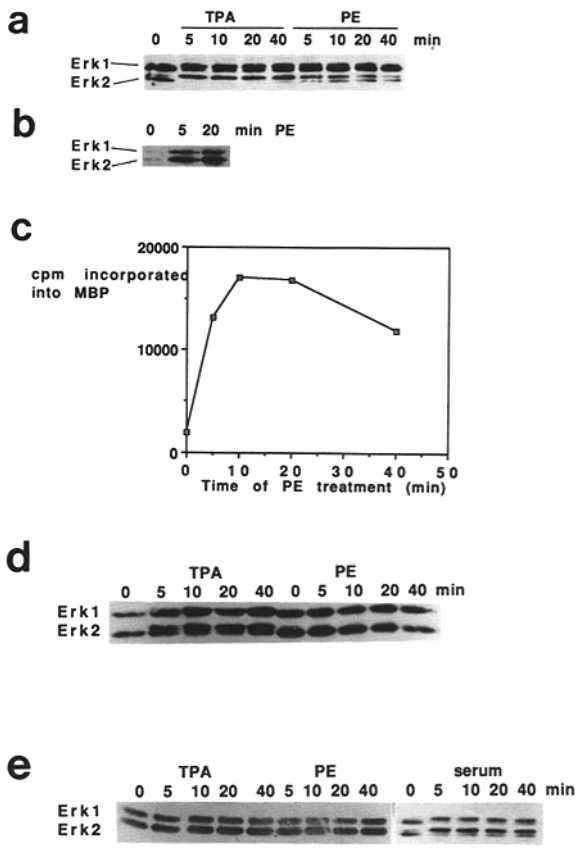

Figure 1. Activation of Erk1 and Erk2 by phenylephrine (PE) in neonatal rat cardiomyocytes. (*a*) Cultured myocytes were treated with 100 μM phenylephrine or 1 μM TPA. After 0, 5, 10, 20, or 40 min cells were harvested and the protein samples obtained were separated on SDS-polyacrylamide gels and Western blotted. The resulting blot was probed with an antibody which recognizes Erk1 and Erk2 indicating that both TPA and phenylephrine treatment retards the mobility of these proteins. (*b*) Phenylephrine-treated cells were harvested and the proteins immunoprecipitated with anti-phosphotyrosine, and then separated and Western blotted using the anti-Erk antibody, indicating that phenylephrine treatment results in increased tyrosine phosphorylation of Erk1 and Erk2. (*c*) Proteins from phenylephrine-treated cells were harvested, immunoprecipitated with the anti-Erk antibody, and used in an in vitro assay using myelin basic protein (MBP) as a substrate. After separation of the labeled protein on a gel, the band corresponding to MBP was excised and counted indicating that there is an increase in Erk kinase activity after phenylephrine treatment. (*d*) Non-myocytes (mostly fibroblasts) were purified from the cultures and treated with phenylephrine or TPA as before. After separation and Western blotting, the mobility of the Erks was determined, showing that phenylephrine does not induce Erk activation of non-myocytes cardiac cells. (*e*) PKC was downregulated by chronic treatment with 1 μM TPA for 24 h (Shubeita et al., 1992). After treatment with TPA, phenylephrine or 10% FBS, the mobility of the Erks on SDS gels was determined by Western blotting as before, showing that PKC downregulation prevents TPA and phenylephrine-induced Erk activation but not serum-induced activation.

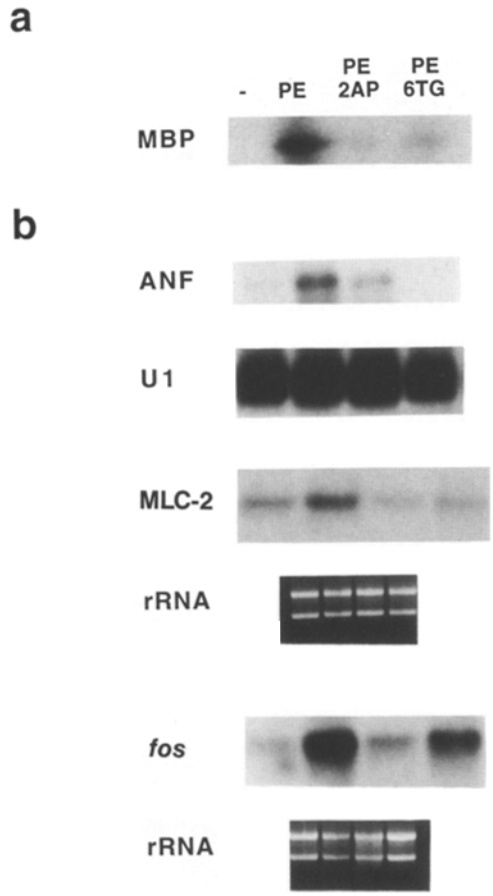

Figure 2. Inhibition of MAP kinase activity by 2AP or 6TG prevents phenylephrine (PE)-induced transactivation of gene expression. (*a*) Cardiac myocytes in serum-free media were treated as described with 10 mM 2AP or 0.5 mM 6TG (Sigma Chem. Co.) for 1 h, and then with 100 μM phenylephrine to induce MAP kinase activity. After ten minutes, the cells were harvested and the MAP kinase activity towards MBP determined by the IP kinase assay. Labeled MBP was separated on a gel which was dried and exposed to film indicating that, as expected (Qiu and Green, 1992), these treatments inhibit MAP kinases. (*b*) Cells were treated with the inhibitors and phenylephrine, and then harvested for RNA analysis. For analysis of c-*fos* expression, cells were harvested after one hour while for analysis of MLC-2 and ANF, they were harvested after 24 h. For measurement of ANF mRNA, ribonuclease mapping was performed using antisense probes against ANF and U1 snRNA as a loading control. For measurement of *fos* and MLC-2 RNA levels, Northern blots were probed with random primed probes made from isolated *fos* or MLC-2 cDNAs. Equal loading of the gels for Northern analysis was confirmed by staining the ribosomal RNA with ethidium bromide.

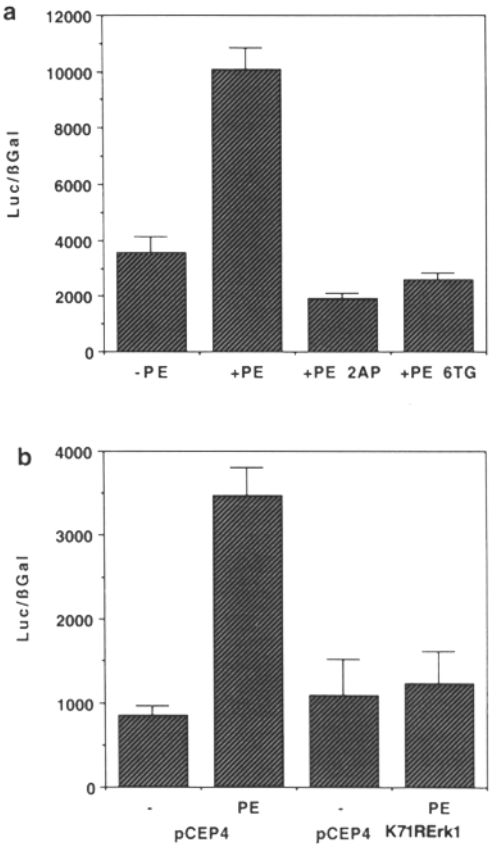

Figure 3. Inhibition of MAP kinase activity prevents phenylephrine (PE)-induced activation of the ANF promoter. (*a*) Transient transfections were performed using ANF-luciferase and CMV-βGal plasmids. After transfection, cells were treated with 10 mM 2AP or 0.5 mM 6TG for one hour as required, and then treated with 100 μM phenylephrine (PE). 48 h later cells were harvested and luciferase and βGal enzyme activities determined. The relative luciferase levels (normalized to βGal) were plotted. Each bar represents the mean ($\pm$SD) from three separate dishes of cells. (*b*) Transient transfection analysis was carried out using ANF-luciferase, CMV βGal, and either pCEP4 (Invitrogen) or pCEP4 K71RErk1 (constructed and generously provided by Dr. T. Geppert, University of Texas, Dallas, TX). After treatment with phenylephrine as described, relative luciferase levels were determined, showing that the mutant Erk1 prevents phenylephrine-induced transactivation of the ANF promoter.

are not specific for these kinases and it is possible that the effect of gene expression is actually due to other effects of the inhibitors. To address this problem, we performed transient transfection experiments using a mutant Erk1 molecule (K71RErk1) which acts as a dominant inhibitor. This molecule is mutated at the ATP-binding site resulting in a nonfunctional kinase which has been shown to be a dominant negative inhibitor capable of preventing Ras-induced activation of the TPA response element (Frost et al., 1994). We do not know the mechanism of action of the kinase deficient mutant in these experiments. Expression of the corresponding kinase-deficient Erk2 molecule (K52RErk2) in CV1 cells inhibits basal and EGF-induced MAP kinase activity (Sontag et al., 1993) while a different dominant negative Erk1 molecule mutated in the activation phosphorylation sites has been shown to prevent activation of endogenous MAP kinases and has been used to show a requirement for MAP kinase activation for growth of fibroblasts (Pages et al., 1993). An ATP-binding site, kinase-deficient dominant negative Raf-1 molecule, has been shown to prevent activation of the endogenous Raf-1 kinase in Xenopus oocytes (MacNichol et al., 1993). Taken together, these data would suggest that the most likely mode of action is that the mutant competes for MAP kinase activators thus preventing activation of the MAP kinases.

Transfections were performed using the ANF-luciferase plasmid plus the mutant Erk1 or the parental expression vec-

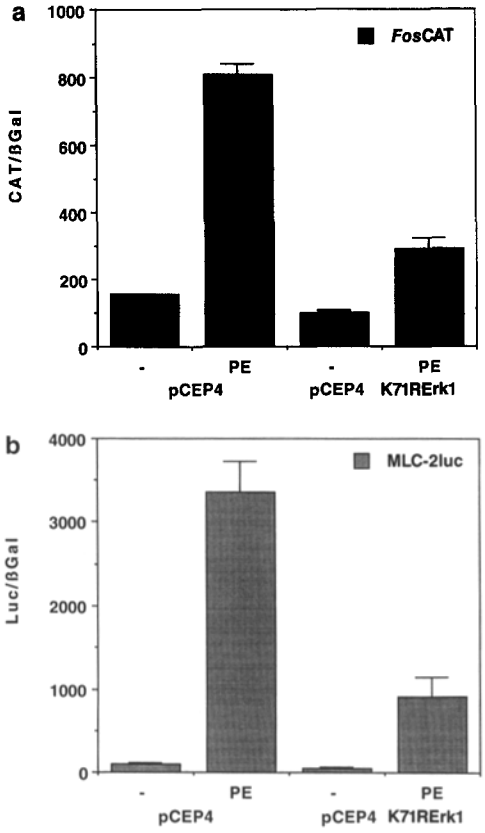

Figure 4. K71R Erk1 prevents phenylephrine-induced transactivation of the *fos* and MLC-2 promoters. Transient transfection analysis was carried out using *fos*CAT (*a*) or MLC-2-luciferase (*b*), SV40βGal and either pCEP4 or pCEP4 K71RErk1 plasmids. After treatment with phenylephrine, relative CAT and luciferase activities were determined, showing that the mutant Erk1 inhibits phenylephrine-induced transactivation of the *fos* and MLC-2 promoters.

tor (pCEP4), and the cells were treated with or without phenylephrine. Fig. 3 *b* shows that phenylephrine activated the ANF promoter when the parental vector was cotransfected but that this transactivation is abolished by cotransfection with the mutant Erk plasmid. No significant effect on basal ANF promoter activity was found. This result therefore confirms that MAP kinase activity is important for the activation of the ANF promoter by phenylephrine treatment. Similar transfection experiments were also performed to determine whether MAP kinase activity was required for activation of the *fos* and MLC-2 promoters by phenylephrine. Fig. 4 shows that expression of the mutant Erk1 molecule also inhibits phenylephrine-induced transactivation of these promoters.

The data outlined above suggests that MAP kinase activity is required for at least some hypertrophic responses to phenylephrine. We were therefore interested in looking at different hypertrophic phenotypes. In the neonatal rat cells, hypertrophy is associated with increased organization of contractile proteins such as MLC-2 (Iwaki et al., 1990) and actin. This effect can be conveniently monitored by staining filamentous actin with fluorescently labeled phalloidin. Fig. 5 shows an experiment where cells were treated with or without phenylephrine in the presence of 2AP. Panel *a* shows cells maintained in serum-free medium with no phenylephrine. These

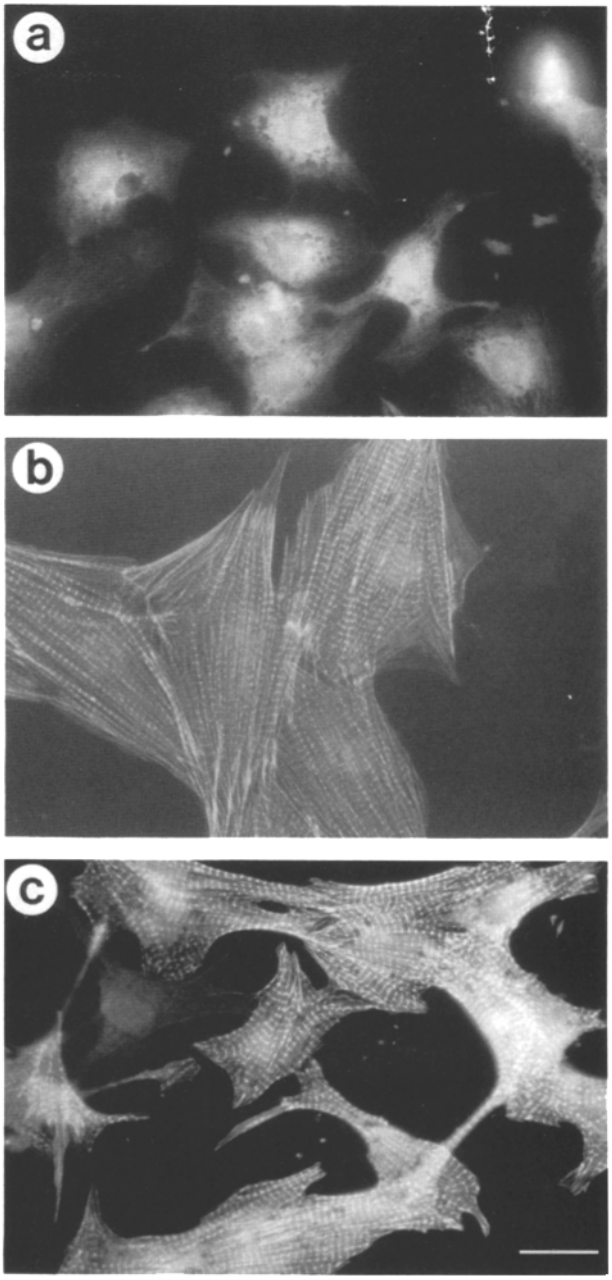

Figure 5. MAP kinase inhibition by 2AP does not prevent phenylephrine-induced organization of actin. Cells were plated onto plastic chamber slides, maintained in serum-free medium, treated with 10 mM 2AP as required, and then with phenylephrine. After 48 h, the cells were washed, fixed, and stained with FITC-conjugated phalloidin to visualize the actin fibers. *a* shows cells which were kept in serum-free medium and have not hypertrophied, they are small and the actin is not organized into contractile units. *b* shows phenylephrine-treated cells, which show the morphology associated with hypertrophy, i.e., they are larger, more regularly shaped and have highly organized actin fibers as shown by the brightly stained bands. *c* shows cells treated with phenylephrine in the presence of 10 mM 2AP. These cells also have highly organized actin visible as bright bands but are not as large or as regularly shaped as the phenylephrine-treated cells in *b*. Bar, 20 μm.

cells are small and their actin is disorganized. Panel *b* shows cells treated with phenylephrine. These cells have increased in size and show highly organized actin visible as discrete fluorescent bands. Panel *c* shows cells treated with both 2AP and phenylephrine. Clearly, these cells also have organized actin, however we note that they seem to be smaller than the phenylephrine-treated cells and often have a spindly shape that is unlike the normal, hypertrophied cells. Cells treated with phenylephrine and 6TG also showed distinct actin organization (data not shown). These data therefore suggest that at least one of the morphological effects associated with hypertrophy (actin organization) does not require MAP kinase activity or any other activity which is also inhibited by 2AP.

To confirm this result, an experiment was performed where the mutant Erk1 expression plasmid was transfected or injected into cells along with a constitutively active RSV-luciferase molecule which was used to identify cells containing the expression plasmids (Thorburn et al., 1993). After treatment with phenylephrine for 48 h, the cells were fixed and stained for luciferase and with FITC-conjugated phalloidin. Fig. 6 shows that cells expressing the mutant kinase as a result of either transfection or microinjection have highly organized actin again suggesting that inhibition of MAP kinase activity does not prevent cytoskeletal rearrangement. As expected, expression of the wild-type Erk1 protein also had no effect on phenylephrine-induced actin organization.

Discussion

This work demonstrates that MAP kinase activation by phenylephrine is important for transactivation of the *fos*, ANF, and MLC-2 genes which are all activated as a result of hypertrophic stimulation. However, MAP kinase activity does not seem to be required for the changes in contractile protein organization which also occurs as a result of phenylephrine treatment.

Agonists which induce the hypertrophic response in neonatal ventricular myocytes activate MAP kinase activity (Fig. 1, Bogoyevitch et al., 1993; Sadoshima and Izumo, 1993*a*; Yamazaki et al., 1993; Bogoyevitch et al., 1994). We

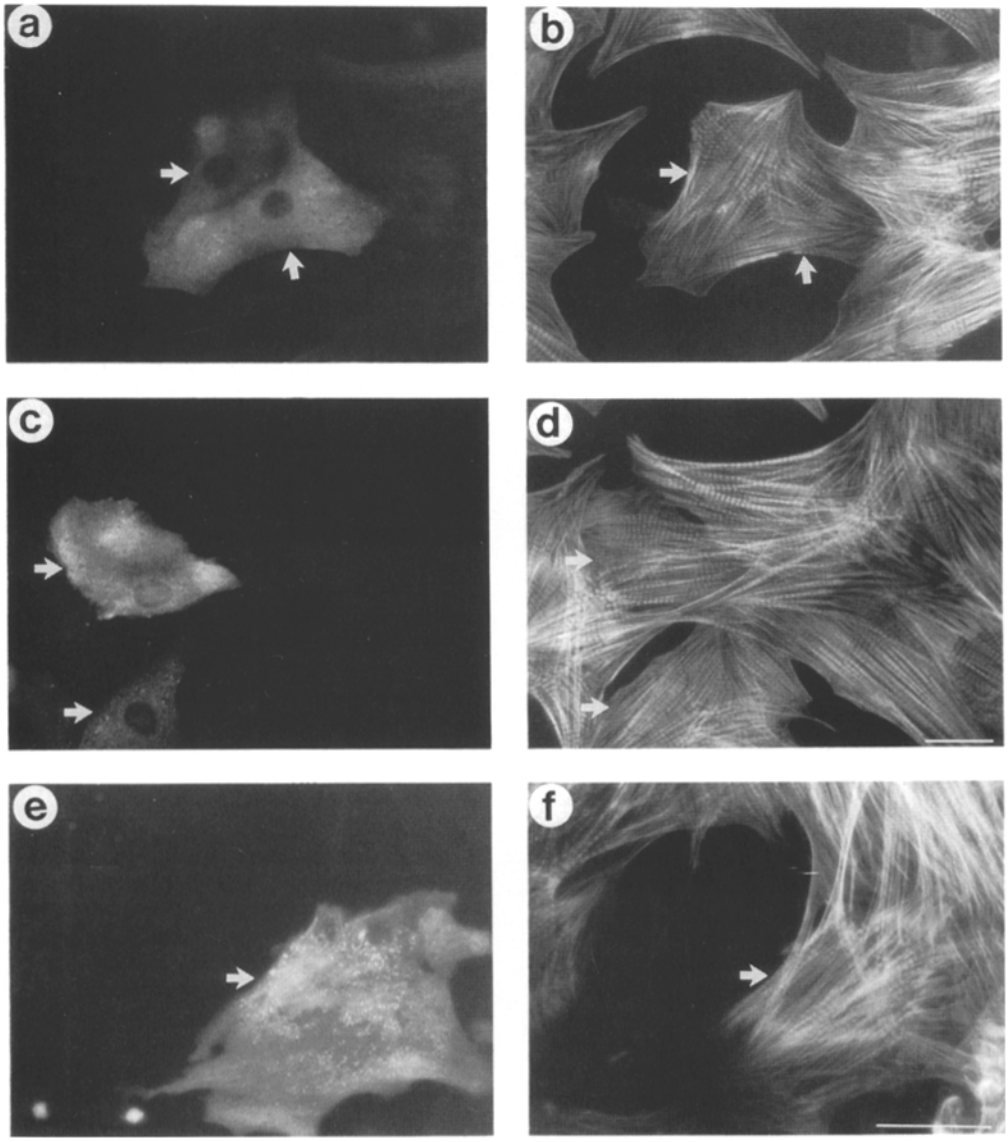

Figure 6. Transfected or microinjected K71RErk1 does not prevent phenylephrine-induced organization of actin. Cells on chamber slides were transfected with pCEP4K71R-Erk1 plus RSV-luciferase (*a* and *b*), or, as a control, with the wild-type pCEP4Erk1 plus RSV-luciferase (*c* and *d*). Alternatively cells were microinjected with pCEP4K71R-Erk1 plus RSV-luciferase (*e* and *f*). After treatment with phenylephrine for 48 h, the cells were fixed and stained with an antibody against luciferase to identify the transfected or injected cells and with FITC-phalloidin to show actin. *a*, *c*, and *e* show luciferase staining which is in a speckled pattern as a result of luciferase transport into peroxisomes while *b*, *d*, and *f* show actin staining of the same fields. Note that all the cells including those that were transfected or injected (*arrowed*) have highly organized actin indicating that expression of the mutant Erk1 does not prevent actin organization. Bars, 20 μm.

have extended these observations to determine the effects of reducing MAP kinase activity in the cell by either treating the cells with pharmacological agents which inhibit MAP kinases or by expressing a dominant inhibitor of the 44-kD MAP kinase, Erk1. Inhibition of MAP kinases by either of these approaches prevents phenylephrine-induced activation of gene expression (Figs. 2, 3, and 4). Using either these pharmacological inhibitors or similar dominant negative mutants, a requirement for MAP kinase activation has also been demonstrated for NGF-induced differentiation of PC12 cells (Qiu and Green, 1992), proliferation of fibroblasts (Pages et al., 1993), and activation of AP1 transcriptional activity (Frost et al., 1994; Pages et al., 1993). We note that expression of the mutant Erk1 molecule is able to completely block phenylephrine-induced transactivation of the ANF promoter. We do not know whether this means that only Erk1 is important for this phenotype or whether the one mutant inhibits more than one of the MAP kinases. A similar effect was found for inhibition of fibroblast proliferation and growth factor–induced AP1 activity using a different Erk1 inhibitor (Pages et al., 1993). We cannot exclude the possibility that the dominant negative Erk1 molecule also inhibits other kinases which are similar to the MAP kinases, and it is therefore possible that some of the effects which we observed may be due to inhibition of these molecules.

The experiments presented here also show that not all phenotypes associated with a particular signaling pathway need necessarily require normal levels of MAP kinase activity since inhibition of MAP kinases by either 2AP treatment or expression of the K71R mutant does not prevent the changes in actin organization which are associated with the hypertrophic response (Figs. 5 and 6). It should be noted that it is unlikely that inhibition of MAP kinase activity by either approach was 100% effective. It is therefore possible that a low level of MAP kinase activity does play a role in actin organization.

Despite this caveat, the simplest explanation of our data is that the diverse effects associated with cardiac cell hypertrophy (i.e., both gene expression and morphological changes) arise from signal transduction pathways which diverge upstream of the MAP kinases. Previously, we showed that activated Ras protein could induce both ANF expression and contractile protein organization (Thorburn et al., 1993) suggesting that the divergence in the signaling pathway leading to both types of phenotype occurs between Ras and MAP kinase. This might be achieved by using different Ras effector molecules for each effect. An attractive idea would be that the Raf-1 kinase is the Ras effector which leads to MAP kinase activation (Howe et al., 1992; Kyriakis et al., 1992; Moodie et al., 1993) and subsequent gene expression changes while another effector molecule such as $p120^{GAP}$ leads to morphological changes perhaps via p190 and the Rho protein (McGlade et al., 1993). Recently, we have found that an estradiol-inducible Raf-1 molecule can induce MAP kinase activation and cause expression from the *fos*, ANF, and MLC-2 promoters in the absence of any other stimuli but is not able to induce actin organization (Thorburn, J., M. McMahon and A. Thorburn, manuscript submitted for publication) supporting this hypothesis.

Ras has been shown to be involved in other heterotrimeric G protein–coupled receptor signaling pathways (Kupperman et al., 1993; LaMorte et al., 1993; van Corven et al., 1993) including MAP kinase activation by lysophosphatidic acid (Cook et al., 1993; Howe and Marshall, 1993) and the acetylcholine muscarinic m2 receptor (Winitz et al., 1993), and so the involvement of the Ras-Raf-MEK-MAP kinase signaling pathway in G protein–coupled systems may be common. However, Ras-independent pathways exist which can lead to MAP kinase activation (Lange-Carter et al., 1993; Burgering et al., 1993), and these pathways may be especially important for heterotrimeric G–protein coupled systems. It is likely that a complicated network of interrelated signaling pathways regulate cellular responses like growth and hypertrophy and it will be necessary to study each putative signaling molecule independently to uncover the relationships between the pathways involved. The strategies used in this study are applicable to other signaling molecules and it should be possible to use this approach to define the various relationships between different signaling pathways. It is not surprising that signal transduction pathways diverge to cause different phenotypes, however the clear separation of the responses associated with cardiac myocyte hypertrophy may make this a particularly useful experimental system in which to analyze such complicated pathways.

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