Specific role of the extracellular signal-regulated kinase pathway in angiotensin II-induced cardiac hypertrophy *in vitro*

Hiroki AOKI*, Mary RICHMOND†, Seigo IZUMO* and Junichi SADOSHIMA†[1]

*Cardiovascular Division, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue SL-201, Boston, MA 02215, U.S.A., and †Weis Center for Research, Pennsylvania State College of Medicine, 100 North Academy Avenue, Danville, PA 17822-2616, U.S.A.

Although MAP (mitogen-activated protein) kinases are implicated in cell proliferation and differentiation in many cell types, the role of MAP kinases in cardiac hypertrophy remains unclear. We examined the role of extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase in angiotensin II (Ang II)-induced hypertrophy compared with phenylephrine-induced hypertrophy in neonatal rat cardiac myocytes. Both Ang II and phenylephrine activated ERKs to a similar extent, whereas phenylephrine caused stronger and more sustained activation of JNK and p38 than Ang II. PD98059, a specific inhibitor of MAPK/ERK kinase (MEK), inhibited Ang II-induced, but not phenylephrine-induced, expression of atrial natriuretic factor (ANF) at both the mRNA and polypeptide levels. SB203580, a specific inhibitor of p38 and some JNK isoforms, did not show significant effects on ANF expression induced by Ang II or phenylephrine. Although PD98059 and dominant-negative MEK1 blocked Ang II-induced activation of the ANF promoter, SB203580 or dominant-negative MEK kinase 1 (MEKK1) showed no effect. Phenylephrine-induced ANF promoter activation was significantly inhibited by SB203580 and dominant-negative MEKK1, but not by PD98059 or dominant-negative MEK1. Dominant-negative Ras inhibited both ERK activation and ANF up-regulation by Ang II, whereas constitutively active forms of Ras and MEK were sufficient to activate the ANF promoter. Dominant-negative Ras also partly inhibited the phenylephrine-induced activation of ANF promoter. PD98059 did not affect other markers of Ang II-induced hypertrophy, such as skeletal α-actin and c-*fos* expression, increases in the rate of protein synthesis or rapid sarcomeric actin organization. These results suggest that Ang II uses ERK for ANF expression, whereas phenylephrine uses other pathways. The Ras/ERK pathway selectively mediates ANF expression in various phenotypes observed in Ang II-induced hypertrophy. The ERK pathway mediates an agonist-specific and phenotype-specific response in cardiac hypertrophy.

Key words: atrial natriuretic factor, ERK, MAP kinase, phenylephrine, Ras.

INTRODUCTION

Mitogen-activated protein kinases (MAP kinases) are signalling molecules conserved throughout evolution, which transduce extracellular stimuli into intracellular responses. Several parallel kinase cascades lead to the activation of different members of the MAP kinase family, including extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Recent studies have shown that ERKs mediate cellular responses initiated by growth factors, whereas JNK and p38 predominantly mediate cellular responses induced by environmental stress or proinflammatory cytokines, although some cross-talk exists between extracellular stimuli and the kinases activated (reviewed in [1–5]). Many growth factors activate ERKs via Ras-dependent mechanisms. Activated Ras recruits the serine/threonine kinase Raf-1 to the plasma membrane, where Raf-1 is activated by phosphorylation [6,7] and conformational changes [8]. Raf-1 phosphorylates and activates MAPK/ERK kinase (MEK), which in turn activates ERK1/2. Activated ERKs phosphorylate serine/threonine residues on cellular substrates, including transcription factors (Elk-1, SAP-1 and c-Myc), RNA polymerase II, translation factors (e.g. PHAS-I), p90RSK, MNK1 and 2, protein phosphatase 2C, phospholipase A2, tyrosine hydroxylase and EGF (epidermal growth factor) receptors. In dividing cells such as cultured fibroblasts, disruption of the ERK pathway typically results in the inhibition of growth responses, whereas its stimulation accelerates cell proliferation [9]. However, the precise role of the ERK pathway in non-dividing cells, such as terminally differentiated cardiac myocytes, has not been fully elucidated.

In cultured cardiac myocytes, ERKs are activated by hypertrophic stimuli such as angiotensin II (Ang II) [10], phenylephrine [11,12], endothelin 1 [12–14] and PMA [13], as well as cellular stresses such as mechanical stretching [15,16] or osmotic shock [17,18]. Although the role of ERKs in phenylephrine-induced cardiac hypertrophy has been studied by several investigators with different methods, reported roles of ERKs in cardiac hypertrophy vary substantially [19–23]. Furthermore, recent evidence indicates that agonists for the G_q-coupled receptor activate not only ERKs but also other members of the MAP kinase family, namely JNK and p38 [17,24–28], and that these MAP kinases are also important in cardiac hypertrophy [25,26,29–32] (reviewed in [5]). However, it should be noted that relative levels of activation of each MAP kinase can differ depending on the type of stimulus; therefore the role of ERKs in

Abbreviations used: ANF, atrial natriuretic factor; Ang II, angiotensin II; βgal, β-galactosidase; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; dnRas, dominant-negative Ras; ERK, extracellular signal-regulated protein kinase; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; Luc, firefly luciferase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEKK1, MEK kinase 1; moi, multiplicity of infection; PKC, protein kinase C; SIE, *sis*-inducing factor element; SKA, skeletal α-actin; STAT, signal transducer and activator of transcription; TCF, ternary complex factor.

[1] To whom correspondence should be addressed (e-mail jsadoshima@psghs.edu).

cardiac hypertrophy must be determined for each hypertrophic stimulus.

Here we report that ERKs selectively mediate the expression of atrial natriuretic factor (ANF), a hypertrophic marker, among the major responses observed in Ang II-induced cardiac hypertrophy. The essential role of ERKs in ANF expression is specific to Ang II, because ERK activity is dispensable for ANF expression by phenylephrine, another G_q-coupled agonist. Our results suggest that the signalling mechanism of cardiac hypertrophy, including the role of each member of the MAP kinase family, might be substantially different in G_q-coupled agonists.

EXPERIMENTAL

Primary culture of neonatal rat ventricular myocytes

Primary cultures of cardiac ventricular myocytes from 1-day-old Crl:(WI)BR-Wistar rats (Charles River Laboratories) were prepared as described previously [33]. In brief, ventricular myocytes were dissociated enzymically and preplated for 1 h to enrich them for myocytes. Cells were cultured in cardiac myocyte culture medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% (v/v) horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite (Gibco BRL), 2 g/l BSA (fraction V), 3 mM pyruvic acid, 15 mM Hepes, 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid and 100 μM 5-bromo-2′-deoxyuridine (Sigma). Culture medium was changed to serum-free at 24 h. Myocytes were cultured under serum-free conditions for 48 h before experiments. In some experiments, myocytes were treated with various concentrations of PD98059 (Parke-Davis), SB208530 (Calbiochem) or vehicle (DMSO) 30 min before and during experiments.

Western blot analyses of activated MAP kinases

Cardiac myocyte cultures were stimulated with 100 nM Ang II or 10 μM phenylephrine supplemented with 2 μM propranolol for the indicated duration. We used 100 nM for Ang II because it caused maximum increases in the ERK phosphorylation (see below and [10]) and 10 μM for phenylephrine because it caused comparable increases in ERK2 phosphorylation (see below). Cells were lysed in lysis buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM dithiothreitol, 1 mM PMSF and 10 μg/ml aprotinin. Equal amounts of total protein from each lysate were resolved by SDS/PAGE [10% (w/v) gel] and transferred on PVDF membranes (Millipore). Activated MAP kinases were detected by using phospho-specific antibodies against ERK (E-4), JNK (G-7) (Santa Cruz) or p38 (New England Biolabs). The probed proteins were detected by Western Blot Chemiluminescence Reagent Plus (NEN) in the linear range of X-ray film.

Immune complex kinase assays of MAP kinases

The activities of MAP kinases (ERK2, JNK1 and p38) were also measured by immune complex kinase assays. Myocytes were stimulated by Ang II (100 nM) or phenylephrine (10 μM) for indicated times, and lysed with 1 ml of lysis buffer containing 25 mM Tris, pH 7.4, 25 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μM okadaic acid, 0.5 mM EGTA, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 10 μg/ml aprotinin and 100 μM leupeptin. Cell debris was separated by centrifugation (18000 ***g*** at 4 °C for 20 min). Immunoprecipitations were performed by incubating samples with 1 μg/ml of rabbit polyclonal antibody raised against ERK2, JNK1 or p38 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 3 h, followed by the addition of 40 μl of Protein A–Sepharose [50% (w/v) slurry] for 1 h at 4 °C. Immunoprecipitates were washed three times with the lysis buffer and once with a kinase assay buffer containing 20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate and 2 mM dithiothreitol. Kinase reactions were started by the addition of 25 μM of unlabelled ATP, 5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) and 4 μg of substrate. Myelin basic protein (Sigma), GST–c-Jun (1–79) and GST–ATF2 (1–96) (Santa Cruz Biotechnology) were used as substrates for ERK2, JNK1 and p38 respectively. After 30 min of incubation at 30 °C, reactions were terminated by adding the Laemmli sample buffer and boiling the samples for 10 min. Samples were resolved by SDS/PAGE [12% (w/v) gel]. Proteins were transferred on PVDF membranes and phosphorylated substrates were detected by autoradiography in the linear range of X-ray film. The membranes were also probed with anti-ERK2, anti-JNK1 or anti-p38 antibodies (Santa Cruz Biotechnology) to confirm equal loading of the immunoprecipitated kinases. The probed proteins were detected by Western Blot Chemiluminescence Reagent Plus in the linear range of X-ray film.

In-gel kinase assays for ERKs

The activities of ERKs were also determined by the immune complex in-gel kinase assay as described previously [10]. ERK activities were measured 5 min after Ang II stimulation because ERKs are maximally activated at this time point. For the immunoprecipitation of ERKs, rabbit polyclonal antibody, which recognizes both ERK1 and ERK2 (Zymed) was used.

Immunofluorescent cell staining

Myocytes grown on glass coverslips were fixed in 3.7% (w/v) formaldehyde for 10 min. ANF was detected by anti-(α-ANP 1–28) antibody (Peninsula Laboratories) (1:200 dilution) followed by FITC-conjugated donkey anti-rabbit IgG antibody (1:50 dilution). Myocytes were identified by staining with anti-(sarcomeric myosin heavy chain) antibody (MF20) followed by Texas Red-conjugated donkey anti-mouse IgG antibody (1:50 dilution). Actin fibres were stained with 40 μg/ml FITC-conjugated phalloidin (Sigma), as described previously [11].

Transient transfection and reporter gene assays

For transient transfection, myocytes were plated at a density of 10^6 cells per well in six-well plates. At 24 h after plating, the medium was changed to DMEM/F12 without supplement. Transfections were performed with 10 μl/ml Lipofectamine (Gibco BRL) in 1 ml of DMEM/F12 medium per well. A plasmid (0.5 μg/ml) containing a 638 bp fragment of the rat ANF promoter linked to firefly luciferase (ANF–Luc, courtesy of Dr K. R. Chien, University of California, San Diego, CA, U.S.A. [34]) was used as a reporter gene. In some experiments, myocytes were co-transfected with 1 μg/ml of an expression plasmid encoding dominant-negative MEK kinase 1 (MEKK1) [dnMEKK1; MEKK1(K1257M)] [35], dominant-negative MEK1 (MEK1(K97M)), constitutively active MEK1 [MEK1 (ΔN3-S218E-S222D)] [36], dominant-negative Ras (N17Ras) or constitutively active Ras (L61Ras) (courtesy of Dr K. L. Guan, University of Michigan, Ann Arbor, MI, U.S.A.). Total amounts

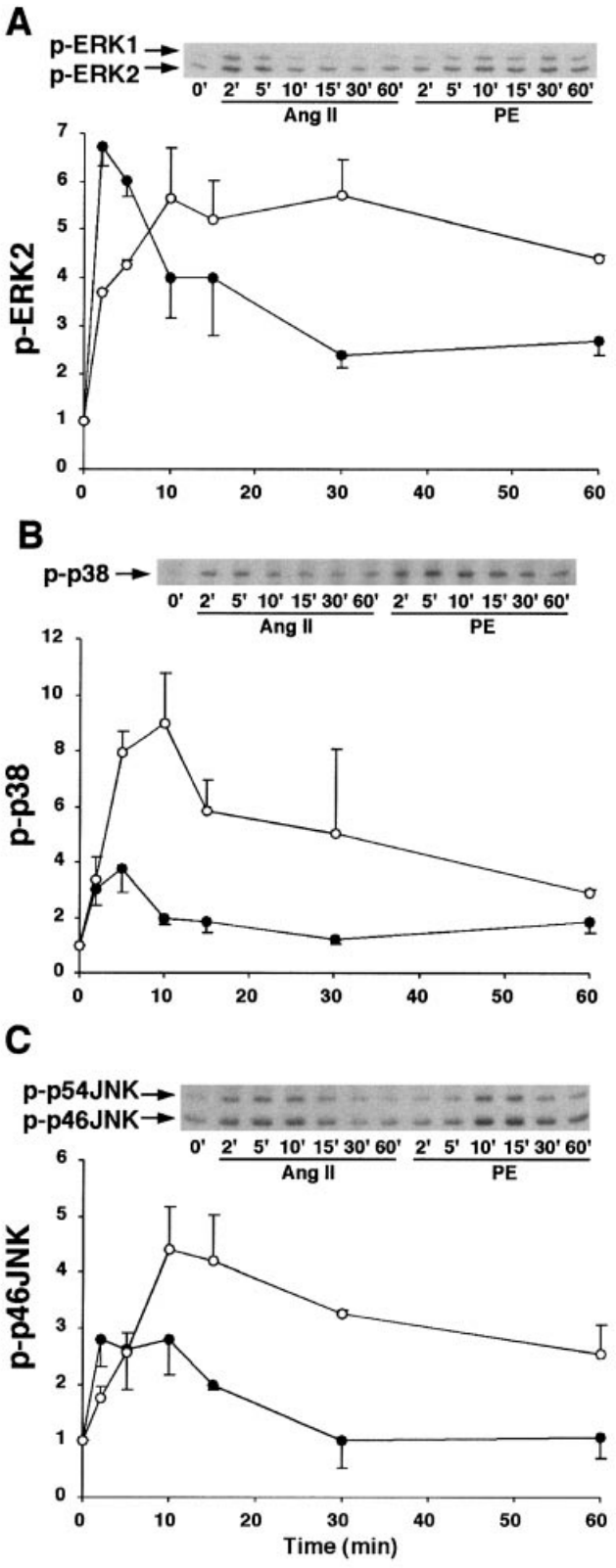

Figure 1 Phosphorylation (activation) profile of MAP kinases by Ang II and phenylephrine

Phosphorylated (activated) ERK1 (p-ERK1) and ERK2 (p-ERK2) (**A**), p38 (p-p38) (**B**) and p46JNK (p-p46JNK) and p54JNK (p-p54JNK) (**C**) in cardiac myocytes stimulated with Ang II (●) or phenylephrine (PE, ○) were quantified by densitometry after Western blotting with phospho-specific antibodies. Representative gel pictures of corresponding kinase(s) are shown in insets. Densitometric analyses for p-ERK1 and p-p54JNK are not shown, for clarity. All results are expressed relative to the control (0 min), which was assigned a value of 1. Results are means ± S.E.M. for three independent experiments.

of DNA were adjusted to 2 μg/ml by the addition of empty vector. At 24 h after the transfection, medium was changed to cardiac myocyte culture medium without serum. Myocytes were cultured for 48 h in serum-free medium and then in the presence or absence of Ang II or phenylephrine supplemented with propranolol in serum-free medium for a further 48 h. Myocytes were then lysed with the Reporter lysis buffer (Promega) and luciferase activities were measured. A cytomegalovirus (CMV) promoter-driven β-galactosidase (βgal) construct (CMV–βgal, 0.5 μg/ml) was co-transfected and βgal activities were determined with Lumi-Gal 530 (Lumigen). The luciferase values were divided by the βgal values to correct for differences in transfection efficiency.

Dominant-negative Ras adenovirus vector

Cardiac myocytes grown in 60 mm dishes were starved of serum for 48 h. Myocytes were infected with either an adenovirus vector encoding the dominant-negative Ras (Ad5.N17Ras) (courtesy of Dr M. D. Schneider, Baylor College of Medicine, Houston, Texas, U.S.A.) or a control adenovirus vector (Ad5/ΔE1sp1B) (courtesy of Dr B. French, Baylor College of Medicine) at a multiplicity of infection (moi) of 10, 30 or 100. Preliminary experiments with adenovirus containing CMV–βgal indicated that more than 95% of cardiac myocytes were stained with X-Gal (5-bromo-4-chloroindol-3-yl β-D-galactopyranoside) when an moi of 100 per myocyte was used for infection (results not shown). All experiments were performed 48 h after infection.

Incorporation of [^{3}H]phenylalanine

[^{3}H]Phenylalanine incorporation was determined as described previously [33]. Myocytes were incubated with 5 μCi/ml [^{3}H]-phenylalanine in the cardiac myocyte culture medium, which contained 0.36 mM unlabelled phenylalanine, then stimulated with Ang II for 24 h. Cells were rinsed three times with PBS and treated with 1 ml of 10% (w/v) trichloroacetic acid for 60 min at 4 °C to precipitate proteins. The precipitate was washed three times with 95% (v/v) ethanol and resuspended in 0.15 M NaOH. Aliquots were counted in a liquid-scintillation counter.

Analysis of mRNA

Total RNA was extracted from myocytes with Trizol (Gibco BRL) (1 ml per 60 mm dish). The RNA was resolved by electrophoresis on 1% (w/v) agarose gels and transferred to nitrocellulose membranes. The probes for c-*fos*, ANF, and skeletal α-actin (SKA) were used as described previously [37].

Statistics

Results are given as means ± S.E.M. Statistical analyses were performed by using ANOVA (analysis of variance). The post-test comparison was performed by the method of Bonferroni. Significance was accepted at $P < 0.05$.

RESULTS

Phosphorylation profiles of MAP kinases are different between Ang II and phenylephrine

We first characterized phosphorylation profiles of MAP kinases in response to Ang II and phenylephrine. We used phospho-

specific antibodies that recognized only dual-phosphorylated (activated) forms of MAP kinases and performed Western blot analyses. Ang II (100 nM) and phenylephrine (10 μM) caused comparable increases in ERK2 phosphorylation (6.3 $\pm$ 0.4-fold compared with 5.4 $\pm$ 0.6-fold; not significant) (Figure 1A). ERK2 phosphorylation by Ang II was more transient than that by phenylephrine. Similar results were obtained for ERK1 phosphorylation (activation) (Figure 1A, inset). The effect of Ang II on p38 and JNK phosphorylation was substantially different from that of phenylephrine. Ang II modestly increased p38 phosphorylation 3.8 $\pm$ 0.9-fold, whereas phenylephrine strongly increased p38 phosphorylation 9.0 $\pm$ 1.8-fold (Figure 1B). Phosphorylation of p38 by phenylephrine persisted longer than that by Ang II. Although both Ang II and phenylephrine increased the phosphorylation of p46JNK, the phosphorylation of p46JNK by phenylephrine (Figure 1C; 4.4 $\pm$ 0.8-fold at 10 min) was stronger and more prolonged than that by Ang II (2.8 $\pm$ 0.6-fold at 10 min). Phosphorylation of p54JNK showed a similar profile to that of p46JNK (Figure 1C, inset). To confirm that MAP kinases were activated by Ang II and phenylephrine, we performed immune-complex MAP kinase assays. Ang II activated ERK2 6.3 $\pm$ 0.9-fold at 5 min ($n = 3$), JNK1 1.9 $\pm$ 0.4-fold at 10 min ($n = 3$) and p38 1.9 $\pm$ 0.3-fold at 10 min ($n = 3$), whereas phenylephrine activated ERK2 8.3 $\pm$ 1.5-fold at 5 min ($n = 3$), JNK1 3.8 $\pm$ 0.7-fold at 10 min ($n = 3$) and p38 2.9 $\pm$ 0.8-fold at 10 min ($n = 3$). Equal loading of each immunoprecipitated kinase was confirmed by Western blot analyses with the respective antibodies (results not shown). These results indicate that Ang II and phenylephrine have distinct profiles of MAP kinase phosphorylation, and hence activation, despite the fact that they are both agonists for the G_q-coupled receptors.

Effects of PD98059 and SB203580 on Ang II- or phenylephrine-induced ANF gene expression

Because Ang II and phenylephrine produce distinct MAP kinase activation profiles, we examined the role of members of the MAP kinase family in Ang II- or phenylephrine-induced ANF mRNA expression, a genetic marker of cardiac hypertrophy. We first examined the role of ERKs by using a specific MEK/ERK pathway inhibitor, PD98059. PD98059 inhibited both Ang II- and phenylephrine-induced ERK1 and ERK2 activation in a concentration-dependent manner with an IC_{50} of 2 μM and complete inhibition at 30 μM (results not shown). As shown in Figures 2(A) and 2(B), both Ang II (2.3 $\pm$ 0.3-fold; $P < 0.05$ compared with control) and phenylephrine (3.7 $\pm$ 0.6-fold; $P < 0.05$ compared with control) induced the expression of ANF mRNA, confirming previous observations [37,38]. Treatment of myocytes with 30 μM PD98059 caused a small suppression of basal expression of ANF mRNA (0.8 $\pm$ 0.1-fold), and complete inhibition of Ang II-induced ANF expression (0.9 $\pm$ 0.1-fold; not significant), whereas it had little effect on phenylephrine-induced ANF expression (3.7 $\pm$ 0.5-fold; $P < 0.05$ compared with control).

Because both Ang II and phenylephrine activated p38 and JNK (Figure 1), we also examined the effect of SB203580, a specific inhibitor of p38 and some isoforms of JNK [27], on the expression of ANF mRNA. As shown in Figure 2(B), treatment of cardiac myocytes with 20 μM SB203580 (hatched columns) showed no significant effect on Ang II-induced (2.3 $\pm$ 0.4-fold; $P < 0.05$ compared with control) or phenylephrine-induced (3.2 $\pm$ 0.1-fold; $P < 0.05$ compared with control) expression of ANF mRNA.

The effects of PD98059 or SB20580 on Ang II- or phenylephrine-induced ANF polypeptide expression were also examined by immunostaining. Ang II (Figure 2C) or phenylephrine (results not shown) caused the perinuclear accumulation of ANF polypeptide in cardiac myocytes. Semi-quantitative scoring of ANF-positive cells (Table 1) revealed that both Ang II and phenylephrine increased the number of ANF-positive myocytes from 16 % at the baseline to 35 % (Ang II) and 42 % (phenylephrine). Treatment of myocytes with 30 μM PD98059 decreased both basal expression and the Ang II-induced accumulation of ANF polypeptide, whereas it did not affect the phenylephrine-induced increases in ANF-positive myocytes. Treatment of cardiac myocytes with 20 μM SB203580 showed no significant effect on basal, Ang II- or phenylephrine-induced ANF polypeptide expression.

These results demonstrate that, at both the mRNA and the polypeptide levels, Ang II-induced ANF expression is dependent on the activity of ERK. Basal ANF expression is also sensitive to PD98059, suggesting that basal activity of ERK might contribute to the low-level expression of ANF under basal conditions. In contrast, the SB203580-sensitive pathway seems to have no significant role in Ang II- or phenylephrine-induced ANF expression.

Involvement of ERK pathway in Ang II- or phenylephrine-induced ANF promoter activation

Because Ang II- and phenylephrine-induced ANF expression showed different sensitivities to PD98059, we examined the involvement of ERK in ANF promoter activation, by using the ANF promoter–luciferase reporter gene construct (ANF–Luc) [39]. As shown in Figure 3(A), Ang II significantly increased ANF promoter activity. Treatment of myocytes with PD98059 abolished Ang II-induced ANF promoter activation. Co-expression of dominant-negative MEK1 (K97M) also abolished Ang II-induced increases in ANF–Luc activity (Figure 3B). In contrast, co-transfection of constitutively active MEK1 [36] stimulated ANF–Luc activity 3.6 $\pm$ 0.5-fold (Figure 3B). Constitutively active MEK1-induced ANF–Luc activation was not inhibited by 30 μM PD98059 (results not shown), confirming that PD98059 acts specifically on MEK1 and does not affect downstream signalling. In contrast, phenylephrine-induced ANF–Luc activation was not affected by PD98059 (Figure 3C) or dnMEK1 (Figure 3D), confirming a previous study [21]. These results indicate that activation of the MEK1–ERK pathway is necessary and sufficient for Ang II-induced activation of the ANF promoter but is dispensable for phenylephrine-induced activation of the ANF promoter.

Involvement of p38 and JNK pathways in Ang II- or phenylephrine-induced ANF promoter activation

The involvement of the SB203580-sensitive pathway in ANF promoter activation was also examined. As shown in Figure 4(A), SB203580 (20 μM) significantly inhibited phenylephrine-induced ANF–Luc activation (4.8 $\pm$ 1.0-fold, 65 % decrease; $P < 0.05$ compared with phenylephrine alone, $n = 6$), confirming previous observations [25]. In contrast, SB203580 failed to inhibit ANF–Luc activity significantly, both before (0.73 $\pm$ 0.23, $n = 6$) and after (1.62 $\pm$ 0.19, $n = 6$) stimulation with Ang II. The fold activations of ANF–Luc by Ang II in the presence and in the absence of SB203580 were comparable (2.4-fold compared with 2.2-fold). SB203580 (5 μM) yielded similar results (results not shown). These results suggest that p38 or SB203580-sensitive JNK does not have an essential role in Ang II-induced ANF promoter activation.

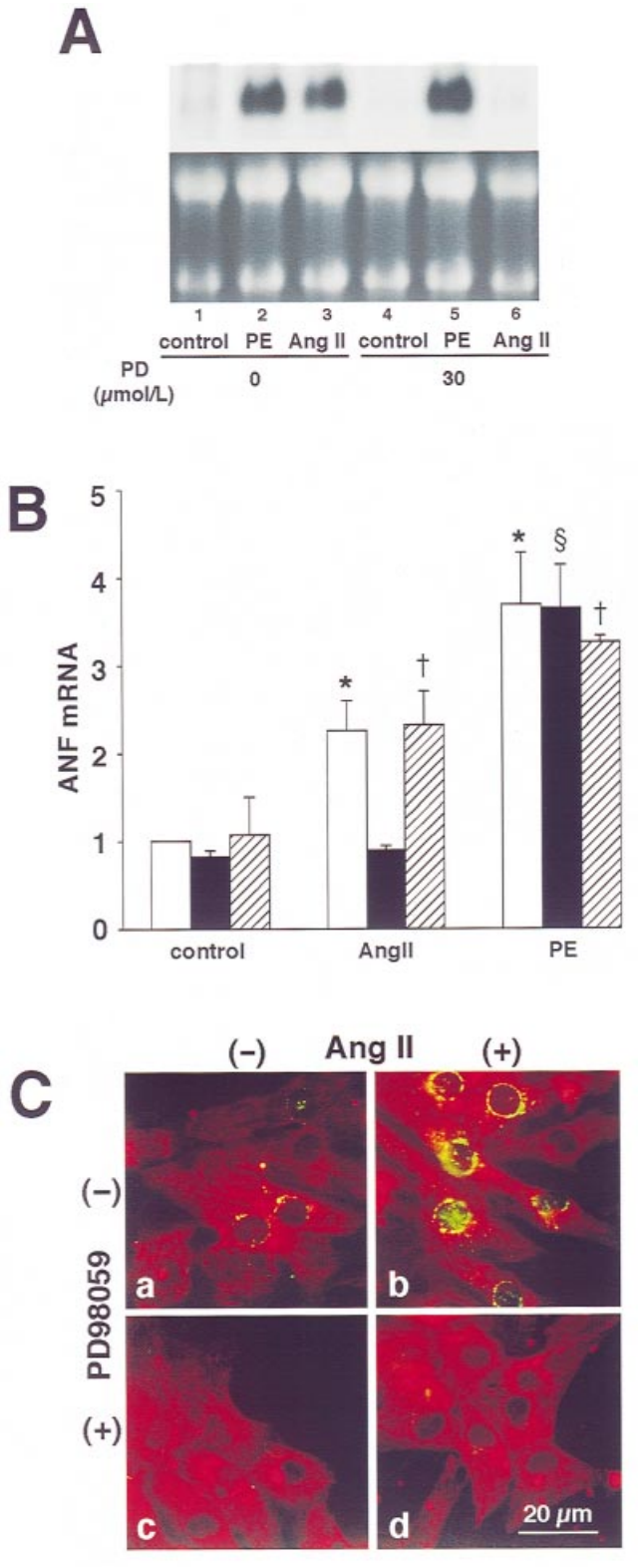

Figure 2 Effect of PD98059 or SB203580 on phenylephrine- or Ang II-induced ANF expression

(**A**) Cardiac myocytes were treated with (lanes 4–6) or without (lanes 1–3) 30 μM PD98059 (PD) and stimulated with 10 μM phenylephrine (PE) (lanes 2 and 5) or 100 nM Ang II (lanes 3 and 6) for 18 h. Northern analyses were performed with a specific oligonucleotide probe for ANF. A representative autoradiogram from four independent experiments is shown with ethidium bromide staining below. (**B**) Quantitative densitometric analyses of Northern blotting in (**A**) are shown with the effect of SB203580 on ANF mRNA expression. Cardiac myocytes were treated with vehicle (open columns), 30 μM PD98059 (filled columns) or 20 μM SB203580 (hatched columns) and stimulated with 100 nM Ang II or 10 μM phenylephrine (PE) for 18 h. ANF mRNA levels are expressed relative to vehicle treatment alone. Results are means $\pm$ S.E.M. for four independent experiments. *, §, †, $P < 0.05$ compared with the control, PD98059 alone and SB203580 alone respectively. (**C**) Cardiac myocytes were treated with (**c**, **d**) or without (**a**, **b**) PD98059 (30 μM) for 30 min, followed by stimulation with (**b**, **d**) or without (**a**, **c**) Ang II (100 nM) for 48 h. Myocytes were double-stained with anti-ANF antibody (green) and anti-(sarcomeric myosin heavy chain) antibody (MF20; red). Results are representative of four independent observations.

Table 1 Effect of PD98059 or SB203580 on PE- or Ang II-induced ANF polypeptide expression

Cardiac myocytes were treated with vehicle, PD98059 (30 μmol/l) or SB203580 (20 μmol/l) for 30 min, followed by stimulation with vehicle (control), 100 nmol/l Ang II or 10 μmol/l PE for 48 h. Myocytes were double-stained with anti-ANF antibody and anti-sarcomeric myosin heavy-chain antibody. Percentages of ANF-positive cells are shown in approx. 500 myocytes counted for each group. Results shown are representative of 4 independent observations.

	Vehicle	PD98059	SB203580
Control	16	7	17
Ang II	35	9	37
PE	42	39	32

To examine the involvement of the JNK pathway, myocytes were co-transfected with ANF–Luc along with either expression vectors encoding dominant-negative MEKK1 [dnMEKK1; MEKK1(K1257M)] [35] or an empty vector. This method has been used to show the involvement of the JNK pathway in cardiac hypertrophic signalling [26,40], although recent evidence suggests that MEKK1 might also feed into the ERK pathway [41]. As shown in Figure 4(B), dnMEKK1 modestly but significantly inhibited phenylephrine-induced increases in ANF–Luc activity [13.4 $\pm$ 1.8-fold (empty vector) compared with 9.1 $\pm$ 1.8-fold (dnMEKK1) (32 % decrease); $P < 0.05$, $n = 5$], whereas it had no significant effects on Ang II-induced increases in ANF–Luc (1.9 $\pm$ 0.3-fold compared with 1.8 $\pm$ 0.4-fold). These results indicate that JNK or p38 are dispensable for Ang II-induced activation of the ANF promoter. In contrast, both SB203580-sensitive mechanisms (presumably p38 and/or some JNK isoforms) and MEKK1-dependent signalling mechanisms (presumably JNK) seem to be involved in phenylephrine-induced ANF–Luc activation.

Involvement of Ras-dependent pathway in Ang II- or phenylephrine-induced gene expression

Because Ras is implicated in activation of the ERK pathway, we examined whether Ras is involved in Ang II-induced ERK activation and ANF expression. We used adenovirus vector harbouring dominant-negative Ras (dnRas) (Ad5.N17Ras) to inhibit Ras specifically. As shown in Figure 5(A), infection of cardiac myocytes with Ad5.N17Ras dose-dependently inhibited the Ang II-induced activation of both ERK1 (26 $\pm$ 9 % of the activity of Ang II alone at 100 moi; $P < 0.05$) and ERK2 (22 $\pm$ 7 % of the activity of Ang II alone at 100 moi; $P < 0.05$), whereas the control adenovirus (Ad5/ΔE1spB) showed no significant effect (84 $\pm$ 11 % for ERK1 and 96 $\pm$ 17 % for ERK2 of Ang II alone at 100 moi). Inhibition of the basal activity of ERKs by Ad5.N17Ras was also noted after autoradiography at longer exposures. This indicates that Ras is involved in the basal and Ang II-induced ERK activity in cardiac myocytes. In the same range of virus titres, both basal and Ang II-induced ANF

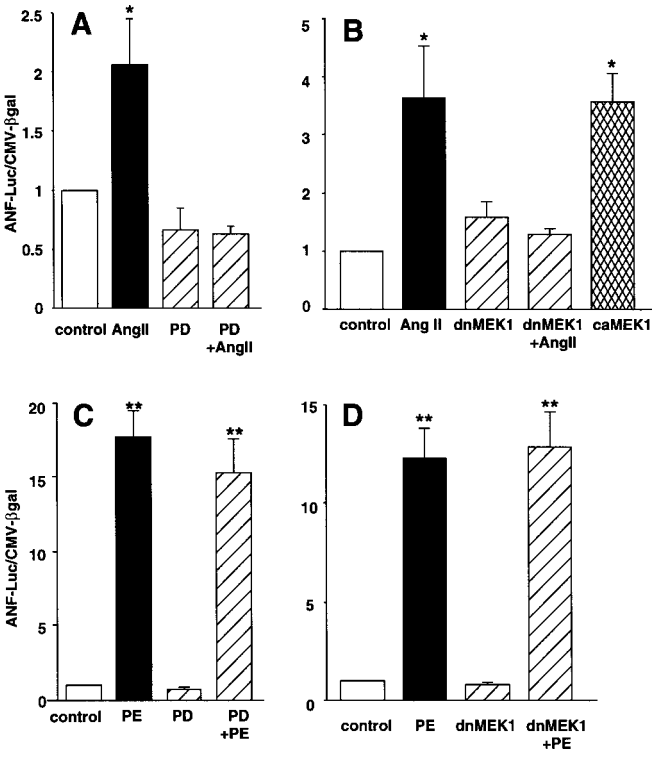

Figure 3 Effect of ERK inhibitors on Ang II- or phenylephrine-induced ANF promoter activation

(**A**) Cardiac myocytes transfected with reporter constructs (ANF–Luc and CMV–βgal) were treated with (hatched columns) or without (open and filled columns) 30 μM PD98059 (PD) for 30 min, then stimulated with Ang II (100 nM) for 48 h; reporter gene activities were determined. Results are means $\pm$ S.E.M. for five independent experiments. (**B**) Cardiac myocytes were co-transfected with the reporter constructs with empty vector (control and Ang II), dominant-negative MEK1 (dnMEK1) or constitutively active MEK1 (caMEK1). At 48 h after transfection, myocytes were stimulated with or without Ang II for 48 h. Results are means $\pm$ S.E.M. for nine independent experiments. (**C**) Cardiac myocytes transfected with reporter constructs were treated with PD98059 (PD, hatched columns) and stimulated with or without 10 μM phenylephrine (PE) for 48 h; reporter gene activities were determined. Results are means $\pm$ S.E.M. for six independent experiments. (**D**) Cardiac myocytes were co-transfected with reporter constructs and dnMEK1 (hatched columns) as described for (**B**) and stimulated with or without 10 μM phenylephrine (PE) for 48 h. Results are means $\pm$ S.E.M. for six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. Results were obtained by dividing the luciferase activities by the corresponding βgal activities and are expressed relative to the control value with no intervention.

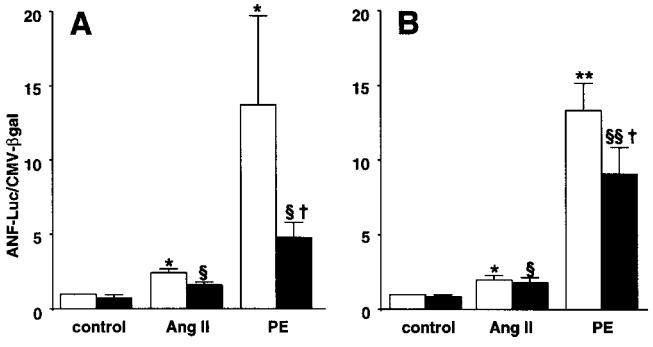

Figure 4 Effect of SB203580 or dominant-negative MEKK1 on Ang II- or phenylephrine-induced ANF promoter activation

(**A**) Cardiac myocytes transfected with reporter constructs (ANF–Luc and CMV–βgal) were treated with (filled columns) or without (open columns) 20 μM SB203580 for 30 min, and stimulated with 100 nM Ang II or 10 μM phenylephrine (PE) for 48 h; reporter gene activities were determined. Results are means $\pm$ S.E.M. for six independent experiments. (**B**) Cardiac myocytes transfected with reporter constructs with either empty vector (open columns) or dominant-negative MEKK1 (filled columns) were stimulated with 10 μM phenylephrine (PE) or 100 nM Ang II for 48 h; reporter gene activities were determined. Results are means $\pm$ S.E.M. for five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control; §$P < 0.05$, §§$P < 0.01$, compared with SB203580 alone (**A**) or dnMEKK1 alone (**B**); †$P < 0.05$ compared with phenylephrine alone. Results were obtained by dividing luciferase activities by the corresponding βgal activities and are expressed relative to the control with no intervention.

mRNA expression were suppressed by dnRas (Figure 5B), whereas Ad5/ΔE1spB showed no effect on either basal or Ang II-induced ANF expression (results not shown). These results suggest that Ras mediates basal and Ang II-induced ANF expression, possibly through the ERK pathway. However, Ad5.N17Ras did not affect Ang II-induced c-*fos* expression (Figure 5C), suggesting that Ad5.N17Ras is not toxic to myocytes and that Ang II-induced c-*fos* expression is mediated by Ras-independent mechanisms.

We further analysed the role of Ras in the Ang II- or phenylephrine-induced activation of ANF–Luc. As shown in Figure 5(D), dnRas suppressed the basal activity of the ANF–Luc promoter and abolished the Ang II-induced activation of the ANF promoter, whereas constitutively active Ras (L61Ras) alone strongly activated the ANF–Luc promoter (25.6 $\pm$ 7.8-fold). Phenylephrine-induced activation of ANF–Luc was partly inhibited by dnRas (Figure 5E), which is consistent with previous reports [42,43]. These results indicate that the activation of Ras is both necessary and sufficient for activation of the ANF promoter by Ang II, whereas Ras is partly involved in phenylephrine-induced activation of the ANF promoter.

Activation of ERK is not required for other aspects of Ang II-induced cardiac hypertrophy

We next examined whether the ERK pathway also mediates other aspects of Ang II-induced cardiac hypertrophy. PD98059 did not significantly affect the Ang II-induced immediate-early expression of c-*fos* in cardiac myocytes (Figure 6A), which was consistent with the results obtained with dnRas (Figures 5A and 5C). We also examined the role of ERK in the mRNA expression of SKA, another 'fetal type' marker gene of cardiac hypertrophy. Ang II significantly increased SKA expression by 1.7 $\pm$ 0.2-fold ($P < 0.05$, $n = 5$) (Figure 6B), which in contrast with ANF expression was not blocked by PD98059 (Figure 6B). These results indicate that the induction of c-*fos* and SKA expression by Ang II is ERK-independent.

Rapid organization of sarcomeres by Ang II [44] was not affected after 30 min of treatment with 30 μM PD98059 (Figure 6C). After 24 h of treatment with Ang II in the presence of PD98059, some disarray of myofibrils was observed, although Ang II caused significant increases in striated myofibrils (results not shown). These results indicate that the ERK pathway is not essential for rapid sarcomere organization but might be involved in the maintenance of organized sarcomeres.

Treatment of myocytes with PD98059 inhibited the basal incorporation of [^{3}H]phenylalanine in a concentration-dependent manner (Figure 6D) ($P < 0.0001$; ANOVA). However, Ang II caused a significant increase in [^{3}H]phenylalanine incorporation even in the presence of 30 μM PD98059 (a 23.8 % increase compared with PD98059 alone; $P < 0.05$). These results suggest

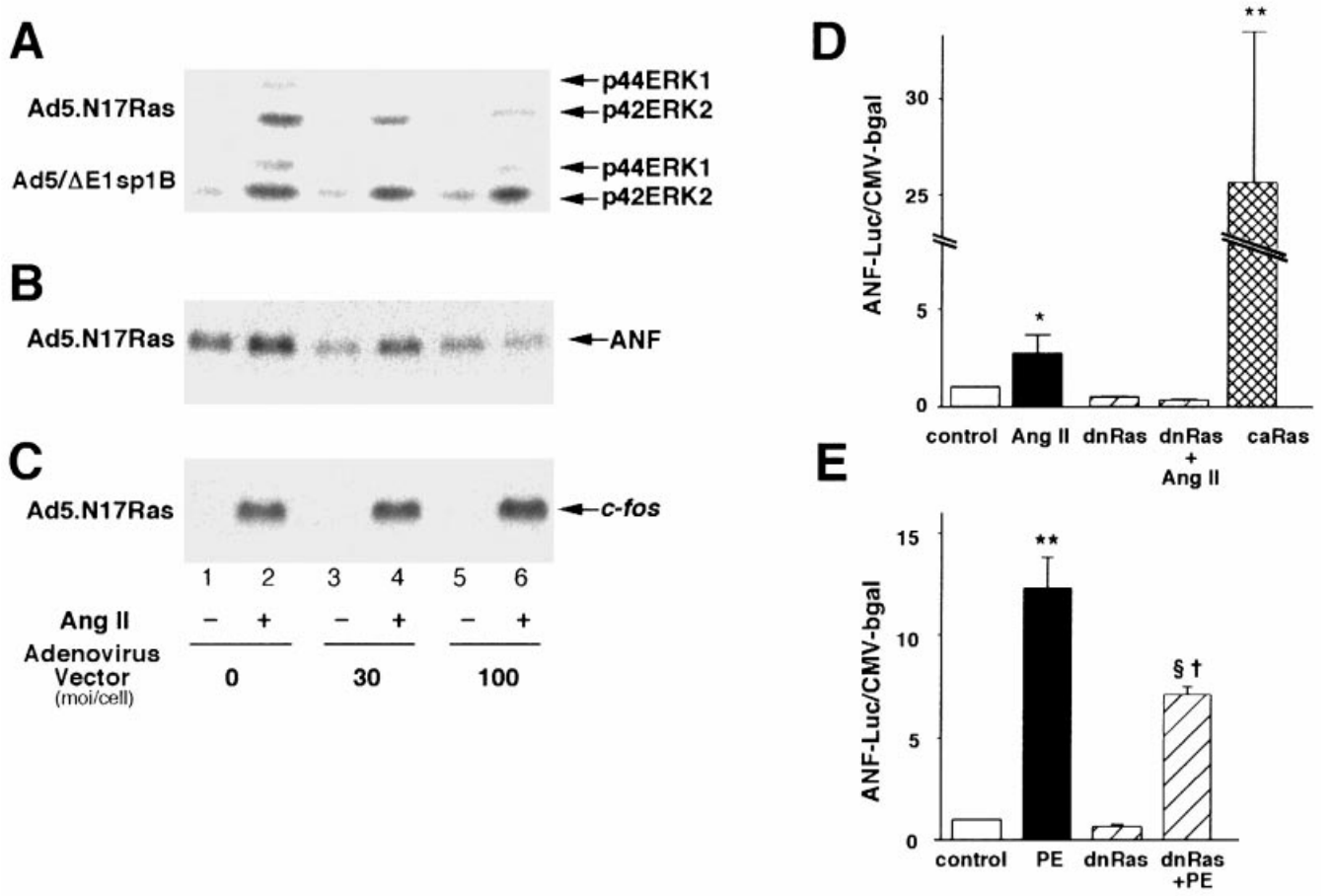

Figure 5 Effect of dominant-negative Ras on Ang II- or phenylephrine-induced gene expressions

(**A**–**C**) Cardiac myocytes were infected with the indicated titres (moi) of adenovirus vector harbouring dominant-negative Ras (Ad5.N17Ras) or control vector (Ad5/ΔE1sp1B), then stimulated with Ang II (100 nM) for 5 min (**A**), 18 h (**B**) or 30 min (**C**). The activities of ERK1 and ERK2 were determined by the immune complex in-gel kinase assay (**A**). Note that the anti-ERK antibody recognized both ERK2 and ERK1. The expression of mRNA for ANF (**B**) and c-*fos* (**C**) was determined by Northern analyses. Results shown are representative of four independent experiments. (**D**) Cardiac myocytes were transfected with ANF–Luc and CMV–βgal with empty vector (control and Ang II), plasmid encoding dominant-negative Ras (dnRas) or constitutively active Ras (caRas). At 48 h after transfection, myocytes were stimulated with or without 100 nM Ang II for 48 h. Results are means $\pm$ S.E.M. for eight independent experiments. (**E**) Cardiac myocytes were transfected with reporter constructs with either empty vector [control and phenylephrine (PE)] or dnRas. At 48 h after transfection, myocytes were stimulated with or without 10 μM phenylephrine for 48 h. Data were obtained by dividing luciferase activities by the corresponding βgal activities and are expressed relative to the control value with no intervention. Results are means $\pm$ S.E.M. for six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the value without stimulation (open column); §$P < 0.05$ compared with dnRas alone; †$P < 0.05$ compared with phenylephrine alone.

that the ERK pathway might be important for the basal rate of protein synthesis, whereas it is not essential for Ang II-induced increases in the rate of protein synthesis.

DISCUSSION

Our results indicate that activation of the ERK pathway is necessary and sufficient for Ang II-induced ANF expression at both the mRNA and polypeptide levels. ERK selectively mediates ANF expression but not other Ang II-induced hypertrophic responses. Furthermore, the involvement of the ERK in Ang II-induced ANF expression contrasts with phenylephrine-induced ANF expression, which does not require the ERK pathway. These results suggest that the signalling mechanisms of cardiac hypertrophy are substantially different among G_q-protein-coupled receptor agonists.

We demonstrated the involvement of the ERK pathway in Ang II-induced ANF expression by using the specific MEK/ERK inhibitor PD98059 and dominant-negative MEK. The effect of specific inhibition of the ERK pathway was examined by using both the ANF–Luc reporter gene and mRNA and polypeptide accumulation. Although specific inhibitors might be justified by careful determination of the dose that inhibits only the target molecule, it is difficult to completely exclude non-specific effects of inhibitors. Furthermore, it has been pointed out that the various degrees of overexpression of signalling intermediates might produce different biological effects [5,28], which might in part explain conflicting reports on the role of ERK and JNK in phenylephrine-induced ANF expression. The signalling mechanism controlling transcription of the reporter gene might also be different from that of the endogenous gene [34,45]. This could be an explanation for the minimal suppression by SB203580 of ANF mRNA and polypeptide expressions induced by phenylephrine, whereas it showed significant suppression of ANF–Luc activity induced by the same agonist. It is therefore important to evaluate the role of signalling molecules in agonist-induced cardiac hypertrophy by using multiple methods. In this regard,

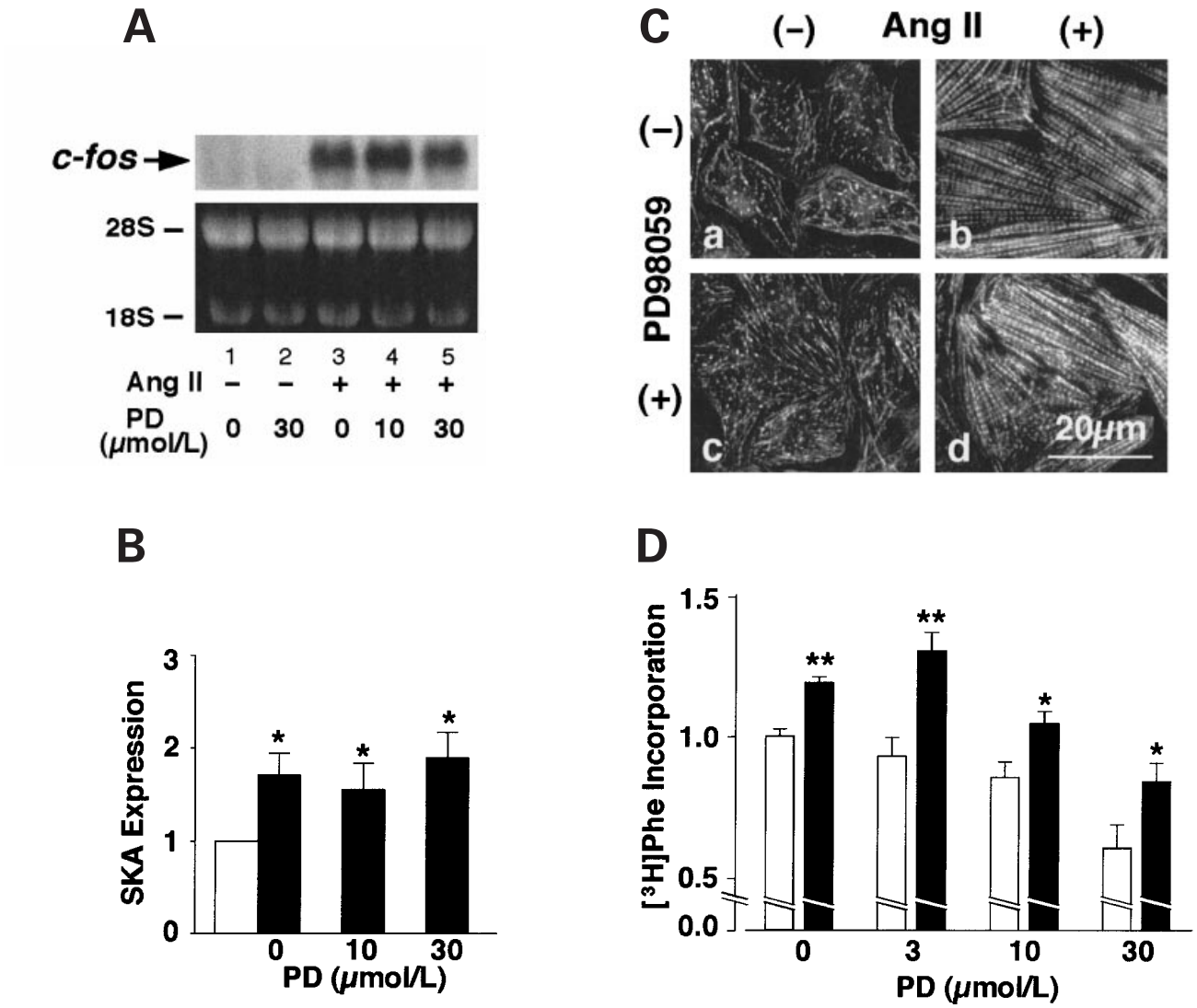

Figure 6 Effect of PD98059 on Ang II-induced c-*fos* and SKA gene expressions, sarcomere organization and increases in the rate of protein synthesis

(**A**) Cardiac myocytes were treated with PD98059 (PD) for 30 min and stimulated with (lanes 3–5) or without (lanes 1 and 2) Ang II (100 nM) for 30 min. Northern analyses were performed with a c-*fos* probe. A representative autoradiogram of three independent experiments is shown with ethidium bromide staining below. (**B**) Cardiac myocytes were treated with PD98059 (PD) for 30 min and stimulated with Ang II (100 nM) for 18 h. Northern analyses were performed with an oligonucleotide probe for SKA. Results of densitometric analyses are shown. The expression level of SKA mRNA in unstimulated myocytes without PD98059 treatment was set at 1 (open column). $^{*}P < 0.05$ compared with basal expression level. Results are means ± S.E.M. for five independent experiments. (**C**) Cardiac myocytes were treated with (**c**, **d**) or without (**a**, **b**) PD98059 (30 μM) for 30 min, then stimulated with (**b**, **d**) or without (**a**, **c**) Ang II (100 nM) for 30 min. Polymerized actin fibres were stained with FITC-conjugated phalloidin. Results are representative of five independent observations. (**D**) Cardiac myocytes were treated with PD98059 (PD) for 30 min, then incubated with [^{3}H]phenylalanine in the presence (filled columns) or the absence (open columns) of Ang II for 24 h. The results are expressed relative to the [^{3}H]phenylalanine incorporation in unstimulated myocytes without PD98059 treatment. Results are means ± S.E.M. for seven independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the value without Ang II stimulation.

all of the results presented in this report are consistent with the concept that the ERK pathway is involved in Ang II-induced ANF expression.

Another important finding in this investigation is that Ang II and phenylephrine use different signalling mechanisms for ANF expression despite the fact that Ang II and phenylephrine are both agonists for the G_q-coupled receptors. Ang II-induced ANF expression is mediated predominantly by the ERK pathway, whereas phenylephrine-induced ANF expression is not. Phenylephrine seems to use signalling pathway(s) insensitive to PD98059 for ANF expression, although a minor contribution of ERK cannot be excluded. MEKK1-mediated cell signals and SB203580-sensitive pathways (presumably p38 and some JNK isoforms [46]) are potential candidates, as demonstrated in previous studies [25,31] as well as this study, although the SB203580-sensitive pathway has only a minor role, if any, in phenylephrine-induced endogenous ANF expression (Figure 2B and Table 1). Although MEKK1 is able to activate both the JNK and ERK pathways, the JNK pathway might be more important for phenylephrine-induced ANF–Luc activation because inhibition of the ERK pathway by PD98059 or dominant-negative MEK1 did not show a significant effect on phenylephrine-induced ANF–Luc activation. The relative importances of the three representative MAP kinase pathways in Ang II- and phenylephrine-induced activation of the −638bp ANF promoter is correlated with the relative activation of the three MAP kinase

pathways by these agonists. Although Ang II and phenylephrine activate ERKs with comparable magnitudes (Figure 1A), the activation of JNK and p38 by phenylephrine is more strong and sustained than that by Ang II (Figures 1B and 1C). Both our observation (Figure 3B) and those by others suggest that activation of each member of the MAP kinases is sufficient, if not equally potent, to stimulate ANF transcription [19,25,30]. Thus the relative contribution of each MAP kinase on ANF expression might depend on the parallel activation of other members of the MAP kinases. Furthermore, the stronger activation of the ANF–Luc by phenylephrine than by Ang II might be due to stronger and more sustained activation of JNK and p38 by phenylephrine (Figures 1B and 1C).

At present we do not know how Ang II and phenylephrine activate substantially different signalling mechanisms, even though both Ang II type 1 receptor (AT1) and α-adrenergic receptors signal through G_q. Differences in the magnitude and the time course of G_q activation between various G_q-coupled receptors might be translated into differences in the intracellular signalling mechanisms activated. Alternatively, sequence-specific direct interaction between G_q-coupled receptors and intracellular signalling molecules might confer the versatile signalling mechanisms on the G_q-coupled receptor (reviewed in [47]).

We have previously shown that the $p62^{TCF}$ (in which TCF stands for ternary complex factor) binding site within the serum response element (SRE) is critical for mediating Ang II-induced c-*fos* expression [48]. ERKs are known to phosphorylate Ser-383 of $p62^{TCF}$ to activate the $p62^{TCF}$/SRF (serum response factor) complex [49]. Our results suggest that the Ras/ERK pathway is not required for Ang II-induced c-*fos* expression. Because $p62^{TCF}$ can be phosphorylated by other members of MAP kinase family [49–51], including JNK and p38, it is possible that Ang II-induced c-*fos* expression is mediated by these kinases. Alternatively, Ang II might activate a $p62^{TCF}$ kinase distinct from these MAP kinases. Such a mechanism might be unique to Ang II because dominant-negative ERK has been shown to inhibit the phenylephrine-induced activation of the c-*fos*-Luc reporter gene [11,19], further supporting the notion that Ang II and phenylephrine use different signalling mechanisms in cardiac myocytes. The contribution of the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway via *sis*-inducing factor element (SIE) in the c-*fos* promoter is another potential mechanism for c-*fos* induction, because this pathway has been shown to be activated by Ang II in cardiac myocytes [52]. However, we have previously reported that SIE is dispensable for Ang II-induced activation of the c-*fos* promoter [48], suggesting that the JAK/STAT pathway is not a major pathway for c-*fos* expression by Ang II.

We have previously shown that Ang II activates ERKs via Ca^{2+}-dependent mechanisms in cardiac myocytes [10]. Ang II activates Ras through Src-family tyrosine kinase-dependent mechanisms [53]. The results presented here suggest that Ras and ERKs are on the same signalling pathway activated by Ang II. It remains to be shown which components of the ERK pathway are Ca^{2+}-dependent. Zou et al. [54] reported that the Ang II-induced activation of ERKs in cardiac myocytes is dependent on protein kinase C (PKC) rather than on tyrosine kinase or Ras. At present we cannot reconcile this discrepancy between their results and ours. In liver GN4 cells, Ang II-induced tyrosine kinase activation is unmasked when the activation of PKC is inhibited [55]. The availability of such 'back-up' systems might be cell-type-specific or dependent on experimental conditions.

In summary, the ERK pathway is critically involved in ANF expression in Ang II-induced hypertrophic responses. In conjunction with our previous papers [10,44,48,53,56], we propose that each characteristic phenotype of Ang II-induced cardiac hypertrophy is controlled by distinct signalling mechanisms (i.e. c-*fos* by PKC, ANF by Ras, RhoA and ERK, protein synthesis by p70S6K, and premyofibril by RhoA). Although the ERK pathway has a central role in cell growth and differentiation in dividing cells, this pathway has an agonist- and phenotype-specific role in terminally differentiated cardiac myocytes. It has been suggested that ERK is critically involved in the cell survival effects of cardiotrophin 1 in cardiac myocytes [57]. It remains to be determined whether ERK-mediated ANF expression is beneficial in Ang II-induced hypertrophy.

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