

Stimulation of the p38 Mitogen-activated Protein Kinase Pathway in Neonatal Rat Ventricular Myocytes by the G Protein-coupled Receptor Agonists, Endothelin-1 and Phenylephrine: A Role in Cardiac Myocyte Hypertrophy?

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Abstract. We examined the activation of the p38 mitogen-activated protein kinase (p38-MAPK) pathway by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine in primary cultures of cardiac myocytes from neonatal rat hearts. Both agonists increased the phosphorylation (activation) of p38-MAPK by ~12-fold. A p38-MAPK substrate, MAPK-activated protein kinase 2 (MAPKAPK2), was activated approximately fourfold and 10 μ M SB203580, a p38-MAPK inhibitor, abolished this activation. Phosphorylation of the MAPKAPK2 substrate, heat shock protein 25/27, was also increased. Using selective inhibitors, activation of the p38-MAPK pathway by endothelin-1 was shown to involve protein kinase C but not G_i/G_o , nor the extracellularly responsive kinase (ERK) pathway. SB203580 failed to inhibit the morphological changes

associated with cardiac myocyte hypertrophy induced by endothelin-1 or phenylephrine between 4 and 24 h. However, it decreased the myofibrillar organization and cell profile at 48 h. In contrast, inhibition of the ERK cascade with PD98059 prevented the increase in myofibrillar organization but not cell profile. These data are not consistent with a role for the p38-MAPK pathway in the immediate induction of the morphological changes of hypertrophy but suggest that it may be necessary over a longer period to maintain the response.

Key words: hypertrophy • cardioprotection • mitogen-activated protein kinases • adrenergic agonists • endothelin-1

THE p38-MAPKs, extracellularly responsive kinases (ERKs)¹ and stress-activated protein kinases/c-Jun NH₂-terminal kinases (SAPKs/JNKs) constitute the three best-characterized subgroups of the mitogen-activated protein kinase (MAPK) superfamily (reviewed by Kyriakis and Avruch, 1996a, 1996b; Lee and Young, 1996;

Cohen, 1997). MAPKs are activated by the dual phosphorylation of a Tyr and a Thr residue within a Thr-Xaa-Tyr motif and the identity of Xaa assists in the classification of subgroup membership. In the p38-MAPKs, Xaa is a Gly residue. Several isoforms of p38-MAPKs have been characterized. The original p38-MAPK (now sometimes called p38-MAPK α ; Han et al., 1993, 1994; Freshney et al., 1994; Lee et al., 1994; Rouse et al., 1994; Zervos et al., 1995) exists as two alternatively spliced isoforms (Lee et al., 1994). Subsequently, the β_1/β_2 (Jiang et al., 1996; Kumar et al., 1997), γ (Li et al., 1996; Mertens et al., 1996), and δ (Goedert et al., 1997; Jiang et al., 1997) isoforms were identified. In many cells, p38-MAPKs are activated by a variety of cellular stresses including inflammatory cytokines, endotoxins, UV irradiation and hyperosmotic shock (reviewed by Lee and Young, 1996; Cohen, 1997). One substrate of p38-MAPK α and p38-MAPK β is MAPK-activated protein kinase 2 (MAPKAPK2; Goedert et al., 1997; Kumar et al., 1997), an enzyme that phosphorylates the small heat

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1. *Abbreviations used in this paper:* ERK, extracellularly responsive kinase; ET-1, endothelin-1; FPLC, fast protein liquid chromatography; GPCR, G protein-coupled receptor; HSP, heat shock protein; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MAPKAPK2, MAPK-activated protein kinase 2; MEK, MAPK/ERK kinase; MBP, myelin basic protein; β -MHC, β -myosin heavy chain; PBS, Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline; PE, phenylephrine; PTX, pertussis toxin; SAPK/JNK, stress-activated protein kinase/c-Jun NH₂-terminal kinase.

shock protein 25/27 (HSP25/27; Rouse et al., 1994). In un-stressed cells, HSP25/27 exists predominantly in an un-phosphorylated state as high molecular mass aggregates (Lavoie et al., 1995). Phosphorylation leads to disaggregation (Lavoie et al., 1995) and is associated with protection of the actin cytoskeleton (Guay et al., 1997; Huot et al., 1997). Several other substrates of the p38-MAPK(s) have been identified (reviewed by Cohen, 1997).

In the intact heart, p38-MAPK is activated by a variety of stresses including ischemia and ischemia/reperfusion (Bogoyevitch et al., 1996; Yin et al., 1997), oxidative stress (Clerk et al., 1998), and hypertensive perfusion pressure (Clerk et al., 1998). The biological functions of the p38-MAPKs in the heart are not understood, although a recent report using an inhibitor (SB203580; Lee et al., 1994; Cuenda et al., 1995) that is selective for the α and β_1 isoforms of p38-MAPK (Goedert et al., 1997; Kumar et al., 1997) suggests that their activation may protect the heart against ischemia (ischemic preconditioning; Weinbrenner et al., 1997). p38-MAPK(s) has also been implicated in hypertrophy of ventricular myocytes (Zechner et al., 1997). This is an important adaptational response to a demand for increased contractile power and is seen in a number of clinical situations in man. Transient transfection of primary cultures of neonatal rat ventricular myocytes with an expression plasmid encoding constitutively activated mutants of MKK6 or MKK3 (the MAPK kinases (MKKs) responsible for the activation of p38-MAPK) induces the transcriptional and morphological changes associated with the development of the hypertrophic phenotype (Zechner et al., 1997; Wang et al., 1998). SB203580 inhibited these changes (Zechner et al., 1997). In addition, SB203580 inhibited the hypertrophic response induced by phenylephrine (PE; Zechner et al., 1997), a powerfully hypertrophic α_1 -adrenergic agonist (Simpson, 1985; Lee et al., 1988; Iwaki et al., 1990; Knowlton et al., 1991).

G protein-coupled receptors (GPCRs) of the $G_{q/11}$ class participate in the cellular responses to α_1 -adrenergic agonists such as PE and endothelin-1 (ET-1; reviewed by Sugden and Clerk, 1997). Like the α_1 -adrenergic agonists, ET-1 is powerfully hypertrophic (Shubeita et al., 1990; Ito et al., 1991) and both α_1 -adrenergic agonism and ET-1 have been implicated in ischemic preconditioning (Banerjee et al., 1993; Wang et al., 1996). ET-1 and PE were first shown to activate the ERKs in cardiac myocytes (Bogoyevitch et al., 1993, 1994; Clerk et al., 1994) and this was the first MAPK cascade to be implicated in cardiac hypertrophy (Thorburn et al., 1994a; Gillespie-Brown et al., 1995). Subsequently, SAPKs/JNKs were shown to be activated in cardiac myocytes by ET-1 and PE (Bogoyevitch et al., 1995a; Ramirez et al., 1997), and these MAPKs have also been implicated in cardiac hypertrophy (Bogoyevitch et al., 1996; Ramirez et al., 1997). Although p38-MAPK has been implicated in PE-induced hypertrophy, there are only very limited data pertaining to the activation of p38-MAPKs in cardiac myocytes by PE (Zechner et al., 1997) and no data for ET-1 or other powerfully hypertrophic agonists such as PMA.

Here, we have characterized extensively the activation of the p38-MAPK pathway by the GPCR agonists, ET-1 and PE, and compared these responses with those to hyperosmotic stress and PMA. We examined the phosphoryla-

tion of p38-MAPK, activation of its substrate MAPKAPK2, and phosphorylation of HSP25/27 in primary cultures of ventricular myocytes isolated from neonatal rat hearts. Using SB203580, we also assessed the role of the p38-MAPK pathway in the development of the morphological changes associated with the hypertrophic response.

Materials and Methods

Materials

Laminin, microcystin LR, myelin basic protein (MBP), pertussis toxin (PTX), sorbitol, ET-1, PE, PMA, protein A-Sepharose and protease inhibitors were from Sigma Chemical Co. (Poole, Dorset, UK). PD98059 and GF109203X were from Calbiochem (Beeston, Nottinghamshire, UK) and were prepared as 25 mM and 10 mM stock solutions in DMSO. SB203580 was a gift from Dr. John C. Lee (SmithKline Beecham, King of Prussia, PA) and a 10 mM stock solution was prepared in DMSO. Lab-Tek tissue culture chamber slides were from Life Technologies (Paisley, Scotland, UK). Other tissue culture materials were from previously described sources (Bogoyevitch et al., 1995b). Prestained protein molecular mass markers, ECL Western blotting reagents, autoradiography film and streptavidin-Texas Red were from Amersham International (Amersham, Buckinghamshire, UK). γ -[32 P]ATP was from NEN Life Sciences (Brussels, Belgium). SDS-PAGE reagents, IEF reagents, and Bradford (Bradford, 1976) protein assay reagent were from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). Nitrocellulose was from Schleicher & Schuell and supplied by Anderman & Co. (Kingston-upon-Thames, Surrey, UK). cAMP-dependent protein kinase inhibitory peptide (sequence TTYADFIASGRTGRRNAIHD) was from Bachem (Saffron Walden, Essex, UK). The MAPKAPK2 substrate peptide (KKLNRTLVA) was synthesized by Severn Biotech (Kidderminster, Worcestershire, UK). The antibody to HSP25/27 (sc-1049) was from Santa Cruz Biotechnology Inc. and supplied by Autogen Bioclear (Deves, Wiltshire, UK). The antibody to the dually phosphorylated form of p38-MAPK was from New England Biolabs (UK) Ltd. (Hitchin, Herts., UK). A mouse monoclonal antibody to β -myosin heavy chain (β -MHC) was from Novocastra (Newcastle-upon-Tyne, Tyne and Wear, UK). Secondary antibodies (horseradish peroxidase-linked, FITC and biotinylated anti-mouse and anti-rabbit IgG antibodies) and fluorescent mounting medium were from Dako (High Wycombe, Buckinghamshire, UK).

Primary Culture of Neonatal Ventricular Myocytes and Sample Preparation

Myocytes were dissociated from the ventricles of neonatal Sprague-Dawley rat hearts by a previously described adaptation (Bogoyevitch et al., 1995b) of the method of Iwaki et al. (Iwaki et al., 1990). To deplete the myocytes of fibroblasts, the cells were preplated in DMEM/M199 (4:1 vol/vol) supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) fetal calf serum and 100 U/ml each of penicillin and streptomycin. The (nonadherent) myocytes were then plated on 60- or 35-mm culture dishes precoated with 1% (wt/vol) gelatin at a density of 1.4×10^3 cells/mm². Myocytes were confluent and beating within 18 h. The cells were incubated for 24 h in serum-free medium in the absence or presence (in some experiments) of 1 μ M PMA or 30 ng/ml PTX, and then exposed to agonists (0.5 M sorbitol, ET-1, PE or 1 μ M PMA) in serum-free medium. For inhibitor studies, the cells were preincubated with inhibitor for 15 min before stimulation with agonist + inhibitor. For phospho-p38-MAPK immunoblot analysis and fast protein liquid chromatography (FPLC), myocytes (60-mm dishes) were washed in ice-cold Ca^{2+}/Mg^{2+} -free Dulbecco's phosphate-buffered saline (PBS) and scraped into 150 μ l/dish extraction buffer A (20 mM β -glycerophosphate, pH 7.5, 50 mM NaF, 2 μ M microcystin LR, 2 mM EDTA, 0.2 mM Na_3VO_4 , 10 mM benzamide, 200 μ M leupeptin, 10 μ M trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM DTT, 300 μ M PMSF, 1% (vol/vol) Triton X-100). The samples were centrifuged (10,000 g, 5 min, 4°C). For Western blotting, supernatants were boiled with 0.33 vol of sample buffer (0.33 M Tris-HCl pH 6.8, 10% (wt/vol) SDS, 13% (vol/vol) glycerol, 133 mM DTT, 0.2 mg/ml bromophenol blue). For FPLC, supernatants were re-centrifuged (10,000 g, 5 min, 4°C) before loading onto the column. Protein concentrations were determined using the Bradford method (Bradford, 1976).

Phospho-p38-MAPK Immunoblot Analysis

Myocyte extracts (25 μ g protein) were immunoblotted for phospho-p38-MAPK according to the manufacturer's instructions. The bands were detected using enhanced chemiluminescence (ECL) with exposure to Hyperfilm MP. Blots were quantified by laser scanning densitometry.

Fast Protein Liquid Chromatography (FPLC) of ERKs and MAPKAPK2

ERKs (four 60-mm dishes/column) were separated on a Mono Q HR5/5 column equilibrated with 50 mM Tris-HCl, pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, 0.03% (vol/vol) Brij-35, 0.3 mM Na_3VO_4 , 1 mM benzamidine, and 4 μ g/ml leupeptin. After a 5-ml isocratic wash, ERKs were eluted using a linear NaCl gradient (20 ml, 0–0.33 M NaCl) at a flow rate of 1 ml/min with collection of 0.5-ml fractions. They were assayed by the incorporation of ^{32}P from γ -[^{32}P]ATP into MBP by the direct method as previously described (Bogoyevitch et al., 1993, 1994), except that in some experiments the assay mix included 0.1% (vol/vol) DMSO or 10 μ M SB203580 (final concentrations).

MAPKAPK2 (two 60-mm dishes/column) was partially purified by FPLC on a Mono S HR5/5 column equilibrated with 25 mM β -glycerophosphate pH 7.3, 2 mM EDTA, 5% (vol/vol) glycerol, 0.1% (vol/vol) Triton X-100 and 0.5 mM DTT. After a 5-ml isocratic wash, MAPKAPK2 was eluted using a linear NaCl gradient (15 ml, 0–0.3 M NaCl) at a flow rate of 1 ml/min with collection of 1 ml fractions. MAPKAPK2 was assayed by the incorporation of ^{32}P from γ -[^{32}P]ATP into KKLNRTLSVA peptide substrate (Rouse et al., 1994) as previously described (Bogoyevitch et al., 1996), except that the incubation time was decreased to 20 min. Total MAPKAPK2 activities were determined by integration of the areas under the FPLC peaks.

Isoelectric Focusing (IEF) and Immunoblotting of HSP25/27

Myocytes (in 35-mm dishes) were scraped into 100 μ l/dish of extraction buffer B (12.5 mM Tris-HCl, pH 7.5, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM DTT, 0.3 mM PMSF, 0.12 mM pepstatin A, 0.2 mM leupeptin, 10 μ M trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane), and centrifuged (10,000 g, 5 min, 4°C). Supernatants were mixed with an equal volume of 8 M urea, 4% (wt/vol) Biolyte 3-10, 1% (vol/vol) Triton X-100. Samples (2 μ l, 2–3 μ g protein) were separated by IEF on 5% polyacrylamide gels containing 4 M urea, 0.2% (wt/vol) CHAPS, 5% (vol/vol) glycerol, 2% (wt/vol) Biolyte 3-10 at 100 V (15 min), and finally 450 V (1 h). Proteins were transferred to nitrocellulose and probed with HSP25/27 antibodies (1/100 dilution). Blots were processed as previously described (Clerk et al., 1994).

Immunocytochemical Staining

Cells were plated in 8-well Lab-Tek tissue culture chamber slides precoated with laminin (20 μ g/ml in PBS) and 1% (wt/vol) gelatin at a density of 5×10^4 cells/well (~ 600 cells/ mm^2) and cultured for 18 h. Serum was withdrawn for 24 h before the experiments. Cells were pretreated (15 min) with 0.1% (vol/vol) DMSO or inhibitors and then left unstimulated or exposed to agonists in serum-free medium containing 0.1% (vol/vol) DMSO or inhibitors. After 4, 8, 24, or 48 h, the medium was removed and the cells washed with ice-cold PBS (3×0.5 ml). The cells were fixed for 10 min with 4% (wt/vol) formaldehyde, permeabilized with 0.3% (vol/vol) Triton X-100 in PBS and nonspecific antibody binding blocked with 1% (wt/vol) BSA in PBS containing 0.3% (vol/vol) Triton X-100. The cells were immunostained with an antibody to β -MHC (1/40 dilution, 1 h, 37°C), using a biotinylated anti-mouse IgG secondary antibody (1/200 dilution, 30 min, 37°C) and streptavidin-Texas Red (1/200 dilution, 15 min, 37°C). All antibody dilutions were in PBS and wells were washed with PBS (3×0.5 ml) between each incubation. Slides were mounted and examined using a Zeiss Axioskop microscope with a 100 \times oil immersion objective. Cells were photographed using Tmax 400 film with a set exposure time (30 s).

Results

Phosphorylation of p38-MAPK

To assess the activation of p38-MAPK in cardiac myocytes, we used an antibody selective for the dually phosphory-

lated (activated) form of the p38-MAPKs for Western blot analysis. Hyperosmotic shock (0.5 M sorbitol) stimulated extensive phosphorylation of p38-MAPK (~ 25 -fold relative to controls; Fig. 1 A, top). This response was rapid and maximal within 5 min (Fig. 1 B). ET-1 (100 nM) and PE (100 μ M) also stimulated a rapid but less extensive phosphorylation of p38-MAPK (~ 12 -fold) which was sustained up to at least 20 min (Fig. 1 A, center and B). The phosphorylation of p38-MAPK in response to PMA (1 μ M) was rapid but was considerably less than with the other agonists (Fig. 1 A, lower and B). We directly compared the relative phosphorylation of p38-MAPK induced by the four agonists after 5 min (Fig. 2, A and B). Taking the response to 0.5 M sorbitol as 100%, the relative levels of

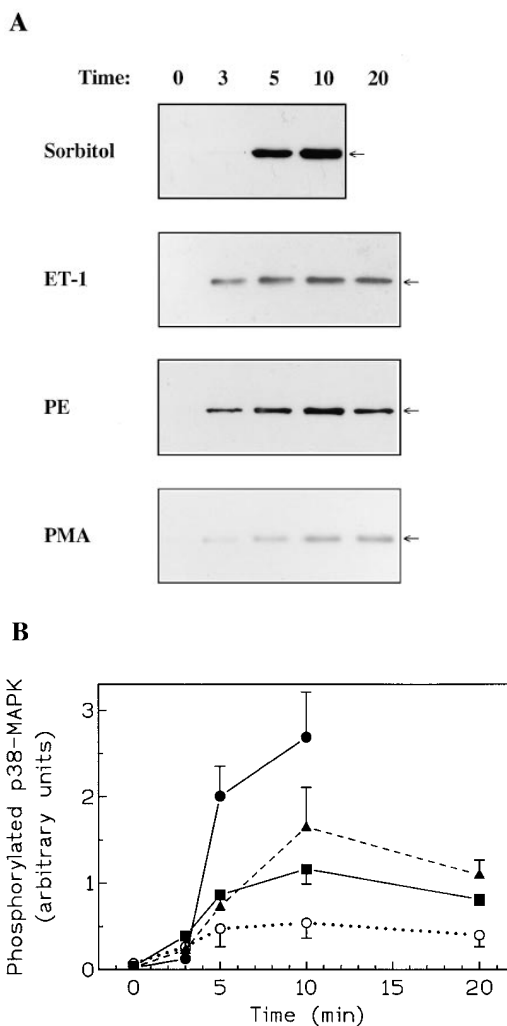


Figure 1. Phosphorylation of p38-MAPK by sorbitol, ET-1, PE, and PMA. Myocytes were exposed to 0.5 M sorbitol, 100 nM ET-1, 100 μ M PE, or 1 μ M PMA for the times indicated. (A) Extracts were immunoblotted for phosphorylated p38-MAPK as described in Materials and Methods. Arrows indicate phosphorylated p38-MAPK. Blots are representative of three independent experiments. (B) Immunoblotted phosphorylated p38-MAPK from myocytes exposed to sorbitol (●, solid line), ET-1 (■, solid line), PE (▲, dashed line), or PMA (○, dotted line) was quantified by laser scanning densitometry. Results are means \pm SEM for three independent experiments.

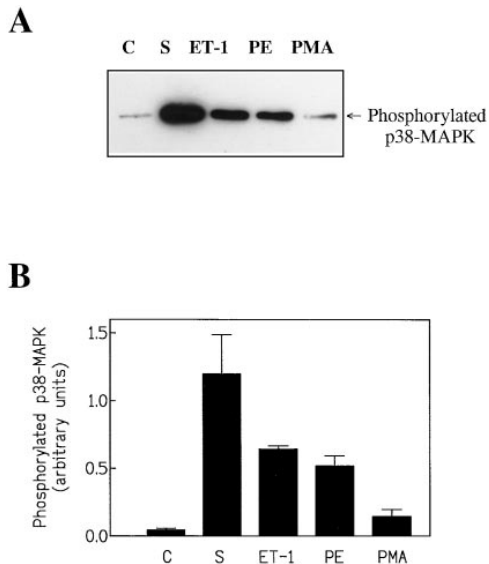


Figure 2. Direct comparison of the degree of p38-MAPK phosphorylation induced by sorbitol, ET-1, PE, and PMA. Myocytes were unstimulated (C) or exposed to 0.5 M sorbitol (S), 100 nM ET-1, 100 μ M PE, or 1 μ M PMA for 5 min. (A) Extracts were immunoblotted for phosphorylated p38-MAPK as described in the Materials and Methods. The blot is representative of three independent experiments. (B) Immunoblotted phosphorylated p38-MAPK was quantified by laser scanning densitometry. Results are means \pm SEM for three independent experiments.

phosphorylation induced by ET-1 (100 nM), PE (100 μ M), and PMA (1 μ M) were 52, 40, and 13%, respectively.

The EC_{50} for stimulation of many biological processes in cardiac myocytes by ET-1 is in the nM range (reviewed by Rubanyi and Polokoff, 1994; Sugden and Bogoyevitch, 1996). Consistent with this, the EC_{50} for ET-1 stimulation of p38-MAPK phosphorylation was 0.46 ± 0.01 nM (mean \pm SEM of three independent experiments; Fig. 3, A and B). In two independent experiments, the ET_A receptor antagonist, BQ123 (10 μ M, 15 min pretreatment), inhibited the phosphorylation of p38-MAPK induced by ET-1 (100 nM, 5 min) as assessed by immunoblotting (results not shown). Quantitative analysis by laser scanning densitometry indicated that this inhibition was $71 \pm 17\%$ (mean \pm SD).

Activation of MAPKAPK2

MAPKAPK2 is a substrate for p38-MAPK (Rouse et al., 1994). In addition to being an important effector in its own right, the stimulation of MAPKAPK2 is a useful indication of p38-MAPK activation. We measured MAPKAPK2 activity in cardiac myocytes after partial purification by Mono S FPLC. Sorbitol (0.5 M) stimulated a very rapid increase in MAPKAPK2 activity that was essentially complete within 5 min and was sustained for up to 30 min (Fig. 4 A). ET-1 (100 nM), PE (100 μ M), and PMA (1 μ M) stimulated modest but sustained increases in MAPKAPK2 activity (Fig. 4 A). The potency of these agonists was compared after 30 min incubation (Fig. 4 B). Sorbitol stimulated an 8.6-fold increase over control values, whereas the stimulation by ET-1, PE and PMA were 5.0-, 3.9-, and 2.8-fold, respectively. Consistent with activation of MAPKAPK2 by p38-MAPK,

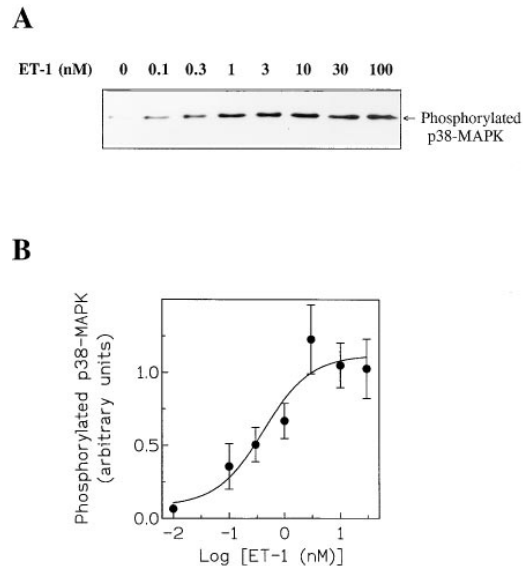


Figure 3. Concentration-dependent phosphorylation of p38-MAPK by ET-1. Myocytes were exposed to the indicated concentrations of ET-1 for 5 min. (A) Extracts were immunoblotted for phosphorylated p38-MAPK as described in Materials and Methods. The blot is representative of three independent experiments. (B) Immunoblotted phosphorylated p38-MAPK was quantified by laser scanning densitometry. Results are means \pm SEM for three independent experiments.

SB203580, a selective inhibitor of p38-MAPK/p38-MAPK β (Goedert et al., 1997; Kumar et al., 1997), essentially completely inhibited the activation of MAPKAPK2 by all four agonists (Fig. 4 B).

MAPKAPK2 was initially identified as a substrate for the ERKs (Stokoe et al., 1992), which are strongly activated in cardiac myocytes by PMA, ET-1 and PE (Bogoyevitch et al., 1993, 1994; Clerk et al., 1994). To confirm that ERKs are not inhibited by SB203580 and the inhibition of MAPKAPK2 activity by this drug reflects its inhibitory effects on p38-MAPK, we purified ERK1 and ERK2 by Mono Q FPLC from myocytes exposed to PMA (1 μ M, 5 min) and assayed activity in the presence of 0.1% (vol/vol) DMSO or 10 μ M SB203580. There was no inhibition of either ERK1 or ERK2 by SB203580 (Fig. 4 C), indicating that p38-MAPK activity is essential for MAPKAPK2 activation in neonatal ventricular myocytes.

Mechanisms of Activation of the p38-MAPK Pathway

Inhibition by PTX. ET-1, in addition to stimulating $G_{q/11}$ -dependent pathways, also couples to G_i in cardiac myocytes (Hilal-Dandan et al., 1994). We investigated the potential involvement of G_i in the activation of the p38-MAPK pathway. Pretreatment of myocytes with 30 ng/ml PTX for 24 h had no effect on the basal levels of phosphorylated p38-MAPK, or on the phosphorylation of p38-MAPK induced by 100 nM ET-1 (Fig. 5 A) or 0.5 M sorbitol (Fig. 5 B). Similarly, neither the basal levels of MAPKAPK2 activity nor the activation of MAPKAPK2 by ET-1 (Fig. 5 C) or sorbitol (Fig. 5 D) was affected by PTX pretreatment. In separate experiments, this regime

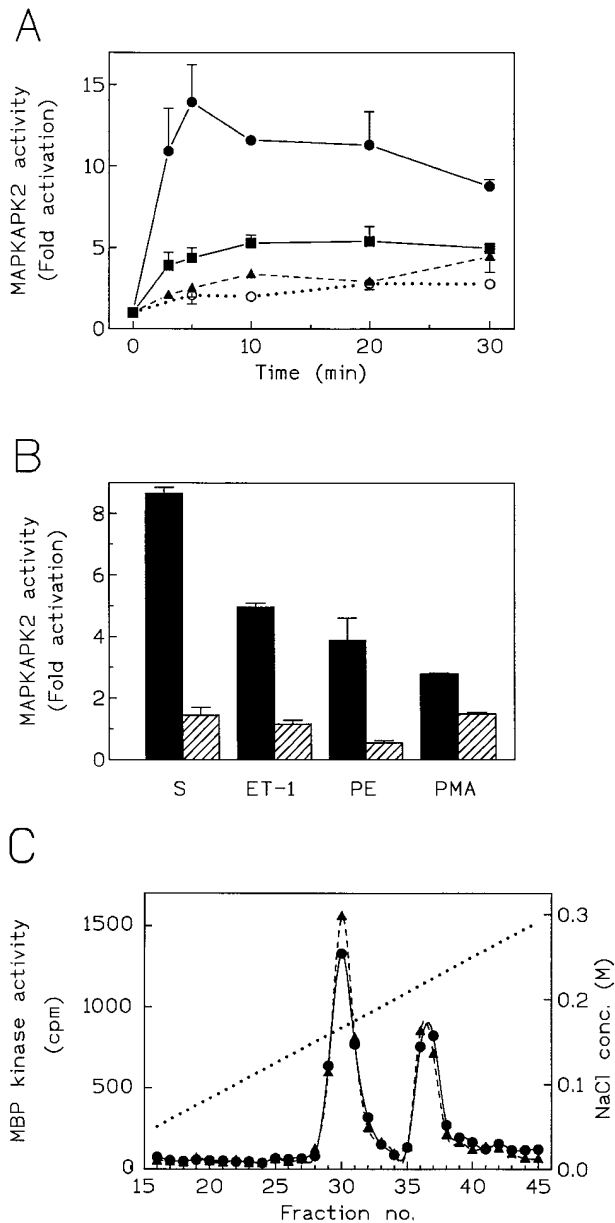


Figure 4. p38-MAPK dependent activation of MAPKAPK2 by sorbitol, ET-1, PE and PMA. (A) Myocytes were exposed to 0.5 M sorbitol (●, solid line), 100 nM ET-1 (■, solid line), 100 μ M PE (▲, dashed line), or 1 μ M PMA (○, dotted line) for the times indicated. MAPKAPK2 activities were measured as described in Materials and Methods. Results are means \pm SEM for three independent experiments. No increase in MAPKAPK2 activity was detected in unstimulated cells over this time (results not shown). (B) Myocytes were exposed to 0.5 M sorbitol (S), 100 nM ET-1, 100 μ M PE, or 1 μ M PMA for 5 min in the presence of 0.1% (vol/vol) DMSO (solid bars) or 10 μ M SB203580 (hatched bars). MAPKAPK2 activities were measured as described in Materials and Methods. Results are means \pm SEM for three independent experiments. (C) Myocytes were exposed to 1 μ M PMA for 5 min and ERK activities were separated on Mono Q FPLC and assayed in the presence of 0.1% (vol/vol) DMSO (●, solid line) or 10 μ M SB203580 (▲, dashed line) as described in Materials and Methods. The NaCl gradient is shown by the dotted line.

of PTX pretreatment inhibited Raf activation by ET-1 (Chiloeches, A., and P.H. Sugden, unpublished observations) indicating the efficacy of this protocol. These data demonstrate that ET-1 stimulation of the p38-MAPK pathway is not mediated through PTX-sensitive G proteins (i.e., G_i or G_o).

Involvement of PKC. Preexposure of myocytes to 1 μ M PMA for 24 h essentially completely downregulates the classical and novel PKC isoforms (Clerk et al., 1995). This protocol increased the basal levels of phosphorylated p38-MAPK (Fig. 5 A) and MAPKAPK2 activity (Fig. 5 C) by two- to threefold. Phosphorylation of p38-MAPK induced by 100 nM ET-1 was inhibited by PMA pretreatment (Fig. 5 A). To evaluate the degree of inhibition, the basal levels of phosphorylated p38-MAPK or MAPKAPK2 activity were subtracted from values obtained in the presence of agonist. The inhibition of ET-1 stimulated p38-MAPK phosphorylation by PMA pretreatment was $79 \pm 8\%$ (mean \pm SEM for three independent experiments). In contrast, the phosphorylation of p38-MAPK by 0.5 M sorbitol was not inhibited by PMA pretreatment (Fig. 5 B). Activation of MAPKAPK2 by ET-1 was inhibited ($47 \pm 7\%$, mean \pm SEM for four independent experiments) by PMA pretreatment (Fig. 5 C), whereas the activation of MAPKAPK2 by sorbitol was unaffected (Fig. 5 D).

We also assessed the effects of a PKC-selective inhibitor, GF109203X (Toullec et al., 1991). This is a more selective PKC inhibitor than staurosporine and, unlike Ro318220 (Beltman et al., 1996), does not strongly activate the SAPKs/JNKs in cardiac myocytes (Clerk, A., and P.H. Sugden, unpublished observations). GF109203X (10 μ M) increased the basal levels of phosphorylated p38-MAPK (Fig. 5 A), although there was no effect on basal MAPKAPK2 activity (Fig. 5 B). GF109203X (10 μ M) had no effect on the phosphorylation of p38-MAPK (Fig. 5 B) or the activation of MAPKAPK2 (Fig. 5 D) by sorbitol, but inhibited the ET-1-stimulated phosphorylation of p38-MAPK (Fig. 5 A) and activation of MAPKAPK2 (Fig. 5 C) by $63 \pm 8\%$ and $64 \pm 7\%$, respectively (means \pm SEM for three or four independent experiments). A lower concentration of GF109203X (1 μ M) had no effect on ET-1 stimulation of either p38-MAPK phosphorylation or MAPKAPK2 activation (results not shown). Published IC_{50} values for inhibition of partially purified PKC by GF109203X are ~ 10 nM (Toullec et al., 1991), considerably less than we observed here. Therefore, using Mono Q FPLC to separate the MAPKs, we assessed the effects of GF109203X on the activation of ERKs in cardiac myocytes by ET-1, a response that is accepted to be predominantly PKC-dependent (Bogoyevitch et al., 1994). ET-1 stimulated two peaks of activity eluting at ~ 0.20 M and 0.25 M NaCl (ERK2 and ERK1, respectively; Fig. 6 A). At 1 μ M, GF109203X had no effect on the activation of ERK2, but inhibited the activation of ERK1 by $\sim 30\%$ (Fig. 6 B). At 10 μ M, activation of both ERK1 and ERK2 were significantly inhibited (Fig. 6 B). This indicates that the IC_{50} for the inhibition of PKC-dependent processes by GF109203X in cardiac myocytes is considerably greater than for purified PKC.

Involvement of the ERK Cascade. PD98059 inhibits the activation of MEK1 and, to a lesser extent, MEK2 (Alessi et al., 1995; Dudley et al., 1995), both of which are involved in ERK activation. We examined the effect of this

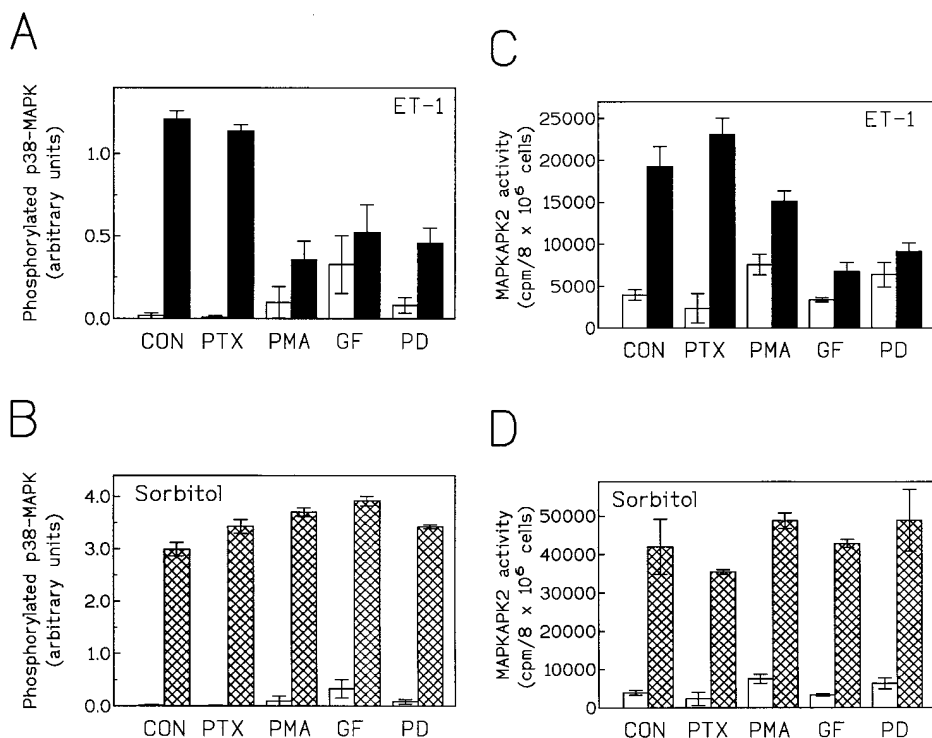


Figure 5. Effects of PTX, PKC inhibition and PD98059 on phosphorylation of p38-MAPK and activation of MAPKAPK2. Myocytes were either not exposed to inhibitors (CON) or pretreated with PTX (30 ng/ml, 24 h), PMA (1 μ M, 24 h), GF109203X (GF; 10 μ M, 15 min) or PD98059 (PD; 50 μ M, 15 min). They were then exposed to 100 nM ET-1 for 5 min (solid bars), 0.5 M sorbitol for 5 min (cross-hatched bars) or left unstimulated (open bars) as described in Materials and Methods. (A and B) Phosphorylation of p38-MAPK by ET-1 (A) or sorbitol (B) was assessed by immunoblotting and laser scanning densitometry as described in Materials and Methods. (C and D) Activation of MAPKAPK2 by ET-1 (C) or sorbitol (D) was measured as described in Materials and Methods. Results are means \pm SEMs for three to nine independent experiments.

drug on the activation of the p38-MAPK pathway to determine whether the same MKKs are involved in the activation of ERKs and p38-MAPK in cardiac myocytes. PD98059 (50 μ M) increased basal levels of phosphorylated p38-MAPK (Fig. 5 A) and MAPKAPK2 activity (Fig. 5 C). The phosphorylation of p38-MAPK and activation of MAPKAPK2 induced by 0.5 M sorbitol were unaffected by 50 μ M PD98059 (Fig. 5, B and D). In contrast, 50 μ M PD98059 inhibited the ET-1 (100 nM) stimulated phosphorylation of p38-MAPK (Fig. 5 A) and activation of MAPKAPK2 (Fig. 5 C) by $50 \pm 3\%$ and $67 \pm 3\%$ (means \pm SEM for four independent experiments), respectively. In two separate experiments, a lower concentration of PD98059 (10 μ M) gave no significant inhibition of MAPKAPK2 activation by ET-1 (results not shown). However, the activation of ERK1 and ERK2 by ET-1 was significantly inhibited ($>80\%$) by 10 μ M or 50 μ M PD98059 (Fig. 6 C). These data indicate that although both ERKs and p38-MAPK may be similarly PKC dependent, the pathways diverge at a level before the MKKs. The results also suggest that whereas ERK activation is probably mediated through MEK1 in these cells (the IC_{50} for MEK1 inhibition by PD98059 is 10 μ M; Alessi et al., 1995), activation of p38-MAPK may be mediated through MEK2 (the IC_{50} for MEK2 inhibition is 50 μ M; Alessi et al., 1995) or another MKK which is sensitive to inhibition by PD98059.

Phosphorylation of HSP25/27. In certain cell types, phosphorylation of HSP25/27 is associated with cytoprotection and stabilization of the actin cytoskeleton (Guay et al., 1997; Huot et al., 1997). HSP25/27 is a known substrate for MAPKAPK2 (Rouse et al., 1994). Therefore, we assessed the phosphorylation state of HSP25/27 in cells exposed to 100 nM ET-1 using IEF followed by immunoblotting. An

increase in the phosphorylation of HSP25/27 increases the negative charge of the protein that allows it to migrate more quickly to the anode during the IEF stage. The individual phosphorylated forms of HSP25/27 are then specifically identified by immunoblotting. Up to three forms of HSP25/27 were detected using this method. In unstimulated cells, HSP25/27 was detected predominantly as bands 1 and 2, the most positively charged (least phosphorylated) forms migrating closest to the cathode (Fig. 7, top). In myocytes subjected to cellular stress (0.5 M sorbitol, 30 min), HSP25/27 was detected principally as bands 2 and 3, the more negatively charged forms. ET-1 stimulated an increase in the levels of band 3 from ~ 5 –8 min with a corresponding decrease in the levels of band 1, indicating an increase in the phosphorylation state of HSP25/27. This change was maximal at ~ 30 min, and was sustained at least up to 1 h. The increase in phosphorylation of HSP25/27 induced by ET-1 was comparable to that induced by 0.5 M sorbitol. SB203580 completely inhibited this response (results not shown), consistent with the stimulation of HSP25/27 phosphorylation by ET-1 being mediated by p38-MAPK/MAPKAPK2.

Effects of p38-MAPK Inhibition on Cardiac Myocyte Hypertrophy/Cell Survival. We investigated the effects of p38-MAPK inhibition on the morphological changes induced in cardiac myocytes by ET-1 and PE over a period of 4–48 h. We compared the effects of SB203580 with those of PD98059, which inhibits the ERK cascade but also partially inhibits the p38-MAPK pathway (Fig. 6 C). Myocytes were cultured in chamber slides and either not stimulated or exposed to agonists in the absence or presence of inhibitors. The myofilaments were immunostained with an antibody to β -MHC. Control cells, cultured in serum-free medium for up to 24 h, were irregular and the myofila-

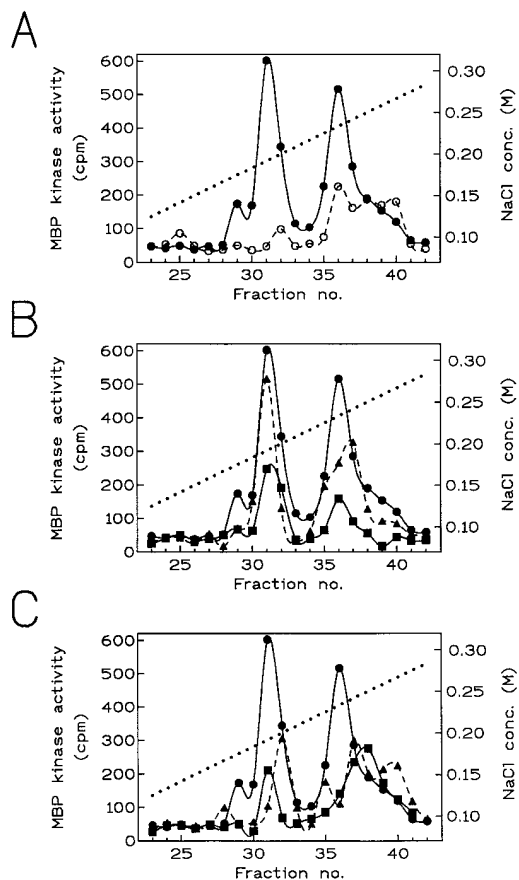


Figure 6. Inhibition of ET-1-stimulated ERK activation by GF109203X and PD98059. (A) Myocytes were pretreated with 0.2% (vol/vol) DMSO for 15 min and either left unstimulated (○, dashed line), or exposed to 100 nM ET-1 for 5 min (●, solid line). ERKs were separated by Mono Q FPLC and assayed as described in Materials and Methods. The NaCl gradient is shown by the dotted line. The experiment was repeated with essentially similar results. (B) Myocytes were pretreated with 0.2% (vol/vol) DMSO (●, solid line), 1 μM GF109203X (▲, dashed line), or 10 μM GF109203X (■, solid line) for 15 min, and then exposed to 100 nM ET-1 for 5 min. ERKs were separated by Mono Q FPLC and assayed as described in Materials and Methods. The NaCl gradient is shown by the dotted line. The experiment was repeated with essentially similar results. (C) Myocytes were pretreated with 0.2% (vol/vol) DMSO (●, solid line), 10 μM PD98059 (▲, dashed line), or 50 μM PD98059 (■, solid line) for 15 min, and then exposed to 100 nM ET-1 for 5 min. ERKs were separated by Mono Q FPLC and assayed as described in Materials and Methods. The NaCl gradient is shown by the dotted line. The experiment was repeated with essentially similar results.

ments were disorganized (Figs. 8 A, 9 A, and 10 A). Myocytes exposed to 50 μM PD98059 (e.g., Figs. 9 D and 10 D) or SB203580 (e.g., Figs. 9 G and 10 G) appeared similar to the control cells at all time points studied.

From as early as 4 h, 40 μM PE (Fig. 8 B) or 40 nM ET-1 (Fig. 8 C) stimulated an increase in cell profile and myofibrillar organization relative to the controls (Fig. 8 A). By 8 h, cells exposed to either PE (Fig. 9 B) or ET-1 (Fig. 9 C) exhibited the typical morphology associated with cardiac

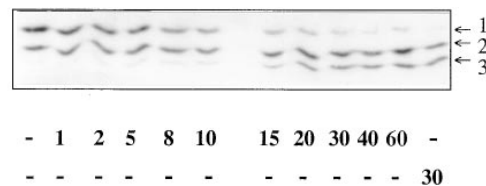


Figure 7. ET-1 stimulation of HSP25/27 phosphorylation. Myocytes were exposed to 100 nM ET-1 or 0.5 M sorbitol for the times indicated. HSP25/27 phosphorylation was assessed by IEF followed by immunoblotting as described in Materials and Methods. The anode is at the bottom of the figure. The blot is representative of three independent experiments.

hypertrophy. The cells were larger, with intense β-MHC staining and clear cross-striations. In myocytes exposed to either agonist in the presence of 50 μM PD98059, the myofibrillar organization was disrupted although there was no apparent decrease in the intensity of β-MHC immunostaining (Fig. 9, E and F). In contrast, 10 μM SB203580 had no effect on the morphology of myocytes stimulated with PE (Fig. 9 H) or ET-1 (Fig. 9 I).

After 24 h, control myocytes maintained in serum-free medium appeared smaller than those at 4 or 8 h, with reduced β-MHC immunostaining (Fig. 10 A), whereas cells exposed to PE (Fig. 10 B) or ET-1 (Fig. 10 C) exhibited a similar morphology to those at 8 h (Fig. 9, B and C, respectively). In myocytes exposed to either agonist in the presence of 50 μM PD98059 for 24 h, the myofibrillar organization was clearly disrupted although there was no apparent decrease in the intensity of β-MHC immunostaining or in cell profile (Fig. 9, E and F). SB203580 (10 μM) had no effect on any aspect of the morphological changes induced in myocytes by PE (Fig. 10 H) or ET-1 (Fig. 10 I) after 24 h.

In addition to inhibiting ERK activation (Fig. 6), 50 μM PD98059 also inhibits the activation of p38-MAPK and MAPKAPK2 by ET-1 (Fig. 5). However, 10 μM PD98059 (which does not significantly inhibit MAPKAPK2 activation, but does inhibit ERK activation [Fig. 6]) also disrupted the myofibrillar organization induced by ET-1 or PE for 8 and 24 h (results not shown). Furthermore, 10 μM SB203580 together with 10 μM (or 50 μM) PD98059 had no additional effect on the morphology of myocytes exposed to ET-1 or PE for 8 or 24 h (results not shown). These results indicate that the effect of PD98059 to disrupt myocyte myofibrillar organization is not attributable its inhibition of the p38-MAPK pathway.

After 48 h in serum-free medium, there were considerably fewer myocytes in the control wells and there was a substantial amount of cell debris (e.g., Fig. 11 A, arrowhead). The remaining myocytes were small, with very little β-MHC staining (Fig. 11 A). The number of myocytes in wells treated with PE or ET-1 appeared similar to the other time points studied (results not shown), suggesting that agonist stimulation promotes survival of cardiac myocytes. Myocytes exposed to PE for 48 h exhibited two patterns of morphology. Approximately 50% of the cells were large and clearly striated with very bright immunostaining for β-MHC (Fig. 11 B). The remaining cells were weakly stained for β-MHC, but were still striated (Fig. 11 C). Myo-

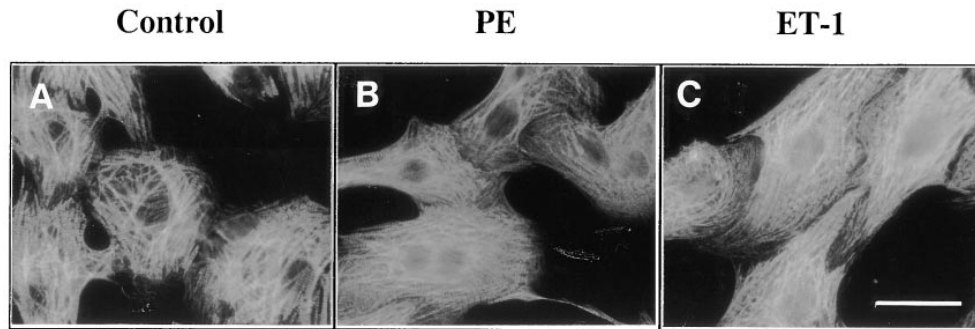


Figure 8. Morphological changes induced by PE or ET-1 in cardiac myocytes after 4 h. Myocytes were either unstimulated (*Control, A*), or exposed to 40 μ M PE (*B*) or 40 nM ET-1 (*C*) for 4 h and immunostained for β -MHC as described in Materials and Methods. Myocytes shown are representative of a typical field within a single experiment. The experiment was repeated on three further occasions with similar results. Bar, 25 μ m.

cytes exposed to PE in the presence of PD98059 appeared weakly stained and the myofilaments were not organized (Fig. 11 *D*). In marked contrast to the results obtained up to 24 h, myocytes exposed to PE in the presence of SB203580 for 48 h were very small with minimal β -MHC immunostaining and no myofibrillar organization (Fig. 11 *E*). Similar results were obtained with ET-1 (results not shown). These data are not consistent with a role for the p38-MAPK pathway in stimulating any of the morphological changes induced by PE or ET-1. Rather, this pathway may be important over a longer period in maintaining the

hypertrophic response. However, inhibition of the ERK cascade by PD98059 clearly disrupts the myofibrillar organization induced by PE or ET-1.

Discussion

Activation of the p38-MAPK Pathway by GPCR Agonists

The p38-MAPK cascade is activated in many cell types in response to cellular stresses including hyperosmotic shock,

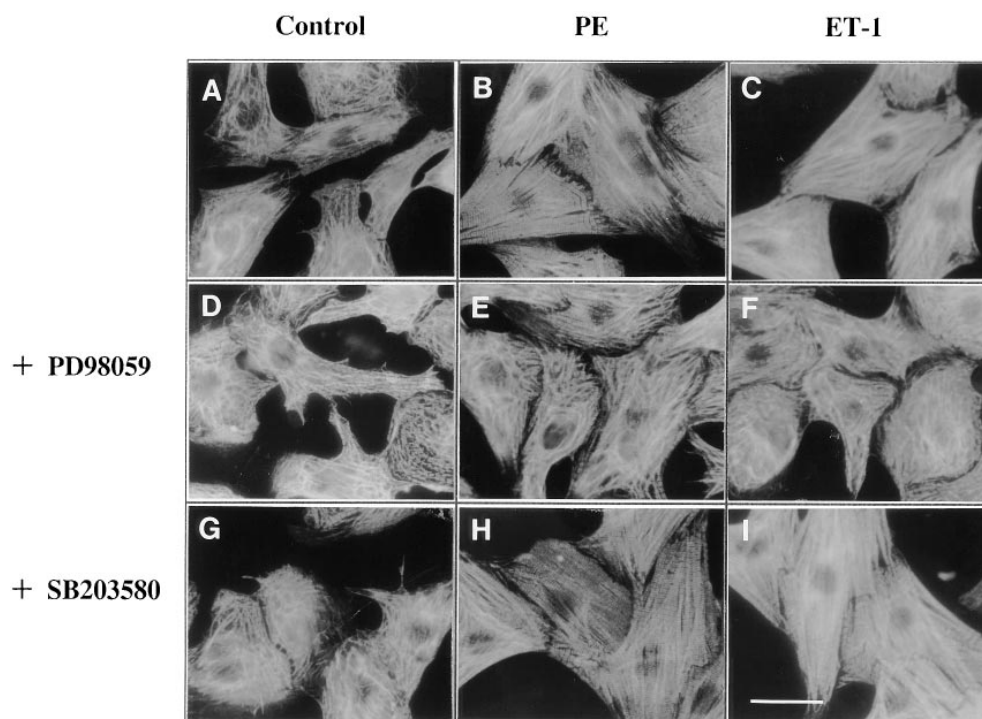


Figure 9. Effects of PD98059 or SB203580 on the morphological changes induced by PE or ET-1 in cardiac myocytes after 8 h. (*A–C*) Myocytes were either unstimulated (*Control, A*), or exposed to 40 μ M PE (*B*) or 40 nM ET-1 (*C*) for 8 h in the absence of inhibitors. (*D–F*) Myocytes were pretreated with 50 μ M PD98059 for 15 min and exposed to 50 μ M PD98059 alone (*D*), to PE in the presence of PD98059 (*E*), or to ET-1 in the presence of PD98059 (*F*) for 8 h. (*G–I*) Myocytes were pretreated with 10 μ M SB203580 for 15 min and exposed to 10 μ M SB203580 alone (*G*), to PE in the presence of SB203580 (*H*), or to ET-1 in the presence of SB203580 (*I*) for 8 h. Myocytes were subsequently immunostained for β -MHC as described in Materials and Methods. Myocytes shown are representative of a typical field within a single experiment. The experiment was repeated on five further occasions with similar results. Bar, 25 μ m.

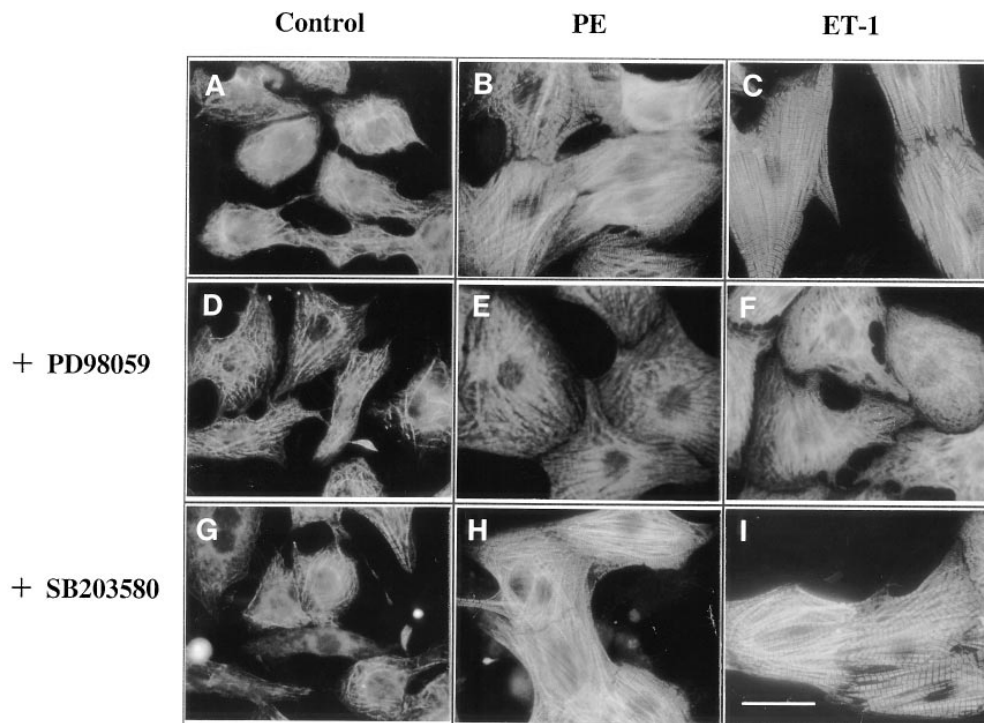


Figure 10. Effects of PD98059 or SB203580 on the morphological changes induced by PE or ET-1 in cardiac myocytes after 24 h. (A–C) Myocytes were either unstimulated (Control, A), or exposed to 40 μ M PE (B) or 40 nM ET-1 (C) for 24 h in the absence of inhibitors. (D–F) Myocytes were pretreated with 50 μ M PD98059 for 15 min and exposed to 50 μ M PD98059 alone (D), to PE in the presence of PD98059 (E), or to ET-1 in the presence of PD98059 (F) for 24 h. (G–I) Myocytes were pretreated with 10 μ M SB203580 for 15 min and exposed to 10 μ M SB203580 alone (G), to PE in the presence of SB203580 (H), or to ET-1 in the presence of SB203580 (I) for 24 h. Myocytes were subsequently immunostained for β -MHC as described in Materials and Methods. Myocytes shown are representative of a typical field within a single experiment. The experiment was repeated on three further occasions with similar results. Bar, 25 μ m.

UV irradiation and exposure to proinflammatory cytokines (reviewed by Kyriakis and Avruch, 1996a, 1996b; Lee and Young, 1996; Cohen, 1997). There are very few reports of activation of the p38-MAPK pathway by GPCR agonists. p38-MAPK is activated in platelets by thrombin (Kramer et al., 1995) and mediates thrombin stimulation of phospholipase A₂ activity (Kramer et al., 1996). The chemotactic peptide *N*-formyl-Met-Leu-Phe also activates p38-MAPK and MAPKAPK2 in neutrophils (Krump et al., 1997). Cotransfection of m1 and m2 muscarinic acetylcholine receptors with an epitope-tagged p38-MAPK has shown that these receptors can activate the pathway (Yamauchi et al., 1997), but since the activation was only approximately twofold, it is difficult to assess its significance. A twofold activation of epitope-tagged p38-MAPK is also seen after β -adrenergic stimulation (Yamauchi et al., 1997). In similar experiments, transfection of a modified ET_B receptor activates ERKs, SAPKs/JNKs and p38-MAPKs (Aquila et al., 1996). In a limited study, PE has been shown to increase the phosphorylation of p38-MAPK twofold in cardiac myocytes after 30 min (Zechner et al., 1997), but this was not studied in detail and the activation of downstream events was not studied. We have previously demonstrated that p38-MAPK is activated in the perfused rat heart by pathophysiological stresses (ischaemia and ischaemia/reperfusion [Bogoyevitch et al., 1996], reactive oxygen species [Clerk et al., 1998] and hypertensive perfusion [Clerk et al., 1998]).

In this study, we have clearly demonstrated that, in addition to cellular stress (hyperosmotic shock), the GPCR agonists ET-1 and PE activate the p38-MAPK pathway in primary cultures of neonatal rat ventricular myocytes. Both agonists induced an \sim 12-fold increase in the phosphorylation (activation) of p38-MAPK (Fig. 2) and a four- to fivefold increase in the activity of the p38-MAPK substrate, MAPKAPK2 (Fig. 4). This represents a significant activation of the pathway and is \sim 50% of the response induced by a severe cellular stress, 0.5 M sorbitol (Figs. 2 and 4). The phosphorylation motif is similar in all the p38-MAPKs and the antibody to the dually phosphorylated form recognizes all isoforms. Thus, from these experiments, it is not possible to determine whether any specific p38-MAPK isoforms were activated by the different agonists. However, activation of MAPKAPK2 was completely inhibited by SB203580, which strongly inhibits p38-MAPK(α) and p38-MAPK β ₁ and is a moderate inhibitor of p38-MAPK β ₂ (Kumar et al., 1997), indicating that at least one of these isoforms is activated in cardiac myocytes. Furthermore, although p38-MAPK(α) is easily detected on immunoblots of myocyte extracts, we have yet to detect any of the other isoforms (Clerk, A., and P.H. Sugden, unpublished data).

From the results presented here, it is now apparent that ET-1 and PE can activate all three of the best-characterized MAPK cascades in the heart. The ERK cascade was first shown to be strongly activated by ET-1 (Bogoyevitch

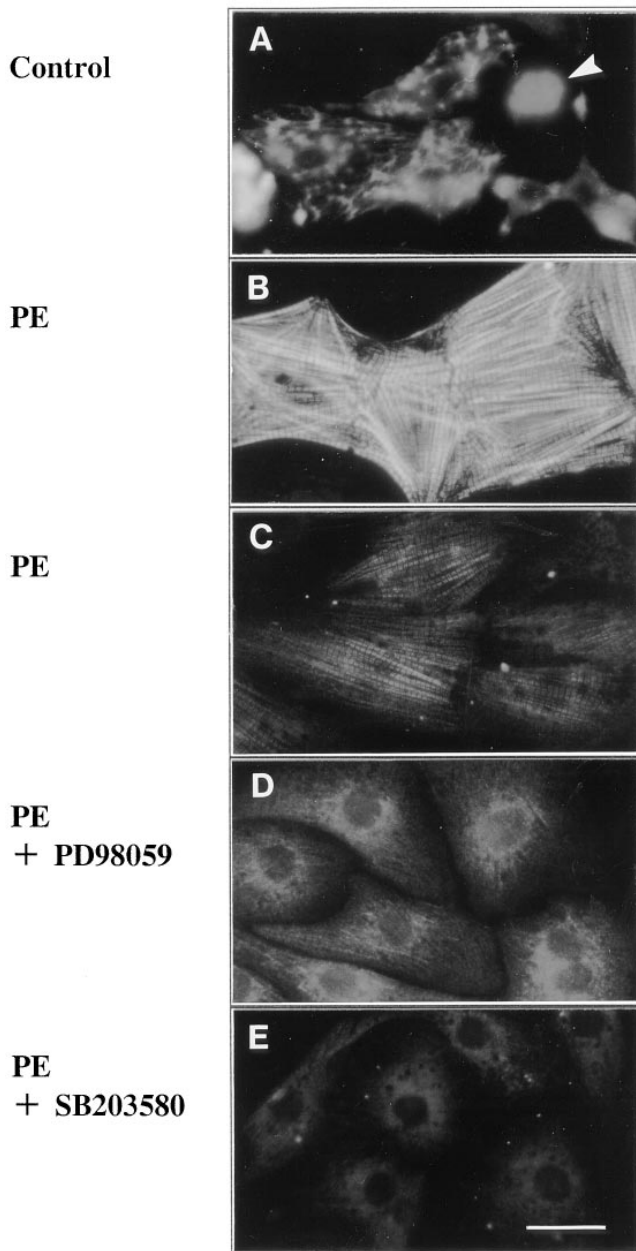


Figure 11. Effects of PD98059 or SB203580 on the morphological changes induced by PE in cardiac myocytes after 48 h. (A–C) Myocytes were unstimulated (*Control*, A), or exposed to 40 μ M PE (B and C) for 48 h in the absence of inhibitors. (D and E) Myocytes were pretreated with 50 μ M PD98059 (D) or 10 μ M SB203580 (E) for 15 min and exposed to PE in the presence of inhibitors for 48 h. Myocytes were subsequently immunostained for β -MHC as described in Materials and Methods. Myocytes shown are representative of a typical field within a single experiment. The experiment was repeated on two further occasions with similar results. Bar, 25 μ m.

et al., 1993, 1994) and, to a lesser extent by PE (Clerk et al., 1994). ET-1 was then demonstrated to activate the SAPKs/JNKs (approximately fivefold) whereas PE induced only an approximately twofold activation of this pathway (Bogoyevitch et al., 1995a). This twofold activation of SAPKs/JNKs by PE was subsequently confirmed by others

(Ramirez et al., 1997). In contrast to the ERKs and SAPKs/JNKs, the activation of the p38-MAPK pathway by PE is comparable to that by ET-1 (Figs. 2 and 4).

Mechanism of Activation of the p38-MAPK Pathway by ET-1

The ET_A receptor mediates most of the effects of ET-1 in cardiac myocytes (reviewed by Sugden and Bogoyevitch, 1996). ET-1-stimulated phosphorylation of p38-MAPK was inhibited by BQ123, a selective ET_A receptor antagonist (see Results), indicating that activation of both pathways is mediated through the same receptor. Consistent with this, the EC₅₀ for p38-MAPK phosphorylation (0.49 nM, Fig. 3) by ET-1 in cardiac myocytes is similar to that for ERK activation (0.2 nM) (Bogoyevitch et al., 1993). Studies of COS cells transfected with ET_B receptors have also indicated that a single receptor subtype can stimulate activation of ERKs, SAPKs/JNKs, and p38-MAPKs (Aquilla et al., 1996), although whether this is through a single G protein subtype remains to be determined. In our study, ET-1-stimulated phosphorylation of p38-MAPK and activation of MAPKAPK2 was not affected by pretreatment with PTX (Fig. 5, A and C), indicating that activation of this pathway in cardiac myocytes is not mediated through G_i/G_o. We have previously shown that PTX inhibits ET-1-induced activation of Raf (Bogoyevitch et al., 1995c), the MKK kinase for the ERK cascade (reviewed by Sugden and Clerk, 1997). This suggests that activation of the ERK and p38-MAPK pathways by ET-1 require activation of different G proteins. As expected, the activation of the p38-MAPK pathway by 0.5 M sorbitol was unaffected by PTX pretreatment (Fig. 5, B and D).

Phosphorylation of p38-MAPK and activation of MAPKAPK2 by ET-1 appeared to be dependent on PMA-sensitive isoforms of PKC since downregulation of these isoforms or inhibition of PKC activity with GF109203X significantly attenuated this response (Fig. 5, A and C). This PKC-dependency was comparable to that of ERK, which is also inhibited by PKC downregulation (Bogoyevitch et al., 1994) and is inhibited by similar concentrations of GF109203X (Fig. 6 B). Activation of the p38-MAPK pathway by hyperosmotic shock (0.5 M sorbitol) was unaffected by either of these treatments (Fig. 5, B and D), indicating that the data do not reflect a nonspecific inhibitory effect of either PMA pretreatment or of GF109203X on the p38-MAPK pathway. Although the activation of p38-MAPK appears to be PKC-dependent, PMA activation of PKC only stimulated approximately a threefold increase in p38-MAPK phosphorylation and in MAPKAPK2 activity. These data suggest that although activation of the p38-MAPK pathway by ET-1 may be PKC-dependent, other factors are necessary for full activation. Activation of PKC is mediated through G_q-stimulation of phospholipase C β (reviewed in Fields and Casey, 1997; Sugden and Clerk, 1997), suggesting that the ERK and p38-MAPK pathways require G_q for full activation by ET-1.

MAPKAPK2 is phosphorylated on multiple Ser/Thr residues and these phosphorylations regulate its activity (Ben-Levy et al., 1995). Although MAPKAPK2 is now generally accepted to be a substrate for p38-MAPK

(Rouse et al., 1994; Ben-Levy et al., 1995), it was originally identified as an ERK substrate (Stokoe et al., 1992) and at least one site (Thr²⁵) may be preferentially phosphorylated by ERKs (Ben-Levy et al., 1995). Whereas phosphorylation of Thr²⁵ does not activate MAPKAPK2 on its own, it may modulate the activation by the other phosphorylation sites (Ben-Levy et al., 1995). PD98059 is a selective inhibitor for MEK1 and, to a lesser extent, MEK2 (Alessi et al., 1995; Dudley et al., 1995), although we are unaware of any testing of PD98059 against other MKKs. PD98059 (50 μ M) partially inhibited the activation of MAPKAPK2 by ET-1 (Fig. 5 C), but also partially inhibited p38-MAPK phosphorylation (Fig. 5 A). At this concentration of PD98059, activation of ERKs by ET-1 was essentially completely inhibited and a lower concentration (10 μ M), which did not significantly inhibit MAPKAPK2 activation (results not shown), almost completely inhibited ERK activation (Fig. 6 C). These data indicate that ERK activation by ET-1 does not contribute to the activation of MAPKAPK2, and provide circumstantial evidence that PD98059 inhibits the activation of the MKK(s) of the p38-MAPK pathway. In addition, we have shown that SB203580 does not inhibit ERK activity (Fig. 4 C), but completely abolishes the activation of MAPKAPK2 by all agonists studied (Fig. 4 B). Although SB203580 does inhibit some isoforms of SAPKs/JNKs in cardiac myocytes (Clerk and Sugden, 1998), there is no evidence currently that these MAPKs phosphorylate and activate MAPKAPK2. Therefore, the data are consistent with phosphorylation of MAPKAPK2 by p38-MAPK being essential for activation.

Role of p38-MAPKs in Hypertrophy or Cell Survival?

p38-MAPKs have been implicated in the development of the hypertrophic phenotype in primary cultures of ventricular myocytes. Transfection of constitutively activated MKK6 (Zechner et al., 1997; Wang et al., 1998) or MKK3 (Wang et al., 1998) induces transcriptional and morphological changes associated with the hypertrophic response, indicating that activation of the p38-MAPK pathway has the potential to promote hypertrophy. However, the situation may be more complex as cotransfection of either MKK3 or MKK6 with p38-MAPK(α) or p38-MAPK β suggest that p38-MAPK(α) promotes apoptosis, whereas p38-MAPK β induces hypertrophy (Wang et al., 1998). Such transfection experiments are not unequivocal since (a) there may be cross-talk between the MAPK pathways, and (b) whereas activation of a single (overexpressed) pathway may induce a particular response, the physiological relevance of such a response cannot be assessed. Furthermore, in these studies, the myocytes are cultured in serum-free conditions for up to 70 h before analysis. Beyond 24 h, as discussed below, it becomes questionable whether studies of this nature are studies of hypertrophy or of myocyte survival.

The hypertrophic response induced by a 48 h exposure to PE is inhibited by SB203580 (Zechner et al., 1997). Here, we have shown that the morphological changes associated with cardiac myocyte hypertrophy induced by ET-1 or PE were apparent from as early as 4 h after stimulation (Fig. 8, B and C), and that between 8 h (Fig. 9, B

and C) and 24 h (Fig. 10, B and C), the cells exhibited the mature hypertrophic phenotype. We also demonstrated that SB203580 had no effect on myocyte morphology over this time period (Fig. 9, G-I and 10, G-I), strongly indicating that the p38-MAPK pathway is not necessary for the induction of the morphological changes associated with cardiac hypertrophy. After 48 h, myocytes exposed to PE (Fig. 11, B and C) or ET-1 (results not shown) maintained their myofibrillar organization and cell profile. However, myocytes exposed to PE or ET-1 in the presence of SB203580 were small, with minimal β -MHC staining and no myofibrillar organization (Fig. 11 E and results not shown). These data at 48 h are essentially similar to those of Zechner et al. (1997) who only studied the effects at this time point, but in view of the absence of any effect of this inhibitor up to 24 h, suggest that the p38-MAPK pathway may be necessary to maintain the cells during hypertrophy rather than have a direct effect in stimulating the hypertrophic response. Consistent with a role for the p38-MAPK pathway in cytoprotection, activation of MAPKAPK2 by ET-1 induced the sustained phosphorylation of HSP25/27 (Fig. 7). Such phosphorylation of HSP25/27 is associated with cytoprotection in other cell types, and is particularly important in the maintenance of the actin cytoskeleton. Even though the activation of the p38-MAPK pathway by ET-1 was only \sim 50% of that by 0.5 M sorbitol (Figs. 2 and 4), the extent of phosphorylation of HSP25/27 induced by either agonist was similar (Fig. 7) and may confer equivalent degrees of cytoprotection. Experiments by others have implicated the p38-MAPK pathway in ischemic preconditioning of the heart, whereby a short period of ischemia protects the heart against a subsequent more prolonged insult (reviewed by Sumeray and Yellon, 1997). Perfusion with SB203580 attenuates this preconditioning (Weinbrenner et al., 1997) suggesting that p38-MAPK, which is activated during ischemia (Bogoyevitch et al., 1996; Yin et al., 1997) plays an essential role in the preconditioning response. Ischemic preconditioning can be mimicked by perfusion with α_1 -adrenergic agonists (Banerjee et al., 1993), ET-1 (Wang et al., 1996), and in the rat heart, activation of PKC may be involved (Speechly-Dick et al., 1994; Li and Kloner, 1995; Mitchell et al., 1995). Our results are entirely consistent with a role for p38-MAPK in ischemic preconditioning. Moreover, since activation of the p38-MAPK pathway by GPCR agonists in cardiac myocytes is attenuated by inhibition of PKC, these data could explain the apparently disparate results implicating both PKC and p38-MAPK in ischemic preconditioning.

The Role of the MAPKs in Cardiac Hypertrophy

The ERKs, SAPKs/JNKs, and p38-MAPKs have all been implicated in cardiac hypertrophy, although the exact roles of each of these pathways has yet to be determined. Activation of the ERK cascade by transfection of myocytes with constitutively activated components of the pathway is not sufficient to induce the myofibrillar organization associated with hypertrophy (Thorburn et al., 1994a, b; Gillespie-Brown et al., 1995). However, in this study, we have shown that PD98059, which inhibits the ERK pathway and partially inhibits the p38-MAPK pathway, selec-

tively inhibits the myofibrillar organization induced by PE or ET-1 (Fig. 9, E and F), suggesting that the activation of ERKs is essential for this response. However, it remains possible that the effects of PD98059 may be mediated through another, as yet unidentified mechanism. PD98059 does not inhibit the transcriptional changes associated with PE-induced cardiac myocyte hypertrophy (Post et al., 1996), although activation of the ERK cascade in transfection experiments can induce these changes (Gillespie-Brown et al., 1995). Such apparently conflicting data are probably indicative of the activation of multiple signaling pathways by physiological agonists. For example, whilst ERK activity may be necessary for myofibrillar organization, other signaling pathways may be necessary for the full response. Indeed, our data are fully supportive of hypertrophy being an integrated response to multiple signaling pathways, where ERKs may be involved in myofibrillar organization and p38-MAPK may be important in potentiating the hypertrophic response. It remains probable that both (and other) pathways are necessary for the full range of transcriptional and morphological changes associated with cardiac hypertrophy.

We thank R.J.A. Taylor for the preparation of the myocyte cultures.

This work was supported by grants from the British Heart Foundation, the Clinical Research Committee of the Royal Brompton Hospital, and the European Union BIOMED2 program.

Received for publication 18 February 1998 and in revised form 3 June 1998.

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