Mitogen-activated protein kinase phosphatase 1 inhibits the stimulation of gene expression by hypertrophic agonists in cardiac myocytes

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The effect of constitutive expression of mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) on gene expression in response to hypertrophic agonists was examined in cultured neonatal rat ventricular myocytes. Luciferase (LUX) reporter genes linked to promoters for atrial natriuretic factor, ventricular myosin light chain 2, β -myosin heavy chain, skeletal muscle α -actin (SkM α -actin) and serum response element-regulated *c-fos* (*c-fos*-SRE) were transfected into cardiomyocytes. Phenylephrine (PE; 10 μ M), phorbol 12-myristate 13-acetate (1 μ M) and endothelin 1 (10 nM) stimulated the expression of these various reporter genes by 2.5–20-fold. MKP-1 inhibited these effects by 60–85%. In contrast, MKP-1 had no effect on the expression of a constitutively active Rous sarcoma virus–LUX reporter gene. A catalytically inactive mutant MKP-1CS (cysteine $\rightarrow$ serine mutation) and the broad-specificity protein tyrosine phosphatase

1B (PTP-1B) had no significant effect on any reporter gene tested. MKP-1 had much less effect on the morphological features accompanying agonist-induced cardiac hypertrophy. PE (10 μ M) increased myocyte area by 59% but this effect was only decreased by one-third by MKP-1 and was also partly decreased (by 25%) by expression of PTP-1B. PE also altered cell shape but this was unaffected by MKP-1. There was also no clear effect of MKP-1 on the organization of the contractile apparatus into sarcomeric structures in the presence of 10 μ M PE. We conclude that the transcriptional responses accompanying cardiac myocyte hypertrophy are dependent on an MKP-1-sensitive step, presumably the activation of one or members of the MAPK family, but that cell size, shape and myofibrillar organization are much less sensitive to inhibition by MKP-1.

INTRODUCTION

The extracellular signal-regulated kinase (ERK) members of the mitogen-activated protein kinase (MAPK) family of serine/threonine protein kinases are intimately involved in the signal transduction processes that lead to the differentiation or proliferation of a wide variety of cell types (reviewed in [1–4]). This action results from their ability to phosphorylate a spectrum of substrates including signalling molecules, transcription factors and structural and other proteins [3,5]. Activation of ERK1 (p44 MAPK) and ERK2 (p42 MAPK) is brought about by phosphorylation on Thr and Tyr residues within a conserved TEY motif by MAPK kinase (ERK kinase; MEK) [6]. MEKs themselves are activated by phosphorylation on Ser residues by MEK kinases including Raf [7–9]. For receptor protein tyrosine kinases the intervening steps between receptor occupancy and activation of the Raf $\rightarrow$ MEK $\rightarrow$ ERK cascade are well established [10,11]. Activation of ERKs by G_i -coupled receptors, but not by G_q -coupled receptors [12], might involve $\beta\gamma$ subunits [12,13]. For G_q -coupled receptors the upstream events are less clear but might involve protein kinase C (PKC) activation and possibly protein tyrosine kinases such as PYK2 [14].

In cardiac myocytes the ERK cascade is activated by fibroblast growth factors acting through receptor protein tyrosine kinases, endothelin 1 (ET-1) and α_1 -adrenergic agonists such as phen-

ylephrine (PE), which interact with G_q -coupled receptors, PKC-activating phorbol esters such as phorbol 12-myristate 13-acetate (PMA) and by stretch [15–21]. These same disparate agents initiate the changes in transcription and cell morphology that characterize the hypertrophic phenotype in these cells and that have prompted the suggestion that activation of the ERK cascade might be a common and obligatory step in the development of cardiac hypertrophy [15,16,22]. Additional evidence for this hypothesis is that overexpression of Raf-1 or constitutively active MEK1 in transient transfection studies has been shown to activate ERK1 and ERK2 and to induce transcriptional responses characteristic of the hypertrophic response in cardiac myocytes [23,24]. Further, the introduction of dominant-negative mutant constructs for Raf-1 [23], MEK1 [24] or ERK1 [23] inhibits the hypertrophic transcriptional responses to PE.

In addition to the transcriptional responses, which include the rapid induction of immediate-early genes (*c-fos*, *c-jun* and *Egr-1* [25,26]), re-expression of foetally expressed genes {atrial natriuretic factor (ANF) [27,28], β -myosin heavy chain (β -MHC) [29] and skeletal muscle α -actin (SkM- α -actin) [30]} and up-regulation of constitutively expressed contractile protein genes {ventricular myosin light chain 2 (vMLC-2) [31] and cardiac muscle α -actin [30]}, hypertrophic agonists induce morphological changes in cardiac myocytes. In response to a hypertrophic stimulus, cultured neonatal rat ventricular myocytes increase in

Abbreviations used: ANF, atrial natriuretic factor; β -gal, β -galactosidase; BK, bradykinin; β -MHC, β -myosin heavy chain; ERK, extracellular signal-regulated kinase; ET-1, endothelin 1; JNK, c-Jun N-terminal kinase; LUX, luciferase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, ERK kinase; MKP-1, MAPK phosphatase 1; MKP-1CS, MKP-1 cysteine $\rightarrow$ serine mutation; PE, phenylephrine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTP, protein tyrosine phosphatase; RSV, Rous sarcoma virus; SAPK, stress-activated protein kinase; SkM- α -actin, skeletal muscle α -actin; SRE, serum response element; vMLC-2, ventricular myosin light chain 2.

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size [25,26,32], increase RNA and protein synthesis [33,34] and display organization of the contractile apparatus into sarcomeric structures [25,26]. Whether activation of the ERK cascade plays such a critical role in these morphological aspects of agonist-induced hypertrophy is much less clear.

Here we have examined the effect of overexpression of MAPK phosphatase 1 (MKP-1) on the transcriptional and morphological responses to hypertrophic stimuli. MKP-1 is a dual-specificity Tyr/Thr phosphatase that displays selectivity for dephosphorylation and inactivation of the ERK subfamily of MAPKs [35–37]. We confirm that activation of the ERK cascade is a critical step for transcriptional activation in response to PE, ET-1 and PMA but propose that the changes in cell size, shape and myofibrillar organization associated with cardiac hypertrophy are much less dependent on ERK activation.

EXPERIMENTAL

General materials

Sprague–Dawley rats were bred within the National Heart and Lung Institute. ET-1 was from Bachem (Saffron Walden, Essex, U.K.). Other agonists, medium 199, Dulbecco's modified Eagle's medium, a mouse polyclonal antibody to β -galactosidase (β -gal) and other biochemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.). Falcon Primaria tissue culture dishes were obtained from Marathon Laboratory Supplies (London, U.K.). Chamber slides and other cell culture products were from Life Technologies (Paisley, Scotland, U.K.). The mouse monoclonal antibody to rat β -MHC was from Novacastra (Newcastle-upon-Tyne, Tyne and Wear, U.K.). Biotinylated anti-mouse IgG and Texas Red-conjugated anti-(mouse IgG) were from Amersham International (Little Chalfont, Bucks., U.K.). Fluorescent mounting medium was from Dako (High Wycombe, Bucks., U.K.). Other laboratory chemicals were from Merck (Lutterworth, Leics., U.K.). PE was prepared freshly on the day of use.

Expression and reporter plasmids

MKP-1, MKP-1CS and protein tyrosine phosphatase 1B (PTP-1B) were subcloned into pCEP4 (Invitrogen). MKP-1 encodes the first 314 amino acid residues of the murine 3CH134 protein [38], and MKP-1CS has an inactivating Cys $\rightarrow$ Ser mutation at position 258 [35,39]. The luciferase (LUX) reporter constructs pANF(–638)LA5' [27], pMLC(–250)LA5' [40], their promoterless control pSVOALA5' [41] and Rous sarcoma virus (RSV)–LUX as well as pON249 [42], in which β -gal is expressed from a constitutive cytomegalovirus (CMV) promoter, were kindly provided by Dr. K. R. Chien (Department of Medicine, University of California, San Diego, CA, U.S.A.) and have been described previously [24,27]. The LUX reporter constructs for β -MHC [43], SkM- α -actin [43–45] and serum response element-regulated *c-fos* (*c-fos*-SRE) [46] were gifts from Dr. M. D. Schneider (Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX, U.S.A.) and have been described previously [24]. Plasmid pNG1 was constructed by subcloning a *Bam*H1–*Not*I fragment of pPD46.21 [47] (a gift from Dr. A. Fire, Carnegie Institute, Baltimore, MD, U.S.A.) encoding a β -gal gene containing a nuclear localization signal into the *Bam*H1–*Not*I sites in pBK-CMV (Stratagene).

Transient transfection of cultured neonatal rat ventricular myocytes

Ventricular myocytes were isolated from the hearts of 1–2-day-old rats as described previously [24]. Myocytes were plated on

gelatin-coated 60 mm tissue culture dishes at a density of 10^6 cells per dish or at a density of 10^5 cells per well in two-well chamber slides (NUNC). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂; 20 h after plating and 4 h before transfection, medium was changed to 4% (v/v) horse serum in maintenance medium: Dulbecco's modified Eagle's medium (Sigma D1152) and medium 199 (Sigma M25200) in a 4:1 (v/v) ratio. Plasmids were diluted in 0.25 M CaCl₂ and an equal volume of 50 mM Bes (pH 6.9)/280 mM NaCl/1.5 mM Na₂HPO₄ was added. After 20 min, 1 ml of this suspension (containing 10 μ g of LUX reporter plasmid, 2 μ g of pON249 and 10 μ g of test plasmid) was added to 4 ml of medium on each plate or 0.4 ml was added to 1.6 ml of medium on chamber slides. After overnight transfection, cells were washed once with 10% horse serum in maintenance medium and twice with maintenance medium before being incubated in maintenance medium in the presence or absence of PE, PMA or ET-1. After 48 h, myocytes were washed three times with ice-cold PBS and extracted on ice with 0.1 M potassium phosphate (pH 7.9)/0.5% (v/v) Triton X-100/1 mM dithiothreitol (0.4 ml) for 15 min. Assays for LUX and β -gal were performed as detailed previously [24]. Results are presented as the means $\pm$ S.E.M. for experiments on four separate preparations of myocytes and using at least two separate preparations of plasmids, purified by polyethylene glycol precipitation [48].

Determination of myocyte size

To assess the area of transfected myocytes, cells were washed twice with ice-cold PBS, fixed with 4% (v/v) formaldehyde in PBS for 10 min and then stained with 0.2 mg/ml 5-bromo-4-chloroindol-3-yl- β -D-galactopyranoside/5 mM K₄Fe(CN)₆/5 mM K₃Fe(CN)₆/2 mM MgCl₂ in PBS. Transfected (blue) cells were randomly selected from all areas of the dishes and imaged with a video hardcopier from which the cell area and perimeter were determined by planimetry.

Immunocytochemistry

Cells were cultured at a density of 10^5 cells per well in Permanox two-well chamber slides pretreated with gelatin (1%, w/v) and laminin (20 μ g/ml). After fixation as for cell sizing, cells were permeabilized with 0.3% Triton X-100 in PBS (for 10 min at room temperature) and non-specific binding sites were blocked with 10% horse serum in 0.3% Triton X-100 (for 10 min at room temperature). Antibodies were diluted in 10% horse serum in 0.3% Triton X-100 in PBS and myocytes were washed three times in PBS between all stages of the immunofluorescence procedure. β -Gal was detected with a mouse polyclonal anti- β -gal primary antibody (Sigma; 1:200 dilution, for 1 h at 37 °C), a biotinylated anti-(mouse IgG) secondary antibody (Amersham; 1:200 dilution, for 30 min at 37 °C) and streptavidin–AMCA (Boehringer Mannheim; 1:400 dilution, for 15 min at 37 °C). After repeating the blocking step, β -MHC was subsequently detected by staining with a mouse monoclonal anti- β -MHC primary antibody (Novacastra; 1:50 dilution, for 1 h at 37 °C) and a Texas Red-conjugated anti-(mouse IgG) secondary antibody (Amersham; 1:100 dilution, for 30 min at 37 °C).

Immunoprecipitation and in-gel myelin basic protein (MBP) kinase assay

Myocytes were plated at a density of 4×10^6 cells per 60 mm culture dish and were transfected with 10 μ g of Myc-tagged ERK2, 2 μ g of pON249 and 10 μ g of MKP-1 or equivalent pCEP4 test plasmid. After 20 h, cells were washed once with

10% horse serum in maintenance medium and twice with maintenance medium before being incubated in maintenance medium for 24 h. Myocytes were then exposed to 100 μ M PE or 1 μ M PMA for 5 min before being washed once with PBS and extracted in 200 μ l of extraction medium [10 mM Tris/HCl (pH 7.4)/5 mM EDTA/50 mM NaF/50 mM NaCl/2 mM Na_3VO_4 /5 mM dithiothreitol/0.1% (w/v) fatty acid-free BSA/1% (v/v) Triton X-100, containing 20 μ g/ml aprotinin, 10 μ M *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 95 μ g/ml leupeptin and 80 μ g/ml pepstatin A]. Control dishes were extracted in the absence of added agonist. Pairs of extracts were pooled and clarified by centrifugation; the supernatants were incubated with 10 μ l of mouse monoclonal anti-c-Myc antibody (9E10; Santa Cruz Biotechnology) for 2 h at 4 $^{\circ}$ C, and for a further 1 h after the addition of 20 μ l of Protein G-Sepharose (1:1 in buffer). Immunoprecipitates were then collected by centrifugation, washed three times in extraction buffer and finally taken up into 50 μ l of extraction buffer. After the addition of 20 μ l of SDS/PAGE sample buffer [10% (w/v) SDS/13% (v/v) glycerol/300 mM Tris/HCl (pH 6.8)/0.2% Bromophenol Blue/0.13 M dithiothreitol], extracts were boiled and then stored at -20°C . In-gel MBP kinase assays with 1 mg/ml MBP polymerized in SDS/10% (w/v) polyacrylamide gels were performed as described elsewhere [15,16].

Statistical analysis

Results are presented as means $\pm$ S.E.M.; statistical significance was assessed by using a paired or unpaired Student's *t* test, as appropriate, with a significant difference taken as being established with $P < 0.05$.

RESULTS

Inhibition of PE-, ET-1- and PMA-stimulated ANF-LUX activity by MKP-1

In cultured neonatal rat ventricular myocytes ANF-LUX expression is stimulated by approx. 20-fold by PE (EC_{50} 10 μ M), by approx. 5-fold by PMA (EC_{50} 10 nM) and by approx. 7-fold by ET-1 (EC_{50} approx. 10 pM) [24], and results not shown). To determine whether activation of the ERK cascade is an obligatory step in the stimulation of ANF expression by these agonists, myocytes were transfected with expression plasmids for MKP-1, a catalytically inactive MKP-1 mutant MKP-1CS, a cytosolic protein tyrosine phosphatase with wide substrate specificity (PTP-1B) [49] or with vector control (pCEP4). ANF-LUX expression in the presence of 10 μ M PE was decreased by 74% after the constitutive expression of MKP-1 (Figure 1A). A similar effect on PE-induced ANF expression was recently reported with CL100, the human homologue of the mouse MKP-1 gene [50]. In contrast, neither the catalytically inactive MKP-1CS mutant nor PTP-1B had any significant effect on PE-induced ANF-LUX expression (Figure 1A). Similarly, ANF-LUX expression in the presence of 10 nM ET-1 (Figure 1B) or 1 μ M PMA (Figure 1C) was inhibited by 68% and 86% respectively by constitutive expression of MKP-1 but neither MKP-1CS nor PTP-1B had any significant effect. As a control for general effects of MKP-1 on gene expression, its effect on RSV-LUX expression was examined. Direct comparison is not possible but the absolute LUX activity in myocytes transfected with the RSV construct lies between that of ANF-LUX expression in response to 1 μ M PMA (approx. 5-fold stimulation) and that in response to 10 μ M PE (approx. 10-fold) (10 nM ET-1 produces an approx. 7-fold stimulation of ANF-LUX expression). In contrast with its effects on ANF-LUX expression,

Figure 1 Inhibition of PE-, ET-1- and PMA-stimulated ANF-LUX expression by MKP-1

Neonatal cardiac myocytes were transfected with pANF(−638)Δ5′ (10 μ g per plate), pON249 (2 μ g per plate) and pCEP4-based expression plasmids (pCEP4, MKP-1, MKP-1CS or PTP-1B; 10 μ g per plate) for 16–20 h and were then exposed to 10 μ M PE (A), 10 nM ET-1 (B) or 1 μ M PMA (C) for 48 h before extraction and assay for LUX and β -gal expression as described in the Experimental section. (D) In these transfections pANF(−638)Δ5′ was replaced by RSV-LUX and the cells were not exposed to agonists. The results are the means from four separate myocyte preparations and are expressed relative to the LUX-to- β -gal ratio with control vector pCEP4. Statistical significance by a paired *t* test: * $P < 0.001$ compared with transfection with pCEP4.

MKP-1 had no effect on LUX expression driven by the constitutive RSV promoter (Figure 1D). This indicates that the effect of MKP-1 is not simply to inhibit transcription in general, but that MKP-1 selectively inhibits the expression of genes associated with the hypertrophic response. We have recently reported that bradykinin (BK), although stimulating the translocation of PKC δ and PKC ϵ and activating p42 MAPK and p44 MAPK, is a poor hypertrophic agonist with only a very modest effect on ANF-LUX induction [51]. Expression of MKP-1 produced a small but non-significant inhibition of ANF-LUX expression in response to 10 μ M BK, and MKP-1CS and PTP-1B were without effect (ANF-LUX/ β -gal expression compared with incubations in the absence of BK (100%) were $191 \pm 45\%$, $133 \pm 24\%$, $256 \pm 52\%$ and $206 \pm 56\%$ for transfections with pCEP4, MKP-1, MKP-1CS and PTP-1B respectively; means $\pm$ S.E.M. for four myocyte preparations). The low level of ANF-LUX induction by BK makes it difficult to assess the significance of this result.

Inhibition of PE-stimulated vMLC-2-LUX, β -MHC-LUX, SkM- α -actin-LUX and c-fos-SRE-LUX activities by MKP-1

The results in Figure 1 show that overexpression of MKP-1 inhibits the induction of ANF expression in response to hypertrophic agonists. To determine whether this inhibition was a consistent feature of the transcriptional changes elicited by hypertrophic agonists, the effect of MKP-1 on other promoters whose expression is up-regulated in response to PE was also investigated. PE (10 μ M) stimulated the expression of vMLC-2-LUX, β -MHC-LUX, SkM- α -actin-LUX and c-fos-SRE-LUX transgenes by 10.4 ± 2.0 -fold, 6.9 ± 1.7 -fold, 3.5 ± 0.3 -fold and 2.6 ± 0.7 -fold respectively (Figure 2). In each case, overexpression of MKP-1 prevented induction of the transgene to an extent that the level of expression was not significantly different

Figure 2 Inhibition of PE-stimulated vMLC2-LUX, β -MHC-LUX, SkM- α -actin-LUX and c-fos-LUX reporter gene activity by MKP-1

Neonatal cardiac myocytes were transfected with LUX reporter constructs (10 μ g per plate) for vMLC2 (A), β -MHC (B), SkM- α -actin (C) or c-fos-SRE (D), together with pON249 (2 μ g per plate) and pCEP4-based expression plasmids (pCEP4, MKP-1, MKP-1CS or PTP-1B; 10 μ g per plate) for 16–20 h. Cells were then exposed to 10 μ M PE for 48 h before extraction and assay for LUX and β -gal expression as described in the Experimental section. The results are the means from four separate myocyte preparations and are expressed as fold induction of the LUX-to- β -gal ratio relative to control transfections with pCEP4 but incubated without PE. Statistical significance by a paired *t* test: ^a*P* < 0.05; ^b*P* < 0.01 compared with transfection with pCEP4 in the presence of PE.

Table 1 Effect of MKP-1 expression on myocyte size

Myocytes were transfected with pANF(–638)LD5' (10 μ g per plate), pON249 (2 μ g per plate) and pCEP4-based expression plasmids (pCEP4, MKP-1, MKP-1CS or PTP-1B; 10 μ g per plate) for 16–20 h and then exposed to 10 μ M PE for 48 h before being stained with 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside to identify transfected cells as described in the Experimental section. Control cells were transfected with pANF(–638)LD5', pON249 and pCEP4 but were incubated in the absence of PE. Myocyte area and perimeter were determined by planimetry on cells from two preparations of myocytes. Statistical significance by a paired *t* test: **P* < 0.001 compared with control; †*P* < 0.01, ††*P* < 0.05 compared with pCEP4 with PE.

Conditions	Vector	<i>n</i>	Area (μ m ²)	Perimeter (μ m)	Area/perimeter
Control	pCEP4	107	1200 $\pm$ 38	192 $\pm$ 5	6.26 $\pm$ 0.11
PE	pCEP4	143	1903 $\pm$ 59*	223 $\pm$ 4	8.40 $\pm$ 0.15*
PE	MKP-1	136	1655 $\pm$ 52†	196 $\pm$ 4	8.31 $\pm$ 0.15*
PE	MKP-1CS	140	1811 $\pm$ 62*	201 $\pm$ 4	8.84 $\pm$ 0.17*
PE	PTP-1B	138	1734 $\pm$ 50††	206 $\pm$ 5	8.32 $\pm$ 0.12*

from that in the absence of PE (i.e. 1-fold). Neither MKP-1CS nor PTP-1B had any statistically significant effect on the stimulation of vMLC2-LUX, β -MHC-LUX, SkM- α -actin-LUX or c-fos-SRE-LUX expression by PE, although there was a small but consistent inhibition of each reporter by MKP-1CS (Figure 2). Although MKP-1CS is catalytically inactive, inhibition could result from binding to phosphorylated ERK and restricting access to its substrates. These results imply that MKP-1 inhibits

a critical step in the stimulation of gene expression by PE and ET-1.

The effect of MKP-1 on myocyte morphology

As well as initiating transcriptional changes, PE and ET-1 induce alterations in cell size and shape, hypertrophied cardiomyocytes being larger and more regular in shape than control cells [25,27,31,52]. To assess the effects of overexpression of MKP-1 on myocyte morphology, cell area and area-to-perimeter ratios were measured as gross indicators of cell size and shape. Treatment of cells with 10 μ M PE for 48 h increased myocyte area by 59 % (Table 1). Expression of MKP-1 decreased the PE-induced increase in myocyte area to 38 %, whereas the MKP-1CS mutant had no significant effect (Table 1). Overexpression of PTP-1B caused a small (25 %) but significant decrease in the PE-induced increase in cell area. PE had very little effect on cell perimeter in these experiments, perimeter lengths being 116 %, 102 %, 105 % and 107 % of control cells (pCEP4 transfected, no PE) in cells transfected with pCEP4, MKP-1, MKP-1CS and PTP-1B respectively (Table 1). In these circumstances the myocyte area-to-perimeter ratio gives a crude indication of cell shape, more regularly shaped cells having a greater ratio. PE increased the area-to-perimeter ratio by 34 %; this was completely unaffected by transfection with MKP-1, MKP-1CS or PTP-1B (Table 1). Thus overexpression of MKP-1 in cardiac myocytes has a much smaller effect on cell size and shape than it does on gene expression.

The effect of MKP-1 on sarcomeric organization

Another characteristic feature of neonatal myocytes cultured in the presence of a hypertrophic agonist is the assembly and organization of contractile proteins into sarcomeric units [25,26,31,51]. To determine whether sarcomerization is dependent on ERK activation, myocytes were transfected under conditions similar to those in which MKP-1 prevents the stimulation of gene expression, except that the β -gal expression vector (pON249) was replaced with pNG1, which expresses β -gal targeted to the nucleus. (Directing the β -gal marker to the nucleus avoids difficulties in cross-reactivity or obscuring that can arise when using two primary mouse antibodies to stain proteins in the same intracellular compartment.) Myocytes were subsequently immunostained for β -gal to identify transfected cells (Figure 3, upper panel) and with an anti- β -MHC antibody to highlight the contractile apparatus (Figure 3, lower panel). MKP-1 had only a modest effect on sarcomeric structural organization in the presence of 10 μ M PE [compare the transfected cell (arrowed) with the surrounding non-transfected cells]. (Note that, because β -MHC expression is inhibited by MKP-1, the intensity of staining in myocytes transfected with MKP-1 might be slightly decreased owing to slow turnover of myofibrillar β -MHC.) Likewise, there was little effect of the MKP-1CS mutant, PTP-1B or the empty backbone vector on sarcomeric organization (results not shown). Owing to the low transfection efficiency, typically 1–2 % [24] but not measured in the current experiments, no attempt was made to quantify the relative degree of sarcomerization. However, some transfected cells were clearly more affected than others. Most probably this represents variability in the amount of MKP-1 plasmid taken up into individual cells. The overall conclusion from these studies is that organization of the contractile apparatus into sarcomeric units is much less dependent on ERK activation than is gene expression.

Figure 3 Effect of MKP-1 on β -MHC organization in response to PE

Myocytes were transfected with pANF(-638)LA5' (4 μ g per well), pNG1 (0.8 μ g per well) and pCEP4-based expression plasmids (pCEP4, MKP-1, MKP-1CS or PTP-1B; 4 μ g per well) for 16–20 h and were then exposed to 10 μ M PE for 48 h before being processed for immunofluorescence as detailed in the Experimental section. Upper panel: anti-(β -gal) staining to highlight the transfected cells. Lower panel: anti-(β -MHC) staining to highlight the contractile apparatus. The arrow indicates the single binucleate transfected cell in the field. Scale bar represents 50 μ m.

Figure 4 Effect of MKP-1 on ERK2 activity

Myocytes were transfected with Myc-tagged ERK2 (10 μ g per dish), pON249 (2 μ g per dish) and pCEP4-based expression plasmids [pCEP4 (lanes 1 and 2), MKP-1 (lane 3), MKP-1CS (lane 4) or PTP-1B (lane 5); 10 μ g per dish] for 16–20 h. After washing and overnight incubation in serum-free medium, myocytes were exposed to 100 μ M PE (lanes 2–5) or left untreated (lane 1) for 5 min before being processed for immunoprecipitation and in-gel MBP kinase assays as described in the Experimental section. The bar graph shows the relative densities of the bands after scanning, with the density in lane 2 set at 100. The result of a single experiment is shown. A similar result was also observed with 1 μ M PMA.

The effect of MKP-1 on ERK2 activity

Finally, to attempt to correlate the effect of MKP-1 on gene expression with ERK activation, myocytes were transfected with an epitope-tagged ERK2 construct and the effect of MKP-1 on immunoprecipitated ERK2 activity was determined by an in-gel MBP kinase assay (Figure 4). Transfection with MKP-1 decreased the stimulation of ERK2 activity by approx. 50% but there was little effect of PTP-1B. In contrast, transfection with the MKP-1CS mutant caused an increase in the immunoprecipitated ERK2 activity. This could be due to the binding of MKP-1CS to the activated ERK2 and preventing its dephosphorylation by endogenous MKP-1 or other phosphatases.

DISCUSSION

The proximal signalling events that occur in PE- and ET-1-induced cardiac myocyte hypertrophy involve binding to G_q -coupled receptors, activation of phospholipase $C\beta$ and hydrolysis of phosphatidylinositol 4,5-bisphosphate. The ensuing generation of *sn*-1,2-diacylglycerol in turn activates classical and novel isoforms of PKC [53–56]. Transient transfection of cardiac myocytes with constitutively active $G\alpha_q$ [32] or one of several constitutively active PKC isoforms is itself sufficient to initiate a hypertrophic response in cardiac myocytes [57–59]. Additional evidence supporting an important role for PKC is that PKC-activating phorbol esters are hypertrophic [60–63] and that ET-1 and PE trigger the translocation (activation) of PKC δ and PKC ϵ , the predominant isoforms detected in neonatal cardiac myocytes [56,64].

The signalling pathway(s) downstream of these initial membrane proximal events are still the subject of debate. One consequence of G_q -coupled receptor agonism and activation of PKC in cardiomyocytes is the stimulation of the Raf $\rightarrow$ MEK $\rightarrow$ ERK cascade [15,16,19,65]. Activation of ERK is also a feature of the response of myocytes to other hypertrophic stimuli including fibroblast growth factors [16], angiotensin II [66] and stretch [21]. This has prompted the suggestion that the activation of ERK might be a convergence point through which diverse stimuli exert a common hypertrophic effect [16]. Initiation of a hypertrophic response in myocytes by transfection with constitutively active MEK is in accord with this view [24]. However, current opinion is divided on whether activation of ERKs is an obligatory requirement. Although some groups, including our own, have demonstrated an inhibition of PE-induced ANF-LUX expression by dominant-negative ERKs and/or MEK1 [19,24], a recent report by another group observed no such inhibition of the response to PE, even though the dominant-negative ERK mutant blocked ANF-LUX expression in response to oncogenic V12Ras [67]. In addition, the latter study documented a discordance between PE-, ET-1-, ATP- and carbachol-induced ERK activation and the induction of the hypertrophic phenotype for which only PE and ET-1 were effective [67]. Part of the discrepancy between these studies might be due to differences in protocol, including the concentration of agonist used and the length of the ANF promoter fragment driving the LUX reporter gene.

MKP-1 dephosphorylates and inactivates ERK and has been used in other systems as a molecular probe to demonstrate a role for ERK in the induction of DNA synthesis by oncogenic Ras [39] or growth factors [68] and to show that ERK is required for the maintenance of the mitotic arrest induced by the depolymerization of spindle microtubules in *Xenopus* eggs [69]. In the present study we used a similar strategy as an alternative approach to examine the role of ERK activation in the response

to hypertrophic agonists. The concentration of PE used in the current experiments gives about half-maximal activation of ANF expression in this system [24] and was chosen for the following reasons. First, this is the concentration at which the response to MKP-1 should be most sensitive. Secondly, supermaximal concentrations of PE might overcome an inhibitory effect, though this subsequently proved unlikely as similar results were obtained with ET-1 and PMA, which were used at concentrations in excess of those needed for a maximum effect. Thirdly, although an inhibition was anticipated in the presence of MKP-1, this protocol allowed for the possibility of an enhanced response to PE, either by MKP-1 or by MKP-1CS or PTP-1B. In this regard, even though the MKP-1CS mutant seemed to increase immunoprecipitated ERK2 activity from cells (Figure 4), it did not enhance gene expression. This is perhaps surprising if MKP-1CS is able to bind to and protect endogenous ERK2 from dephosphorylation. However, MKP-1CS bound to activated ERK2 could equally prevent access of ERK2 to its substrates and hence be expected to have an inhibitory effect. Indeed, a modest decrease in gene expression was sometimes seen.

It is also important to note the different protocols of the immunoprecipitation and gene expression studies. Because of the very low transfection efficiency of cardiac myocytes, conditions were chosen to maximize the potential to see a differential effect of MKP-1 and PTP-1B on ERK2 activity. Thus, cells were plated at four times the usual density for transfection, pairs of plates were pooled for analysis, and ERK2 activity was determined 5 min after the addition of PE, the time when ERK2 activity is maximal in response to hypertrophic agonists [51,56]. The relationship between this transient increase in ERK activity and the longer-term effects on gene expression in response to hypertrophic agonists is still unclear. However, our recent observation that an anti-sense to MKP-1 can enhance gene expression in response to hypertrophic agonists supports a causal link between these two effects [70].

Overexpression of MKP-1 inhibited the transcriptional responses to PE, ET-1 and PMA by 60–80% but had a much smaller effect on cell size, shape and organization of the contractile apparatus into sarcomeric structures. The reasons for the smaller sensitivity of the morphological aspects of the hypertrophic response to inhibition by MKP-1 might be severalfold. Most obviously, activation of the ERK pathway might play a less critical role in this response. Alternatively, the morphological aspects might result from one of several signalling pathways simultaneously activated by hypertrophic agonists, including the ERK cascade, any one of which might be sufficient. In this regard the choice of agonist concentration might be important because higher concentrations might activate additional signalling pathways.

Another possible explanation for the current results is that MKP-1 might have greater access to ERKs that translocate to the nucleus. Activation of transcription factors such as Elk-1 by ERKs is a nuclear event, whereas phosphorylation of structural proteins, several protein kinases and other enzymes takes place in the cytosol [5,71,72]. Several, but not all [73], members of the MKP-1 family are known to localize to the nucleus including MKP-1 itself [68], CL100 and its *Xenopus* homologue XCL100 [74] and the haemopoietic cell-specific MAPK phosphatase PAC-1 [75,76]. However, when MKP-1 is overexpressed it might not be confined to its endogenous cellular distribution. Attempts to demonstrate specific nuclear staining in myocytes transfected with MKP-1 were unsuccessful (results not shown).

Overexpression of MKP-1 in COS cells results in the selective dephosphorylation of ERK2 from the spectrum of tyrosine-phosphorylated proteins in the cell [35]. In addition, 30 times

more recombinant MKP-1 is required to inactivate the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) family than ERK2 [39], and in REF-52 cells MKP-1 inhibits ERK activation in response to PMA but not the activation of JNK1 in response to UV radiation [39]. However, recent results suggest that in some cell types MKP-1 might dephosphorylate the JNK/SAPK and/or p38/RK members of the MAPK family in addition to the ERKs [77,78] and therefore we cannot unequivocally attribute the effects of MKP-1 solely to dephosphorylation and inhibition of the ERKs. Our own recent work has shown that activation of the JNK/SAPK and p38/RK members of the MAPK family might also be important in cell signalling in the heart [79,80]. Thus the current results support a role for one or more of the classes of MAPK in the mediation of the hypertrophic response but the observation that MKP-1 inhibits the effect of PMA (which strongly activates ERKs but not SAPKs in cultured neonatal myocytes [80]) implies a principal role for ERK in this response. Inhibition of ANF-LUX expression in response to PE by using an anti-sense oligonucleotide to ERK reinforces this conclusion [81].

In summary, the current results suggest that activation of ERKs and/or other members of the MAPK family is a critical step in the activation of gene expression in response to hypertrophic agonists but that the morphological aspects of the hypertrophic response might be less dependent on ERK activity. The ability of MKP-1 to reveal this dichotomy makes it a valuable tool with which to dissect out the signalling components associated with each of these responses. The extent to which individual members of the MAPK family are activated in response to different agonists might help to explain some of the discrepancies that bear on the importance of ERK activation in cardiac hypertrophy.

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REFERENCES

- Blumer, K. J. and Johnson, G. L. (1994) Trends Biochem. Sci. **19**, 236–240
- Marshall, C. J. (1994) Curr. Opin. Genes Dev. **4**, 82–89
- Robbins, D. J., Zhen, E., Cheng, M., Xu, S., Ebert, D. and Cobb, M. H. (1994) Adv. Cancer Res. **63**, 93–116
- Cobb, M. H. and Goldsmith, E. J. (1995) J. Biol. Chem. **270**, 14843–14846
- Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556
- Ahn, N. G., Seger, R. and Krebs, E. G. (1992) Curr. Opin. Cell Biol. **4**, 992–999
- Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R. and Avruch, J. (1992) Nature (London) **358**, 417–421
- Howe, L. R., Leever, S. J., Gomez, N., Nakielnny, S., Cohen, P. and Marshall, C. J. (1992) Cell **71**, 335–342
- Dent, P., Haser, W., Haystead, T. A. J., Vincent, L. A., Roberts, T. M. and Sturgill, T. W. (1992) Science **257**, 1404–1407
- McCormick, F. (1993) Nature (London) **363**, 15–16
- Marshall, C. J. (1995) Cell **80**, 179–185
- Koch, W. J., Hawes, B. E., Allen, L. F. and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 12706–12710
- Ito, A., Satoh, T., Kaziro, Y. and Itoh, H. (1995) FEBS Lett. **368**, 183–187
- Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B. and Schlessinger, J. (1995) Nature (London) **376**, 737–745
- Bogoyevitch, M. A., Glennon, P. E. and Sugden, P. H. (1993) FEBS Lett. **317**, 271–275
- Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) J. Biol. Chem. **269**, 1110–1119
- Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Lazou, A., Marshall, C. J. and Sugden, P. H. (1993) Biochem. Soc. Trans. **21**, 358S
- Yamazaki, T., Tobe, K., Hoh, E., Maemura, K., Kaida, T., Komuro, I., Tamemoto, H., Kadowaki, T., Nagai, R. and Yazaki, Y. (1993) J. Biol. Chem. **268**, 12069–12076

- 19 Thorburn, J., Frost, J. A. and Thorburn, A. (1994) *J. Cell Biol.* **126**, 1565–1572
- 20 Bogoyevitch, M. A., Andersson, M. B., Gillespie-Brown, J., Clerk, A., Glennon, P. E., Fuller, S. J. and Sugden, P. H. (1996) *Biochem. J.* **314**, 115–121
- 21 Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Mizuno, T., Takano, H., Hiroi, Y., Ueki, K., Tobe, K. et al. (1995) *J. Clin. Invest.* **96**, 438–446
- 22 Lazou, A., Bogoyevitch, M. A., Clerk, A., Fuller, S. J., Marshall, C. J. and Sugden, P. H. (1994) *Circ. Res.* **75**, 932–941
- 23 Thorburn, J., McMahon, M. and Thorburn, A. (1994) *J. Biol. Chem.* **269**, 30580–30586
- 24 Gillespie-Brown, J., Fuller, S. J., Bogoyevitch, M. A., Cowley, S. and Sugden, P. H. (1995) *J. Biol. Chem.* **270**, 28092–28096
- 25 Iwaki, K., Sukhatme, V. P., Shubeita, H. E. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 13809–13817
- 26 Shubeita, H. E., McDonough, P. M., Harris, A. N., Knowlton, K. U., Glembotski, C. C., Brown, J. H. and Chien, K. R. (1990) *J. Biol. Chem.* **265**, 20555–20562
- 27 Knowlton, K. U., Baracchini, E., Ross, R. S., Harris, A. N., Henderson, S. A., Evans, S. M., Glembotski, C. C. and Chien, K. R. (1991) *J. Biol. Chem.* **266**, 7759–7768
- 28 Sei, C. A., Irons, C. E., Sprengle, A. B., McDonough, P. M., Brown, J. H. and Glembotski, C. C. (1991) *J. Biol. Chem.* **266**, 15910–15916
- 29 Waspe, L. E., Ordahl, C. P. and Simpson, P. C. (1990) *J. Clin. Invest.* **85**, 1206–1214
- 30 Long, C. S., Ordahl, C. P. and Simpson, P. C. (1989) *J. Clin. Invest.* **83**, 1078–1082
- 31 Lee, H. R., Henderson, S. A., Reynolds, R., Dunnmon, P., Yuan, D. and Chien, K. R. (1988) *J. Biol. Chem.* **263**, 7352–7358
- 32 LaMorte, V. J., Thorburn, J., Absher, D., Spiegel, A., Brown, J. H., Chien, K. R., Feramisco, J. R. and Knowlton, K. U. (1994) *J. Biol. Chem.* **269**, 13490–13496
- 33 McDermott, P. J. and Morgan, H. E. (1989) *Circ. Res.* **64**, 542–553
- 34 McDermott, P. J., Rothblum, L. I., Smith, S. D. and Morgan, H. E. (1989) *J. Biol. Chem.* **264**, 18220–18227
- 35 Sun, H., Charles, C. H., Lau, L. F. and Tonks, N. K. (1993) *Cell* **75**, 487–493
- 36 Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M. and Cohen, P. (1995) *Curr. Biol.* **5**, 283–295
- 37 Duff, J. L., Monia, B. P. and Berk, B. C. (1995) *J. Biol. Chem.* **270**, 7161–7166
- 38 Charles, C. H., Abler, A. S. and Lau, L. F. (1992) *Oncogene* **7**, 187–190
- 39 Sun, H., Tonks, N. K. and Bar-Sagi, D. (1994) *Science* **266**, 285–288
- 40 Henderson, S. A., Spencer, M., Sen, A., Kumar, C., Siddiqui, M. A. and Chien, K. R. (1989) *J. Biol. Chem.* **264**, 18142–18148
- 41 deWet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R. and Subramani, S. (1987) *Mol. Cell Biol.* **7**, 725–737
- 42 Cherrington, J. M. and Mocarski, E. S. (1989) *J. Virol.* **63**, 1435–1440
- 43 Abdellatif, M., MacLellan, W. R. and Schneider, M. D. (1994) *J. Biol. Chem.* **269**, 15423–15426
- 44 Brand, T., MacLellan, W. R. and Schneider, M. D. (1993) *J. Biol. Chem.* **268**, 11500–11503
- 45 MacLellan, W. R., Lee, T.-C., Schwartz, R. J. and Schneider, M. D. (1994) *J. Biol. Chem.* **269**, 16754–16760
- 46 Parker, T. G., Chow, K.-L., Schwartz, R. J. and Schneider, M. D. (1992) *J. Biol. Chem.* **267**, 3343–3350
- 47 Fire, A., Harrison, S. W. and Dixon, D. (1990) *Gene* **93**, 189–198
- 48 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 49 Charbonneau, H. and Tonks, N. K. (1992) *Annu. Rev. Cell Biol.* **8**, 463–493
- 50 Thorburn, J., Carlson, M., Mansour, S. J., Chien, K. R., Ahn, N. and Thorburn, A. (1995) *Mol. Biol. Cell* **6**, 1479–1490
- 51 Clerk, A., Gillespie-Brown, J., Fuller, S. J. and Sugden, P. H. (1996) *Biochem. J.* **317**, 109–118
- 52 Thorburn, A., Thorburn, J., Chen, S. Y., Powers, S., Shubeita, H. E., Feramisco, J. R. and Chien, K. R. (1993) *J. Biol. Chem.* **268**, 2244–2249
- 53 Kaku, T., Lakatta, E. and Filburn, C. (1991) *Am. J. Physiol. Cell Physiol.* **260**, C635–C642
- 54 Shubeita, H. E., Martinson, E. A., Van Bilsen, M., Chien, K. R. and Brown, J. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1305–1309
- 55 Bogoyevitch, M. A., Parker, P. J. and Sugden, P. H. (1993) *Circ. Res.* **72**, 757–767
- 56 Clerk, A., Bogoyevitch, M. A., Andersson, M. B. and Sugden, P. H. (1994) *J. Biol. Chem.* **269**, 32848–32857
- 57 Kariya, K., Karns, L. R. and Simpson, P. C. (1991) *J. Biol. Chem.* **266**, 10023–10026
- 58 Shubeita, H. E., Martinson, E. A., Van Bilsen, M., Chien, K. R. and Brown, J. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1305–1309
- 59 Decock, J. B. J., Gillespie-Brown, J., Parker, P. J., Sugden, P. H. and Fuller, S. J. (1994) *FEBS Lett.* **356**, 275–278
- 60 Fuller, S. J. and Sugden, P. H. (1989) *FEBS Lett.* **247**, 209–212
- 61 Henrich, C. J. and Simpson, P. C. (1988) *J. Mol. Cell. Cardiol.* **20**, 1081–1085
- 62 Dunnmon, P. M., Iwaki, K., Henderson, S. A., Sen, A. and Chien, K. R. (1990) *J. Mol. Cell. Cardiol.* **22**, 901–910
- 63 Allo, S. N., McDermott, P. J., Carl, L. L. and Morgan, H. E. (1991) *J. Biol. Chem.* **266**, 22003–22009
- 64 Clerk, A., Bogoyevitch, M. A., Fuller, S. J., Lazou, A., Parker, P. J. and Sugden, P. H. (1995) *Am. J. Physiol. Heart Circ. Physiol.* **269**, H1087–H1097
- 65 Sadoshima, J. and Izumo, S. (1993) *EMBO J.* **12**, 1681–1692
- 66 Sadoshima, J., Qiu, Z., Morgan, J. P. and Izumo, S. (1995) *Circ. Res.* **76**, 1–15
- 67 Post, G. R., Goldstein, D., Thuermer, D. J., Glembotski, C. C. and Brown, J. H. (1996) *J. Biol. Chem.* **271**, 8452–8457
- 68 Brondello, J. M., McKenzie, F. R., Sun, H., Tonks, N. K. and Pouyssegur, J. (1995) *Oncogene* **10**, 1895–1904
- 69 Minshall, J., Sun, H., Tonks, N. K. and Murray, A. W. (1994) *Cell* **79**, 475–485
- 70 Fuller, S. J. and Davies, E. L. (1996) *Circulation* **94**, I–530
- 71 Chen, R. H., Sarnecki, C. and Blenis, J. (1992) *Mol. Cell Biol.* **12**, 915–927
- 72 Seth, A., Gonzales, F. A., Gupta, S., Raden, D. L. and Davis, R. J. (1992) *J. Biol. Chem.* **267**, 24796–24804
- 73 Mourey, R. J., Vega, Q. C., Campbell, J. S., Wenderoth, M. P., Hauschka, S. D., Krebs, E. G. and Dixon, J. E. (1996) *J. Biol. Chem.* **271**, 3795–3802
- 74 Seger, R. and Krebs, E. G. (1995) *FASEB J.* **9**, 726–735
- 75 Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Kruttsch, H., Siebenlist, U. and Kelly, K. (1993) *Science* **259**, 1763–1766
- 76 Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J. and Kelly, K. (1994) *Nature (London)* **367**, 651–654
- 77 Liu, Y., Gorospe, M., Yang, C. and Holbrook, N. J. (1995) *J. Biol. Chem.* **270**, 8377–8380
- 78 Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J. and Kelly, K. (1996) *J. Biol. Chem.* **271**, 6497–6501
- 79 Bogoyevitch, M. A., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996) *Circ. Res.* **79**, 162–173
- 80 Bogoyevitch, M. A., Ketterman, A. J. and Sugden, P. H. (1995) *J. Biol. Chem.* **270**, 29710–29717
- 81 Glennon, P. E., Kaddoura, S., Sale, E. M., Sale, G. J., Fuller, S. J. and Sugden, P. H. (1996) *Circ. Res.* **78**, 954–961