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Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activities in ventricular cardiomyocytes

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ET-1 stimulated MBP kinase activity in cultured cardiomyocytes. Maximal activation (3.5-fold) was at 5 min. EC_{50} was 0.2 nM. PMA or PE also increased MBP kinase (4- or 2.5-fold, respectively). Pre-treatment with PMA down-regulated the subsequent response to ET-1 or PMA. ET-1- or PMA-stimulated MBP kinase was resolved into 2 major (peaks II and IV) and 2 minor peaks by FPLC on Mono Q. Peaks II and IV were inactivated by either LAR or PP2A. Renatured MBP kinase activities following SDS-PAGE in MBP-containing gels and immunoblot analysis showed that peak II was a p42 MAP kinase and peak IV was a p44 MAP kinase.

Endothelin; Phorbol ester; α_1-Adrenergic agonist; Mitogen-activated protein kinase; Cultured neonatal rat ventricular cardiomyocyte; Cardiac hypertrophy

1. INTRODUCTION

The ventricular cardiomyocyte cultured from neonatal rats is a valuable model system which, following exposure to appropriate agonists, exhibits many of the features of the hypertrophic response in the adult heart in vivo [1]. Hypertrophic agonists include the endothelins [2–4], α_1-adrenergic agonists [5–7] and tumour-promoting phorbol esters [7–9]. Both ET-1 and the α_1-adrenergic agonists stimulate phosphoinositide hydrolysis [2,3,10], raise diacylglycerol concns. [2,11] and activate PKC [7,12,13] in (mainly neonatal cardiomyocyte) cardiac preparations. These findings suggest an important role for PKC in the signal transduction mechanism of cardiac hypertrophy [1], a hypothesis supported by PKC transfection experiments [14]. However, details of the signalling events downstream from PKC remain poorly defined. Recently, the MAP kinases have been identified as ubiquitous [15] Ser/Thr protein kinases which are acutely activated in a variety of cells in response to mitogens [16–18] and which probably play a central role in diverse growth-related responses [19]. For full activation, MAP kinases require phosphorylation on both Ser/Thr and Tyr residues [20,21]. Here, we demonstrate that hypertrophic agonists rapidly stimulate the activity of two MAP kinases as well the activity of two uncharacterized MBP kinases.

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Abbreviations: ECL, enhanced chemiluminescence; ET, endothelin; FCS, foetal calf serum; FPLC, fast protein liquid chromatography; LAR, leukocyte common antigen-related protein tyrosine phosphatase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PBST, 0.05% (v/v) Tween-20 in phosphate-buffered saline; PE, phenylephrine; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PP2A, protein serine/threonine phosphatase 2A; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

2. EXPERIMENTAL

2.1. *Myocyte isolation and culture*

Hearts of 1- to 2-day-old rats were digested with trypsin (Sigma type I) then with collagenase (Worthington type II) [22]. The resulting cell suspension (in medium 199 with Hank's salts (Gibco) supplemented with 5 mg/ml glucose, 10% (v/v) heat-inactivated FCS and 0.1 mM bromodeoxyuridine) was enriched for cardiomyocytes by a preliminary plating (30 min) during which time non-myocytes attached to the culture plates. Non-adhering cells were plated at density of 4.2×10^3 cells/mm^2 in culture dishes (60 or 35 mm, Primaria, Falcon) and they began to beat spontaneously after 1–2 d. The cardiomyocytes were maintained for 4 d before bromodeoxyuridine was withdrawn. After a further 2 d, FCS was replaced with insulin, transferrin, and vitamin B_{12} (Sigma, each at a final concentration of 1 mg/100 ml) for 20–24 h before use. Cardiomyocytes were exposed to agonists (all Sigma) as specified in serum-free medium at 37°C. The plates were rinsed 3 × with ice-cold Dulbecco's phosphate buffered saline and scraped into ice-cold extraction buffer (20 mM β-glycerophosphate, 20 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 10 mM benzamidine, 25 μg/ml leupeptin, 50 μg/ml PMSF, 0.3% (v/v) mercaptoethanol, pH7.5). Extracts were centrifuged for 10 min at 10 000 × g and 4°C, and the supernatant fractions retained.

2.2. *Assay of MBP kinase activity*

Protein kinase activity against MBP (Sigma) was assayed at 30°C in a final volume of 50 μl containing 15 mM β-glycerophosphate, 0.28 mg/ml MBP, 10 mM $MgCl_2$, 50 mM NaF, 2 mM EDTA, 0.3 mM Na_3VO_4, 2 μM cAMP dependent-protein kinase inhibitory peptide (TTYADFIASGRTGRRNAIHD, Bachem) and 120 μM [γ-^{32}P]ATP (Amersham, 40–100 Ci/mol, with which the reaction was initiated) at a final pH of 7.5. Reactions were terminated by spotting 40 μl of the

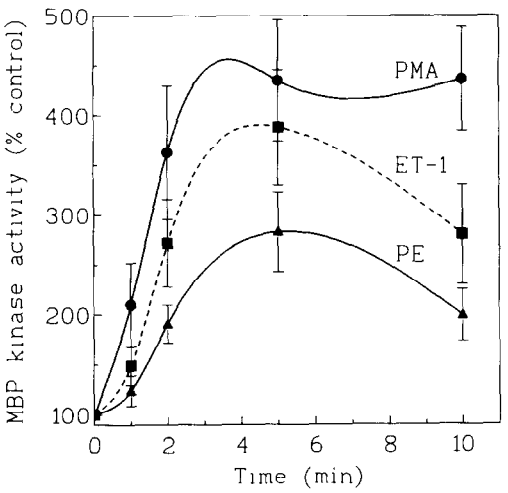


Fig. 1. Time course of MBP kinase stimulation in extracts of cultured neonatal cardiomyocytes. Cardiomyocytes (one 35 mm plate for each observation) were exposed to 1 μM TPA (●, solid line), 100 nM ET-1 (■, dashed line) or 50 μM PE (▲, solid line). Extracts were prepared and MBP kinase was assayed as described in section 2. Results (means ± S.E., n = 3–4) are expressed relative to MBP kinase in extracts from untreated cardiomyocytes.

mixture onto 3MM papers (Whatman) that were immediately immersed in 20% (v/v) TCA. Papers were washed (1 × 5 min, 3 × 30 min) in 20% TCA, then counted in 10 ml of Fluoran HV (Merck).

3. RESULTS

3.1. *Activation of protein kinase activity in cardiomyocyte extracts*

Pre-treatment of cardiomyocytes with PMA, ET-1 or PE stimulated MBP kinase activity maximally by 2.5- to 4.5-fold in subsequently-isolated extracts (Fig. 1). Maximal activation occurred at 5 min. For ET-1 or PE, the MBP kinase activity declined toward basal levels thereafter, whilst activation by PMA was sustained. The EC_{50} for ET-1 was 0.2 nM (Fig. 2). This value is similar to its K_d for its receptors [23,24] and its EC_{50} for stimulation of PI hydrolysis [2,3] and diacylglycerol accumulation [2].

Chronic treatment of cells with PMA down-regulates PKC, a manoeuvre that is useful in demonstrating the involvement of PKC in signalling pathways [25,26]. Pre-treatment of cardiomyocytes with PMA (1 μM) for 24 h attentuated the stimulation of MBP kinase activity by ET-1 or PMA (Table I). Although these findings are consistent with a role for PKC in the activation of MBP kinase, other unknown effects of PMA (e.g. were it down-regulates the ET receptor) may complicate this interpretation.

Table I

Effect of pre-treatment of cardiomyocytes with PMA for 24 h on ET-1 and PMA-stimulation of MBP kinase activity

Pre-treatment with PMA (1 μM)	Agonist	MBP kinase activity (% control)
–	None	(100)
+	None	121 ± 8[d]
–	ET-1 (100 nM)	293 ± 18[c]
+	ET-1 (100 nM)	177 ± 18[b,c]
–	PMA (1 μM)	375 ± 52[c]
+	PMA (1 μM)	206 ± 23[b,e]

Cardiomyocytes were pre-treated with PMA for 24 h when appropriate. One 35 mm plate was used for each observation Medium was removed and replaced with medium containing the appropriate agonist. After 5 min, extracts were prepared as described in section 2.1 and assayed for MBP kinase activity as described in section 2.2. Results are means ± S.E. for 3–4 separate preparations. Statistical significance [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs. cardiomyocytes never exposed to PMA or other agonists; [d]$P < 0.05$, [e]$P < 0.01$ for the effect of pre-treatment with PMA on the acute response to ET-1 or PMA (2-tailed Student's t-test, n = 3–4).

3.2. *FPLC of MBP protein kinase activities*

FPLC of cardiomyocyte extracts on Mono Q columns revealed that two major (II and IV) and two minor (I and III) peaks of MBP kinase activities were stimulated by pre-treatment with 100 nM ET-1 (Fig. 3). Peak II eluted at 0.20 M NaCl and activity was stimulated 4.1 ± 0.5-fold (mean ± SE, n = 5). Peak IV eluted at 0.25 M NaCl and was stimulated 5.7 ± 0.7-fold (n = 5). Peaks I and III eluted at 0.185 and 0.22 M NaCl, respectively. Apart from a small amount eluting in the flow-through before the start of the gradient, no other activity was eluted by up to 1 M NaCl (not shown). Extracts from cardiomyocytes exposed to 1 μM PMA for 5 min showed an identical profile (not shown) and stimulation was similar to ET-1 (Peak II: 4.7 ± 0.5-fold, Peak IV: 5.3 ± 0.8-fold, n = 3). Peak II or IV did not phosphorylate histone IIIS, casein or protamine

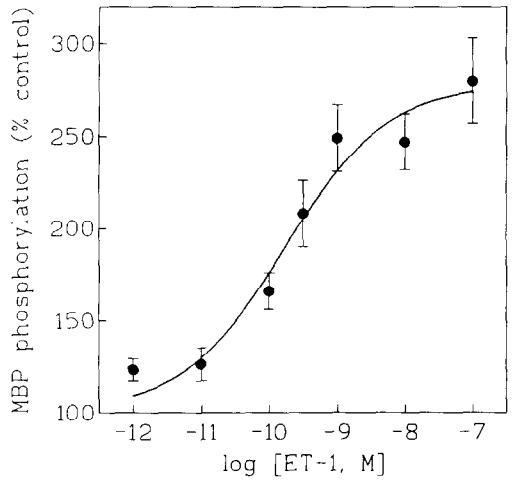


Fig. 2. Dependence of MBP kinase stimulation on ET-1 concentration. Cardiomyocytes (one 35 mm plate for each observation) were exposed to ET-1 for 5 min. Extracts were prepared and MBP kinase was assayed as described in section 2. Results (means ± S.E., n = 5) are expressed relative to activities in extracts from untreated cardiomyocytes.

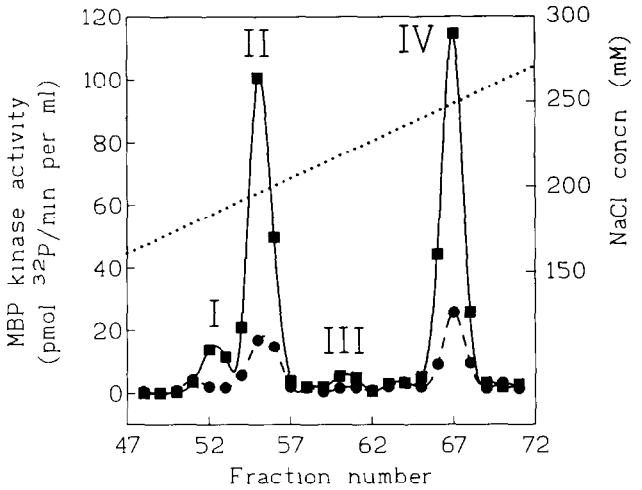


Fig. 3. FPLC of ET-1-stimulated MBP kinase. Cardiomyocytes were exposed to 100 nM ET-1 for 5 min. Extracts (0.5 ml total from four 60 mm plates) were prepared as described in section 2.1 and were applied to a Mono Q HR 5/5 column (Pharmacia LKB) equilibrated with 15 mM β-glycerophosphate, 1.5 mM EDTA, 0.1 mM Na_3VO_4 and 1 mM dithiothreitol, pH 7.3 (Buffer A) at a flow rate of 1 ml/min. After washing with 5 ml of Buffer A, MBP kinase activity was eluted with a linear gradient of NaCl (0–0.35 M, dotted line) in Buffer A. Fractions (0.5 ml) were collected and assayed for MBP kinase as described in section 2.2. Typical elution profiles are shown for cells exposed to ET-1 (■, solid line) or control conditions (●, dashed line). Similar elution profiles were obtained with 10 separate preparations of cardiomyocytes.

(each at 5 mg/ml, Sigma, not shown). Because of their low activities, Peaks I and III were not examined further.

3.3. *Inactivation of MBP kinase activities by protein phosphatases*

Peak II or IV MBP kinase was inactivated by incubation with either the protein Tyr phosphatase LAR or with the protein Ser/Thr phosphatase PP2A (Fig. 4). This suggests phosphorylation of both Tyr and Ser/Thr residues is important in activation of these kinases, a characteristic of the MAP kinases [20,21].

3.4. *Identification of peaks II and IV MBP kinases as p42 and p44 MAP kinases*

Fractions around Peaks II and IV from FPLC of ET-1-stimulated cardiomyocytes (Fig. 3) were examined for their ability to phosphorylate MBP in gels [27,28] in situ (Fig 5a). Peak II (fraction 55) and Peak IV (fractions 67/68) had molecular masses of 42 and 44 kDa, respectively (Fig. 5a). After concentration, SDS-PAGE and immunoblotting, Peaks II and IV showed immunoreactivity with an antiserum raised against an oligopeptide (ITVEEALAHPYLEQYYDPTFDEPV) corresponding to a common epitope in p42- and p44-MAP kinases [29]. Thus Peak II is probably a p42 MAP kinase and Peak IV is a p44 MAP kinase. Because > 90% of the MBP kinase in ET-1- or PMA-stimulated cardiomyocytes (Fig. 3) is MAP kinase (Fig. 5), results from the PKC down-regulation experiments (Table I) suggest that activation of PKC is necessary for the activation of MAP kinase.

4. DISCUSSION

The potent vasoconstrictor peptide ET-1 was initially isolated from the medium of aortic endothelial cell cul-

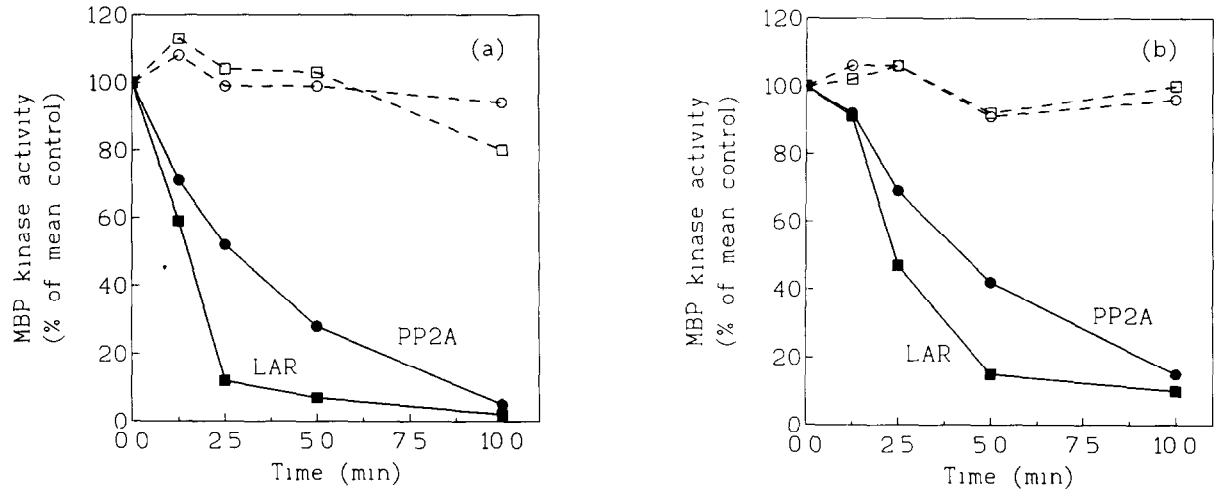


Fig. 4. Inactivation of MBP kinases by incubation with protein phosphatases. MBP kinase Peaks II or IV (Fig. 3) were buffer-exchanged by centrifugation through Sephadex G50 Superfine (Pharmacia NICK Spin columns) equilibrated with 50mM Tris/HCl, 0.15 mM EGTA, 0.1% (v/v) mercaptoethanol and 1 mg/ml bovine serum albumin, pH 7.0, at 4°C in accordance with the manufacturer's instructions. Samples were incubated at 30°C (final volume: 100 μl) with PP2A (30 U/ml final, ○, ●; 1 U of PP2A dephosphorylates 1 nmol of phosphorylase *a*/min) in the presence (○) or absence (●) of 2 μM okadaic acid; or with LAR (0.1 mg/ml final, □, ■) in the presence (□) or absence (■) of 1 mM Na_3VO_4. Samples (20 μl) were withdrawn and 0.1 vol. of 40 μM okadaic acid (PP2A) or 20 mM Na_3VO_4 (LAR) added. MBP kinase was assayed immediately as described in section 2.2 for peak II (Fig. 4a) or peak IV (Fig. 4b). Results are expressed relative to the mean of activities in assays containing the appropriate protein phosphatase inhibitors (okadaic acid or Na_3VO_4). Results from a single experiment are shown and similar results were obtained with another preparation of MBP kinases.

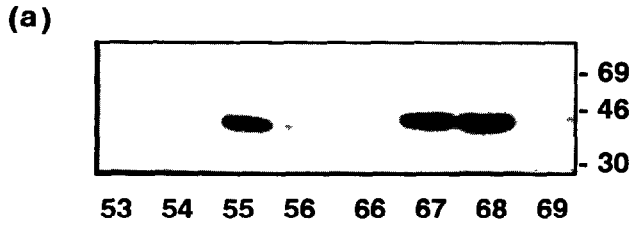
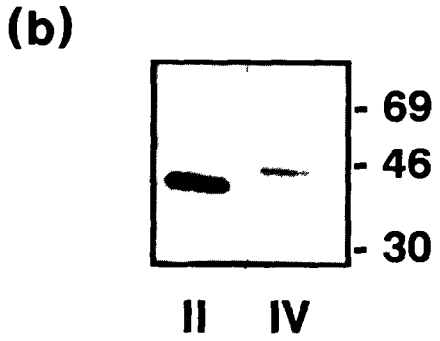


Fig. 5. Identification of MBP kinase peaks II and IV as MAP kinases. The numbers on the right of the panels refer to the molecular masses of marker proteins in kDa. In Fig 5a, MBP kinases were detected in situ in MBP-containing gels [27,28]. Fraction numbers (53–56 and 66–69) are indicated across the bottom. Cardiomyocytes were exposed to 100 nM ET-1 for 5 min and extracts were analysed by FPLC (Fig. 3). SDS-PAGE sample buffer [20 μl containing 10% (w/v) SDS, 13% (v/v) glycerol, 0.3 M Tris/HCl, 0.2% (w/v) bromophenol blue, 0.13 M dithiothreitol, pH 6.8] was added to samples (40 μl) of fractions with subsequent heating at 100°C for 5 min. Samples were then subjected to SDS-PAGE in a 10% polyacrylamide gel containing 0.5 mg/ml MBP. After electrophoresis, SDS was removed from the gel by washing with 20% (v/v) 2-propanol in 50 mM Tris-HCl (pH 8.0), then 5 mM mercaptoethanol in 50 mM Tris-HCl (pH 8.0). Proteins were further denatured by washing the gel in 6 M guanidine HCl, then renatured by extensive washes in 50 mM Tris-HCl (pH 8.0) containing 0.04% (v/v) Tween-40 and 5 mM mercaptoethanol at 4°C. After pre-incubation of the gel at 20°C for 1 h in 40 mM HEPES, 2 mM dithiothreitol, 10 mM $MgCl_2$, pH 8.0, in situ phosphorylation of MBP was performed in 40 mM HEPES, 0.5 mM EGTA, 10 mM $MgCl_2$, 2 μM TTYADFIASGRTGRRNAIHD, 40 μM [γ-^{32}P]ATP (5 μCi/ml, 25 μCi/gel), pH 8.0 at 20°C for 3 h. After extensive washing in 5% (w/v) TCA and 10% (w/v) sodium pyrophosphate, gels were dried and autoradiographed.



In Fig 5b, MBP kinases were immunoblotted. Peaks II or IV from Mono Q chromatography of ET-1-stimulated cardiomyocytes (Fig. 3) were concentrated approx. 10-fold (Amicon microconcentrators), then subjected to SDS-PAGE in 10% (w/v) acrylamide gels. Gels were transferred to nitrocellulose using a Semi-Dry Transfer Cell (Bio-Rad) and immunoblotted with an antibody that recognises both p42 and p44 MAP kinases [29]. Non-specific sites were blocked with 5% non-fat milk in PBST for 1 h at room temperature, then primary antibodies (1/500 dilution in the blocking solution) were incubated with the nitrocellulose overnight at 4°C. After washing (3×5 min) in PBST, nitrocellulose filters were incubated for 1 h at room temperature with the horse-radish-peroxidase-labelled secondary antibody (1/5000 dilution in 1% non-fat milk in PBST). After repeating the washing as described above, bound antibody was detected by the ECL method (Amersham) according to the manufacturer's instructions. Immunoblotting of samples from an independent experiment gave similar results.

tures [30]. In addition to its vasoconstrictor action, ET-1 has numerous trophic effects on cells (including the cardiomyocyte [1]) and has recently been shown to activate MAP kinases in mesangial cells [26]. An attractive hypothesis is that one role of ET-1 in vivo is to function as a paracrine agent coupling local mechanical stimuli in the vascular bed (e.g. increases in pressure or flow) to the necessary hypertrophic adaptation of the cardiomyocyte [1]. ET-1 may also have an autocrine function in the cardiomyocyte [31]. Equally, a common feature of conditions which induce cardiac hypertrophy in vivo is an increase in sympathetic tone and a role for α_1-adrenergic agonism has been proposed [32,33]. ET-1, α_1-adrenergic agonists and PMA induce responses characteristic of hypertrophy in cultured neonatal cardiomyocytes (increased protein per cell, accumulation and assembly of sarcomeres, activation of primary response genes, transcriptional activation of genes normally expressed only in foetal heart, transcriptional activation of constitutively-expressed contractile protein genes) [1]. In adult cardiomyocytes, ET-1, α_1-adrenergic agonists and PMA each acutely stimulate translation [34–36]. PKC is thus strongly implicated as a participant in the hypertrophic response [1]. In this regard, we have recently demonstrated that ET-1, adrenaline and PMA each activate the major isoform of PKC (PKC-ε) present in adult cardiomyocytes [13].

MAP kinases participate in a protein phosphorylation cascade that is important in the transduction and transmission of extracellular signals to the nucleus [19], even though the phenotype following activation of MAP kinases may, for unknown reasons, be cell type-specific [19]. Several growth factors (e.g. nerve growth factor, epidermal growth factor) activate MAP kinases in other cell types [37,38]. We have now shown here ET-1, PE and PMA activate p42 and p44 MAP kinases in cultured neonatal cardiomyocytes. There is also consistent activation of two further MBP kinases (Peaks I and III in Fig. 3) which we have not yet been able to characterize because of the small amounts of activity available. We cannot exclude the possibility that these are further members of the extended MAP kinase family [17]. Down-regulation of PKC attenuates activation of MAP kinases by ET-1 or PMA and thus activation of PKC may be one pre-requisite for activation of MAP kinase under these conditions. Our results imply that activation of MAP kinases may be important in the development of cardiac hypertrophy.

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