BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 328–333 (1998)
ARTICLE NO. RC989476

Differential Regulation of Parallel Mitogen-Activated Protein Kinases in Cardiac Myocytes Revealed by Phosphatase Inhibition

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Received September 4, 1998

Previous studies have suggested that the contribution of inducible phosphatases to ERK MAPK deactivation is both cell-type- and agonist-specific. The aim of this study was to define the role of inducible phosphatases in ERK MAPK regulation in cardiac myocytes. We examined the kinetics of activation/deactivation of ERK MAPKs following the exposure of cardiac myocytes to endothelin-1 or phorbol ester. Deactivation was prevented by inhibition of protein synthesis indicating a contribution of inducible phosphatases. In contrast, okadaic acid failed to prolong ERK MAPK activation, but activated three myelin basic protein kinases (MBPKs, 55, 62, and 87 kDa) and two c-Jun kinases (46 and 55 kDa). Although the identity of the MBPKs is unknown, the c-Jun kinases corresponded to JNK MAPKs. Simultaneous exposure of cardiac myocytes to okadaic acid and osmotic shock potentiated JNK MAPK activation. Thus, inducible phosphatases regulate ERK MAPK deactivation, whereas okadaic acid-sensitive phosphatases regulate JNK MAPKs and three novel MBPKs.

The mitogen-activated protein kinases (MAPKs) are a family of protein kinases that are regulated by the phosphorylation of specific Thr- and Tyr- residues within a conserved Thr-X-Tyr motif (where X is Glu, Pro or Gly) in the protein kinase subdomain VII [1]. The MAPKs include the Extracellular Signal-Regulated Kinases (ERK MAPKs) which have been traditionally characterised as protein kinases activated after exposure of cells to growth factors acting through either tyrosine kinase- or heterotrimeric G protein-coupled-receptors [2]. Activation of ERK MAPKs is the result of phosphorylation of residues within its Thr-Glu-Tyr motif by MEK (for MAPK or ERK Kinase) [3]. The MAPKs also include the c-Jun N-terminal Kinases (JNK MAPKs) and p38/Reactivating Kinase which are activated upon exposure of cells to inflammatory cytokines and cellular stresses [4]. Activation of JNK MAPKs is a result of phosphorylation of residues within its Tyr-Pro-Thr motif by distinct dual-specificity kinases (MKK4 and MKK7) whereas p38/Reactivating Kinase is activated by phosphorylation of residues within its Thr-Gly-Tyr motif by dual-specificity kinases (MKK3 and MKK6) [1].

The dephosphorylation of either the regulatory phospho-Thr or the phospho-Tyr residue deactivates the MAPKs [5]. Thus, ERK MAPKs may be deactivated *in vitro* by either Ser/Thr phosphatases or Tyr phosphatases [6]. Recent studies employing protein synthesis inhibitors have implicated a family of inducible dual-specificity phosphatases capable of dephosphorylating both Ser/Thr and Tyr residues in the regulation of ERK activity *in vivo* [7, 8]. However, other phosphatases may deactivate ERKs in different cell types [9, 10]. Thus it has been suggested that the involvement of different phosphatases in the regulation of MAPK pathways may be dependent on the type of cell as well as the type of stimulus [9, 10].

We report here that inducible phosphatases control deactivation of ERKs in endothelin-1 and phorbol ester-treated cultured neonatal rat cardiac myocytes. In contrast, okadaic acid, a potent inhibitor of the Ser/Thr phosphatase, protein phosphatase 2A, does not prevent ERK MAPK deactivation, but activates the JNK MAPKs and other potentially novel renaturable myelin basic protein (MBP) kinases.

Abbreviations used: ERK, extracellular signal-regulated protein kinase; ET1, endothelin-1; GST, glutathione S-transferase; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK (or ERK) Kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

0006-291X/98 $25.00

EXPERIMENTAL PROCEDURES

Materials. Endothelin-1 (ET1), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), okadaic acid and protein synthesis inhibitors were from Sigma. Sources of other reagents have been described [6, 11, 12]. Recombinant c-Jun(1-135) or full length $p42^{mapk}$ were expressed as glutathione S-transferase (GST) fusion proteins and purified by glutathione-Sepharose chromatography. These fusion protein clones were gifts from Professor C. J. Marshall, Chester Beatty Laboratories, Institute of Cancer Research, London.

Cultured cardiac ventricular myocytes. Cardiac myocytes, dissociated from the ventricles of neonatal rat hearts, were plated (1.0×10^3 cells/mm^2) on gelatin-coated dishes and were confluent and beating within 18 h [12]. Serum was withdrawn for 24 h before treatment. Protein synthesis was determined by rates of incorporation of [^{3}H]phenylalanine [13].

Characterization of protein kinase activities. Cultured myocytes were exposed to agonists in serum-free medium at 37°C, washed with cold phosphate-buffered saline, then lysed in cold Buffer A [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 phenylmethanesulphonyl fluoride, 0.3% (v/v) mercaptoethanol, pH 7.5]. Lysates were centrifuged (10,000 × g, 4°C, 10 min) and the supernatants retained.

Activities of renaturable MBP or c-Jun(1-135) protein kinases were assayed by the in-gel method [6, 14, 15]. Briefly this method requires the polymerization of the substrate of choice within the SDS–PAGE gel matrix. Following electrophoresis, SDS is removed from the gel. Then the proteins resolved within the gel matrix are completely denatured then renatured. Kinase activity is assayed by the incorporation of ^{32}P from [γ-^{32}P]ATP into the substrate within the gel, thus indicating the relative molecular mass of the renatured protein kinase.

Immunoblotting with ERK antiserum used the enhanced chemiluminescence method [14]. MEK activity was assayed by the activation of GST-$p42^{mapk}$ [14].

RESULTS AND DISCUSSION

Sustained activation of ERK MAPKs in the presence of protein synthesis inhibitors. When myocytes were treated with ET-1 or TPA and soluble extracts examined by in-gel MBP kinase assays, 42 and 44 kDa MBP kinases changed dramatically in intensity with treatment (Fig. 1A). MBP kinases of 65 and 107 kDa (and a weaker MBP kinase of 97 kDa) did not change consistently (Fig. 1A). We have previously established that the 42 and 44 kDa MBP kinases correspond to ERK2 and ERK1, respectively [6, 14]. Activation of the ERK MAPKs coincided with a decrease in their electrophoretic mobilities revealed by immunoblotting (results not shown). The time-course of ERK MAPK activation was examined by quantification of in-gel MBP kinase assays (Figs. 1B and 1C). Both TPA and ET-1 maximally activated ERK MAPKs within 5 min. For TPA, inactivation was relatively slow, with activities returning to control values within 120 min (Fig. 1B). For ET-1, inactivation occurred more rapidly (within 60 min, Fig. 1C). Importantly, inactivation occurred despite the continued presence of the agonists. We were interested in determining the possible mechanisms involved in the differential regulation of ERK MAPK deactivation under these conditions.

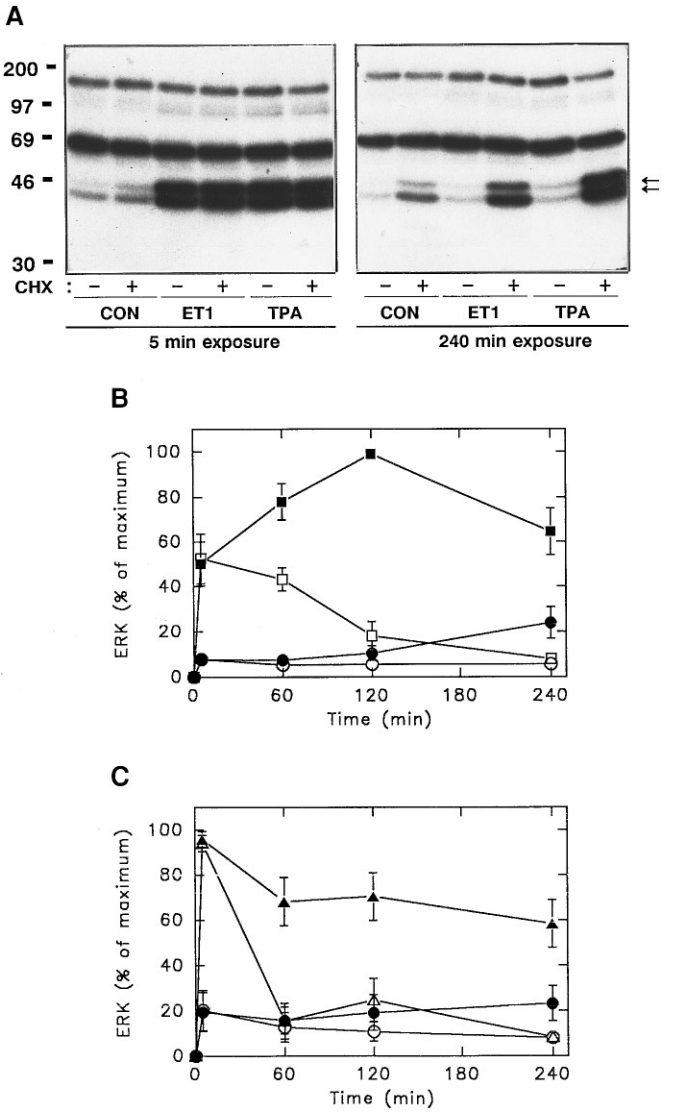

FIG. 1. ERK MAPK deactivation is prevented by cycloheximide. Ventricular myocytes were stimulated for the times indicated with control serum-free medium (CON, ○), 20 μM cycloheximide (CHX, ●), 1 μM TPA (□), 1 μM TPA + 20 μM cycloheximide (■), 100 nM ET1 (△), or 100 nM ET1 + 20 μM cycloheximide (▲). In A, ERK MAPK activity in soluble extracts of these cells was assayed as in-gel 42 and 44 kDa MBP kinases as described under Experimental Procedures. The arrows to the right of the panels indicate the positions of 44 kDa ERK1 and 42 kDa ERK2. In B and C, results from laser scanning densitometry of autoradiographs (means ± S.E.M., $n = 5$) are expressed relative to maximal 42 and 44 kDa MBP kinases activated by TPA and ET1, respectively.

The dual-specificity MAPK phosphatases MKP-1 and MKP-2 have been previously reported to be expressed in rat heart [8] and may play a major role in the deactivation of MAP kinases in a variety of cell types [8, 16]. These protein phosphatases have been distinguished by their rapid induction following the exposure of cells to many of the agents that activate ERK MAPKs (e.g., [17]). However, this is a complex

family of protein phosphatases with multiple family members now identified [18]. We therefore chose to investigate the potential role of this large group of inducible MAPK phosphatases in the inactivation of ERK MAPKs by the use of protein synthesis inhibitors. Myocytes were incubated in the presence of 20 μM cycloheximide which decreases the basal or TPA-stimulated incorporation of [^{3}H]phenylalanine in the cultured myocyte protein by more than 80% (results not shown). Incubation with cycloheximide did not affect the activation of ERK MAPKs by ET1 or TPA after a 5 min exposure and did not significantly increase basal ERK MAPK activity until after 4 h (Fig. 1). After a 2-h exposure to 1 μM TPA in the presence of cycloheximide, ERK MAPK activity was elevated approximately 10-fold over the activity achieved after a 2 h exposure to TPA alone (Fig. 1B). Thus, the presence of cycloheximide not only maintained but enhanced ERK MAPK activation by TPA.

To investigate the contribution of inducible phosphatases to the rapid deactivation of ERK MAPKs observed during ET1 exposure, we incubated myocytes with ET1 in the presence of cycloheximide. In contrast to the situation for TPA exposure, no enhanced ERK MAPK activation was achieved by ET1 (Fig. 1C). ERK MAPK activity elicited by ET1 exposure was sustained at 60 to 80% of its maximal values for more that 2 h when cycloheximide was present (Figs. 1A and 1C). Thus, cycloheximide allows the maintenance of high levels of ERK MAPK activation after either ET1 or TPA exposure. The IC_{50} for the effects of cycloheximide on ERK MAPK activation by TPA (2 h exposure) or ET1 (1 hour exposure) was approximately 0.5 μM (two separate observations). This cycloheximide concentration is similar to that required to inhibit protein synthesis by 50% in these cells (IC_{50} approximately 0.2 μM, two separate observations). Other inhibitors of protein synthesis, including 40 μM anisomycin, 20 μM puromycin, or 20 μM emetine also maintained ERK MAPK activation by ET1 or TPA (results not shown). These results suggest that, as in other cell types [18], inducible protein phosphatases contribute to, but can not completely account for, the inactivation of ERK MAPKs in the cardiac myocyte.

We tested that the effect on protein synthesis inhibition was specific for the activation of ERKs within the ERK MAPK signalling cascade. The activation of MEK, the immediate upstream regulator of ERK MAPKs, by TPA was rapid and relatively sustained (Fig. 2A). The maximal activation of MEK by ET1 was only approximately 50 % of that achieved after TPA exposure and was relatively transient, returning to control values within 30 min (Fig. 2B). However the kinetics of activation of MEK by either TPA or ET1 were not significantly affected by the presence of cycloheximide (Figs. 2A and 2B). This indicates the specificity for the effect of cycloheximide on ERK MAPK

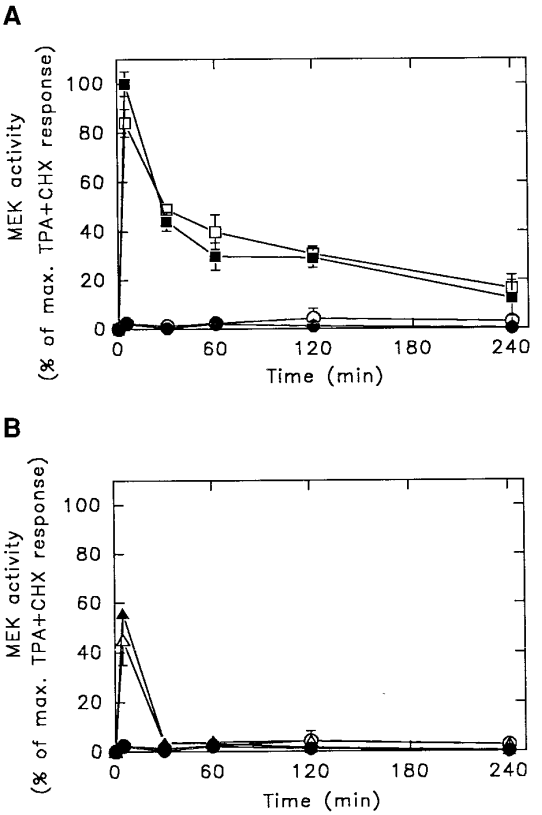

FIG. 2. Cycloheximide does not affect MEK activation by ET1 or TPA. Ventricular myocytes were stimulated for the times indicated with control serum-free medium (○), 20 μM cycloheximide (●), 1 μM TPA (□), 1 μM TPA + 20 μM cycloheximide (■), 100 nM ET1 (△), or 100 nM ET1 + 20 μM cycloheximide (▲). MEK activity was assayed using the activation of recombinant GST-p42^{mapk} and the subsequent incorporation of ^{32}P from [γ-^{32}P]ATP into MBP. Results (means ± S.E.M., n = 3 independent observations) are expressed relative to the maximal MEK activated by exposure to TPA for 15 min.

activity. We propose that the enhanced activation of ERK MAPKs by the exposure to TPA in the presence of cycloheximide (Fig. 1) may be the result of the sustained activation of MEKs by TPA together with an inhibition of phosphatases in the presence of cycloheximide.

Okadaic acid does not alter ERK deactivation. Recent studies have suggested that the rapid deactivation of ERK MAPKs may be the result of protein phosphatases such as Protein Phosphatase 2A [9]. We therefore investigated whether this Ser/Thr phosphatase may contribute to the rapid loss (20–40%) of the ERK MAPK activity in the myocytes treated with ET1 and cycloheximide (Fig. 1). Okadaic acid is a potent, membrane-permeable inhibitor of protein phosphatase 2A and protein phosphatase 1 [19]. Exposure to okadaic acid alone resulted in low levels of ERK MAPK activation (Figs. 3 and 4). ERK MAPKs have been previously shown to be slowly activated in okadaic acid-treated B-lymphocytes and fibroblasts [20, 21].

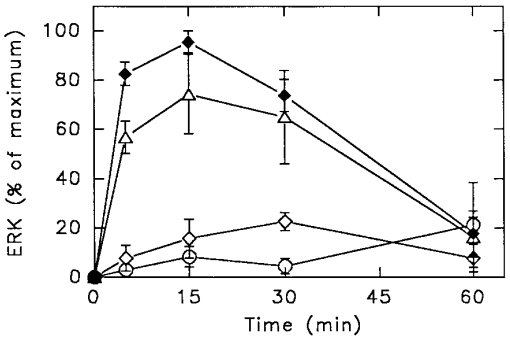

FIG. 3. ERK MAPK deactivation is not prevented by okadaic acid. Ventricular myocytes were exposed to control medium (○), 100 nM ET1 (△), 1 μM okadaic acid (◇), or 100 nM ET1 + 1 μM okadaic acid (◆). MBP kinases were assayed as described in Fig. 1. Results from laser scanning densitometry of autoradiographs (means ± S.E.M., n = 4 independent observations) are expressed relative to maximal 42 and 44 kDa MBP kinase activities.

The simultaneous exposure of myocytes to 100 nM ET1 in the presence of 1 μM okadaic acid did not prolong ERK MAPK activation (Fig. 3). However, there was a small effect of okadaic acid to potentiate the ERK MAPK activation by 5 min ET1 exposure. Thus, phosphatases sensitive to okadaic acid inhibition may contribute to the early phase of ERK MAPK deactivation following ET1 exposure but the long-term deactivation is mediated by one or more members of the large family of inducible MAPK phosphatases.

Okadaic acid exposure activates other potentially novel renaturable MBP kinases. Studies using okadaic acid or the simian virus small t antigen, both of which inhibit phosphatase 2A, have suggested that this phosphatase may regulate many intracellular functions [19, 22]. Although the exposure of cultured myocytes to okadaic acid caused only low levels of ERK MAPK activation, activation of MBP kinases of 87, 62, and 55 kDa was observed after 15 to 30 min exposure to okadaic acid alone (Fig. 4). A variable activation (of up to 2-fold) of a 130 kDa MBP was observed on several occasions. The identity of these kinases and the functions that they may play in the cardiac myocyte is unknown. Previous studies have however reported a 63 kDa MBP kinase that is activated in okadaic-treated or v-src-transformed chicken embryo fibroblasts [23]. Furthermore, v-src-transformation also activated a 87 kDa MBP kinase [23]. This suggests that the 87 and 62 kDa MBPKs revealed by the use of okadaic acid in this study may provide important links in growth factor signalling.

JNKs are amongst the protein kinases revealed after okadaic acid exposure. The molecular mass of the smallest MBP kinase (55 kDa) activated upon okadaic acid exposure suggested that it might be related to the stress-activated JNK MAPKs [15, 24]. We performed in-gel analysis using either MBP (Fig. 5A) or the c-Jun N-terminal domain (amino acids 1 to 135) as a substrate (Fig. 5B). For myocytes treated with okadaic acid, osmotic shock induced by sorbitol, or okadaic acid and sorbitol, we observed activation of a number of MBP kinases in addition to the ERK MAPKs. Okadaic acid activated 55, 62, and 87 kDa MBP kinases (MBPK-55, MBPK-62, and MBPK-87, respectively) within 15–30 min although it did not greatly stimulate the 107 and 65 kDa MBP kinases. The autoradiograph in Fig. 5 has been overexposed to show the weak activation of ERKs by okadaic acid. Shorter exposure times allowed resolution of the 55, 62, and 65 kDa MBP kinases (results not shown). MBPK-87 was resolved into two bands on some occasions, and variable activation (up to 2-fold) of 130 and 200 kDa MBP kinases was observed. In contrast to the weak and transient activation of ERK MAPKs, the strong activation of MBPK-55, -62, and -87 was maintained for at least 120 min (Fig. 4). Myocytes did not survive longer exposures to okadaic acid (unpublished observations).

Two c-Jun kinases (46 and 55 kDa) that correspond to JNK1 and JNK2 that are activated by sorbitol exposure [15] were activated by okadaic acid (Fig. 5B). This activation was potentiated by incubation with okadaic acid and sorbitol (Fig. 5B). No kinase activity was observed in gels that contained no protein substrates (results not shown).

GENERAL CONCLUSIONS

This study demonstrates that the inactivation of the parallel ERK and JNK MAPK cascades in cardiac myocytes following their exposure to ET1, phorbol esters, or osmotic shock may be differentially regulated. The ERK MAPK pathway (which is acti-

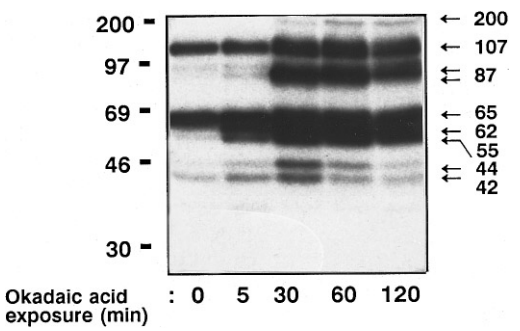

FIG. 4. Okadaic acid activates MBPKs other than the ERK MAPKs. Ventricular myocytes were exposed to 1 μM okadaic acid for 5 to 120 min as indicated. MBP kinases were assayed as described in the legend to Fig. 1. The gel is representative of three independent experiments. The molecular masses (kDa) of marker proteins are indicated by the numbers to the left of the panel. The numbers to the right of the panel indicate the positions of the MBP kinases (including the 44 kDa ERK1 and the 42 kDa ERK2) that were activated by okadaic acid.

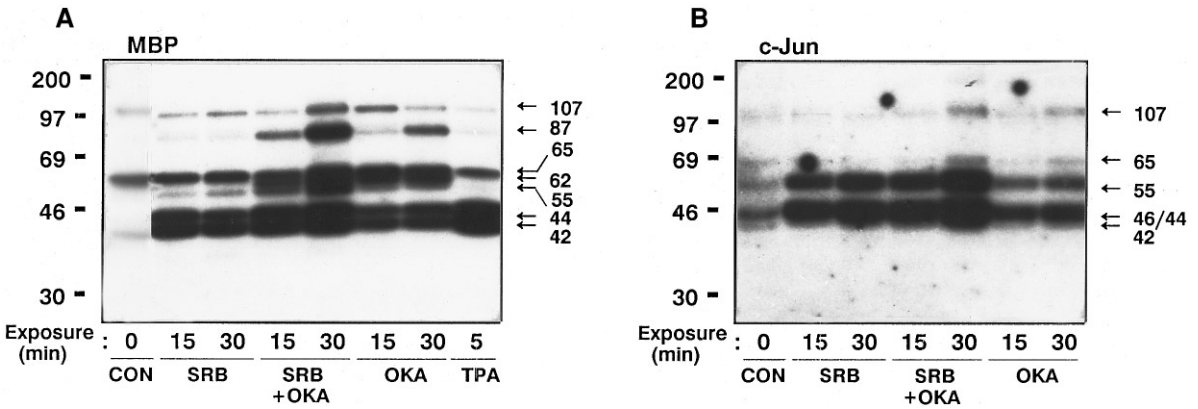

FIG. 5. Okadaic acid and osmotic shock activate MBP kinases and JNK MAPKs. Ventricular myocytes were exposed to control medium (CON), 0.5 M sorbitol (SRB), 1 μM OKA, or 0.5 M sorbitol + 1 μM OKA (SRB + OKA) for 15 or 30 min as indicated or to 1 μM TPA for 5 min. MBP kinases (A) or JNKs (B) in soluble extracts were assayed by the in-gel method using (A), MBP or (B), GST-c-Jun(1-135) as substrate. Gels are representative of three independent experiments. The molecular masses (kDa) of marker proteins are indicated by the numbers to the left of each panel. The numbers to the right of the panels indicate in kDa the positions of the MBP kinases (A) or JNK MAPKs (B) that were activated.

vated by ET1, and TPA) is subject to regulation by inducible dual-specificity protein phosphatases and is relatively unaffected by okadaic acid. Within 24–48 h, ET1 and TPA induce the morphological and transcriptional changes in the cardiac myocyte that typify the hypertrophic phenotype and we have previously proposed that ERK MAPKs participate in this response [6]. Our current findings imply that dual-specificity phosphatases may also play a role in the development of the hypertrophic phenotype. Indeed, the overexpression of the dual-specificity MAPK phosphatase-1, prevents the changes in gene expression (such as increased atrial natriuretic factor and myosin light chain-2 expression) induced by phenylephrine during the hypertrophic adaptation [25, 26].

In contrast, the activation of JNK and ERK MAPKs following exposure of myocytes to osmotic shock appears to be influenced by okadaic acid-sensitive protein phosphatases. It is not possible to study whether the signal transduction pathways activated by osmotic shock or okadaic acid may lead to an induction of a hypertrophic response because myocytes do not survive exposure to these agonists for the period (24–48 h) required to observe the hypertrophic changes (unpublished observations). The results of the present study demonstrate that phosphatases play a critical role in the regulation of ERK and JNK MAPKs as well as a series of potentially novel renaturable MBP kinases.

ACKNOWLEDGMENTS

This work was supported by grants from The Wellcome Trust and the British Heart Foundation to Dr. P. H. Sugden (National Heart and Lung Institute, Imperial College of Science, Technology and Medicine, Dovehouse St., London, UK) and a grant (0035566) from the National Health & Medical Research Council to M.A.B. We thank Nicola J. Haward for the preparation of myocyte cultures.

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