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# Characterization of Protein Kinases Activated during Treatment of Cells with Okadaic Acid

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**Six renaturable protein kinases that utilize the myelin basic protein (MBP) as a substrate were activated during prolonged exposure of cardiac myocytes to okadaic acid (OA). We characterized the substrate preference and activation of these kinases, with particular emphasis on 3 novel kinases-MBPK-55, MBPK-62 and MBPK-87. The transcription factors c-Jun, Elk, ATF2, and c-Fos that are used to assess mitogen-activated protein kinase activation were all poor substrates for these three kinases. MAPKAPK2 was also not phosphorylated. In contrast, Histone IIIS was phosphorylated by MBPK-55 and MBPK-62. These protein kinases were activated in cultured cardiac fibroblasts, H9c2 cardiac myoblasts, and Cos cells. High concentrations (0.5 to 1 μM) of OA were essential for the activation of the protein kinases in all of the cell types examined, whereas calyculin A [an inhibitor of protein phosphatase 1 (PP1) and PP2A], cyclosporin A (a PP2B inhibitor), and an inactive OA analog all failed to activate these kinases. The high dose of okadaic acid that is required for kinase activation was also required for phosphatase inhibition, as assessed by immunoblotting whole cell lysates with anti-phosphothreonine antibodies. A variety of chemical inhibitors, including PD98059 (MEK-specific), genistein (tyrosine kinase-specific) and Bisindolylmaleimide I (protein kinase C-specific), failed to inhibit the OA activation of these kinases. Thus, MBPK-55 and MBPK-62 are also Histone IIIS kinases that are widely expressed and specifically activated upon exposure to high OA concentrations.**

**Keywords:** Protein kinases, MAPKs, Phosphatase inhibition, Okadaic acid.

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## Introduction

The regulation of intracellular signaling cascades is the result of a complex interplay between phosphorylation catalyzed by protein kinases, and dephosphorylation catalyzed by protein phosphatases (Hunter, 1995). Taking the mitogen-activated protein kinases (MAPKs) as an example, it is clear that this family of protein kinases is 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 (Cano *et al.*, 1995). The dephosphorylation of either the regulatory phospho-Thr or the phospho-Tyr residue deactivates the MAPKs. It is now widely accepted that a family of dual-specificity phosphatases catalyzes this dephosphorylation, although other phosphatases may deactivate ERK MAPKs in different cell types (Wu *et al.*, 1994; Alessi *et al.*, 1995). Thus, it has been suggested that the involvement of different phosphatases in the regulation of protein kinase pathways may be dependent on the protein kinase pathway, the cell type, and the type of stimulus.

A number of specific phosphatase inhibitors are now available to evaluate the role of phosphatases in regulating signal transduction pathways and ultimate cellular outcomes. One such compound that has been widely used is okadaic acid (OA), a potent inhibitor of the serine/threonine phosphatases protein phosphatase 2A (PP2A) and PP1 (*see reviews*, Cohen *et al.*, 1990; Shenolikar, 1994). OA is hydrophobic and crosses cellular membranes, making it useful in delineating the role of phosphatases in intact cells (Cohen *et al.*, 1990). We recently described three novel protein kinases (MBPK-55, MBPK-62 and MBPK-87) which are activated during the exposure of cardiac myocytes to 1 μM OA (Andersson *et al.*, 1998). This suggests that protein phosphatases, such as protein phosphatase 2A (PP2A) and/or protein phosphatase 1 (PP1), play an important role in signal transduction in these cardiac cells. Recent studies with other inhibitors of PP2A and PP1, such as fostriecin and calyculin A, suggest an important role for these phosphatases during survival following ischemic injury (Armstrong *et al.*, 1992; Weinbrenner *et al.*, 1998). To

further delineate the role that OA-regulated protein kinases may play, we characterized their substrate preference, activation in different cell types, and demonstrated that the effect of OA to activate these kinases is not mediated by a variety of known intracellular signaling events.

## Materials and Methods

**Materials** 2-*O*-Tetradecanoylphorbol-13-acetate (TPA), sorbitol, myelin basic protein, and Histone IIIS were from Sigma. Okadaic acid (potassium salt, Calbiochem) was dissolved to a stock concentration of 100 μM in a sterile tissue culture medium and stored in aliquots at −20°C until use. Cyclosporin A and calyculin A (Calbiochem) were dissolved to stock concentrations of 10 mM in ethanol and stored in aliquots at −20°C until use. Suramin (Calbiochem) was prepared in a medium just prior to use. Bisindolylmaleimide I HCl, PD98059, genestein and okadaic acid methyl ester (Calbiochem) were all prepared as stock solutions in DMSO and stored in aliquots at −20°C until use. The phosphotyrosine antibody (clone 4G10) was from Upstate Biotechnology Inc. The phosphothreonine antibody and the phospho-ERK MAPK antibody were from New England Biolabs. Sources of other reagents have been described (Bogoyevitch *et al.*, 1995a; Bogoyevitch *et al.*, 1996). Recombinant c-Jun (1-135), ATF2 (19-96), Elk (307-428), Fos (210-313), and MAPKAPK2 (46-400) were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and purified by glutathione-Sepharose chromatography.

**Cultured cells** Cardiac myocytes, dissociated from the ventricles of neonatal rat hearts, were plated ($4 \times 10^6$ cells) on gelatin-coated 60 mm dishes and were confluent and beating within 18 h (Bogoyevitch *et al.*, 1995a). Cardiac fibroblasts as well as the other established cell lines (H9c2, rat embryonic heart myoblast; Cos, simian fibroblast; 293, human embryonic kidney) were grown under standard tissue culture conditions in 10% (v/v) fetal calf serum in Dulbeccos Modified Essential Medium. They were then plated onto 60-mm dishes and grown until approximately 90% confluent. Serum was withdrawn 24 h before any further treatment.

**Characterization of protein kinase activities** Cells were exposed to agonists in a serum-free medium at 37°C, washed with a cold phosphate-buffered saline, then scraped into an ice-cold lysis buffer [20 mM HEPES pH 7.7, 20 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl supplemented with 0.05% (v/v) Triton X-100, 500 μM dithiothreitol, 100 μM $Na_3VO_4$, 20 μg/ml leupeptin, 20 μg/ml leupeptin]. The lysates were centrifuged (10,000 × g, 4°C, 10 min) and the supernatants retained.

The activities of the renaturable protein kinases were determined by in-gel assays (Bogoyevitch *et al.*, 1994). In this method, the substrate of choice is polymerized within the SDS-PAGE gel matrix. We used the following final concentrations of substrates for these assays-MBP (0.25 mg/ml), Jun/Elk/ATF2/Fos/MAPKAPK2 (0.2 mg/ml), and Histone IIIS (0.4 mg/ml). As a control, we also evaluated gels in which the protein substrates were omitted. Following electrophoresis of the samples, the SDS was removed from the gel. The proteins that were resolved within the gel matrix were then completely denatured, then renatured. Finally, the protein kinase activity of the renatured protein kinases was assayed by the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$] ATP into the substrate within the gel, thus indicating the relative molecular mass of the renatured protein kinase.

**Immunoblotting for phosphothreonine, phosphotyrosine, and phosphorylated ERK MAPKs.** The cell lysates (typically 50 μg of protein per sample) were separated by SDS-PAGE, and transferred to nitrocellulose membranes. They were then blocked for 60 min with gentle agitation with either 5% (w/v) non-fat milk powder in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% v/v Tween 20; pH 7.5) for phosphothreonine or phospho-ERK detection, or 1%(w/v) bovine serum albumin in TBST for phosphotyrosine detection. The membranes were incubated with the appropriate primary antibody (1/1000 dilution of the appropriate antibody in their block solution) overnight at 4°C with agitation. The nitrocellulose membrane was then washed in several changes of TBST, and incubated with a secondary antibody that was conjugated to horseradish peroxidase (diluted in 1% (w/v) non-fat milk powder in TBST for phosphothreonine or phospho-ERK, or in 0.2% (w/v) bovine serum albumin for phosphotyrosine) for at least 2 h. The membranes were treated with Supersignal Chemiluminescent Substrates for 5 min, and exposed to Hyperfilm for <5 min.

**Inhibitor treatments** To begin to elucidate the events that lead to activation of the 55 kDa, 62 kDa, and 87 kDa MBP kinases, a series of experiments were undertaken in which the Cos cells were pretreated for specific chemical inhibitors. The inhibitors were chosen to target upstream signal transduction components with a focus on a number of specific protein kinases. Specifically, we tested each of the following compounds individually for their effects: genestein (100 μM), Bisindolylmaleimide I (1 μM), PD98059 (30 μM), as well as cycloheximide (20 μM) or suramin (600 μM). For genestein, Bisindolylmaleimide I, and PD98059, stock solutions were prepared in dimethylsulfoxide and stored at −20°C. Dilutions of these stocks into a culture medium ensured that the concentration of dimethylsulfoxide did not exceed 0.1% (v/v). Suramin and cycloheximide were made fresh daily in a culture medium and used immediately. The rationale behind the specific choices of inhibitors was to be able to inhibit tyrosine kinases, protein kinase C, ERK MAPK kinase, protein synthesis, and growth factor receptors, respectively. In all cases, we preincubated the cells in the inhibitor at the specified concentration for 45 min, then maintained this inhibitor during the exposure to OA. Previously we have shown that these concentrations of agents are effective signal transduction inhibitors under our incubation conditions (Ng *et al.*, 2000; Ng *et al.*, 2001).

## Results

**Substrate preferences of the renaturable protein kinases activated in okadaic acid-stimulated cells** When the myocytes were treated with 1 mM OA and soluble extracts that were examined by in-gel MBP kinase assays, 55 kDa, 62 kDa, and 87 kDa MBP kinases were activated during the 30

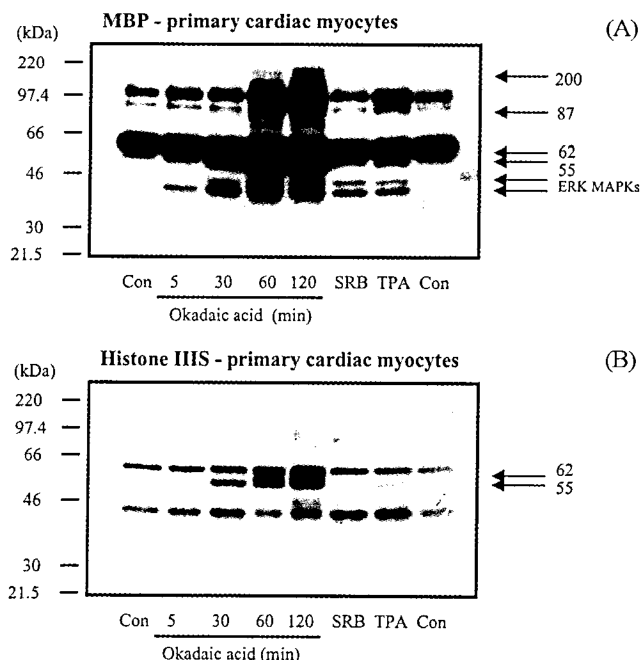


**Fig. 1.** Exposure of cardiac myocytes to okadaic acid stimulates renaturable MBP and Histone IIIS kinases. Cultured ventricular myocytes were stimulated for the times indicated with the control serum-free medium (Con), or 1 μM okadaic acid. As controls we also included cells that were treated for 15 min with either 0.5 M sorbitol (SRB), or 1 μM TPA. Protein kinase activity was assayed by the in-gel method, as described in the Materials and Methods. The substrates used were (a) MBP and (b) Histone IIIS. The arrows to the right of the panels indicate the positions of 55 kDa, 62 kDa, 87 kDa, and 200 kDa renaturable kinases, as well as the ERK MAPKs (42 and 44 kDa). This experiment was repeated with similar results.

and 60 minutes of treatment (Fig. 1A and Andersson *et al.*, 1998). These kinases remained elevated at 120 min of exposure, and additional MBP kinases >100 kDa were also observed during this prolonged treatment (Fig. 1A). As we reported previously, the ERK mitogen-activated protein kinases (ERK MAPKs) are also activated by this treatment with OA (Fig. 1B and Andersson *et al.*, 1998).

We examined the substrate preference of these protein kinases by performing in-gel assays on similar extracts of cardiac myocytes that were exposed to OA. As summarized in Table 1, we observed no significant phosphorylation of the four transcription factor substrates (c-Jun, Elk, ATF2, and c-Fos), or the protein kinase MAPKAPK-2 that are phosphorylated by members of the mitogen-activated protein kinase family. In contrast, Histone IIIS was a good substrate for the 55 and 62 kDa protein kinases (Fig. 1A and Table 1).

**Activation of renaturable kinases in other okadaic acid-treated cell types** To assess whether the activation of these novel renaturable kinases by OA is a cardiac myocyte-specific phenomenon, we evaluated the activation of these kinases in other cell types. Given the potential importance of PP2A in the regulation of the cardiac function (Armstrong *et al.*, 1992; Weinbrenner *et al.*, 1998), we first chose to examine the regulation of these kinases in a different cell type that was derived from the heart, namely the cardiac fibroblast. Clearly the same protein kinases were activated in these cells following only a 5-min exposure to OA, and continued to increase in activation up to the 120 min of exposure (Fig. 2A). The 87 kDa protein kinase was also activated with maximal activation at 120 min (Fig. 2A).

**Table 1.** Substrate preference of renaturable kinases

| Substrate | 55 kDa | 62 kDa | 87 kDa | ERK MAPK | JNK MAPK |
|---|---|---|---|---|---|
| MBP | + | + | + | + | - |
| c-Jun | - | - | - | - | + |
| Elk | - | - | - | + | + |
| ATF2 | - | - | - | - | + |
| c-Fos | - | - | - | - | - |
| MAPKAPK2 | - | - | - | + | - |
| Histone IIIS | + | + | - | - | - |

Cardiac myocytes were exposed to 1 μM OA for 5 to 120 min, then protein kinase activity was assayed by the in-gel method, as described in the Materials and Methods. The substrates used were MBP (0.25 mg/ml), GST fusion proteins of c-Jun, Elk, ATF2, c-Fos, or MAPKAPK2 (0.20 mg/ml) or Histone IIIS (0.4 mg/ml). The ability of MBPK-55, MBPK-62, and MBPK-87 to phosphorylate the tested substrates was judged in the lysates that were prepared from myocytes treated with okadaic acid for 60 or 120 min. Activation of JNK MAPKs was assessed by treating myocytes with osmotic shock (Bogoyevitch *et al.*, 1995b). These results summarize the observations taken from 2 to 3 independent experiments with each substrate.

In contrast, the cardiac myoblastic cell line H9c2 failed to significantly activate the 87 kDa protein kinase, even after prolonged OA exposure (Fig. 2B). However, the 55 and 62 kDa protein kinases were clearly activated within 60 min (Fig. 2B). These results suggest that OA will modulate the activity of protein kinases in a number of heart-derived cells.

We further examined two cell lines that were not of cardiac origin. First, we examined the human embryonic kidney cell line HEK293. High doses of OA (0.1 to 1 μM) were toxic, killing these cells within 15 to 30min (results not shown). The activation of the renaturable MBPKs was not observed at the lower non-toxic OA doses, therefore, this cell line proved unsuitable for further analysis (results not shown). In contrast, the simian fibroblast COS cell line could be stimulated with 1 μM OA, so that the 55, 66, and 87 kDa protein kinases were activated within 30 min of exposure (Fig. 3A, and seen clearer in the shorter film exposure shown in Fig. 3B). Furthermore, an additional 38 kDa OA-stimulated protein kinase was observed when the autoradiograph of OA-stimulated Cos cells was exposed for longer periods of time (Fig. 3A).

The failure of lower non-toxic doses of OA to stimulate

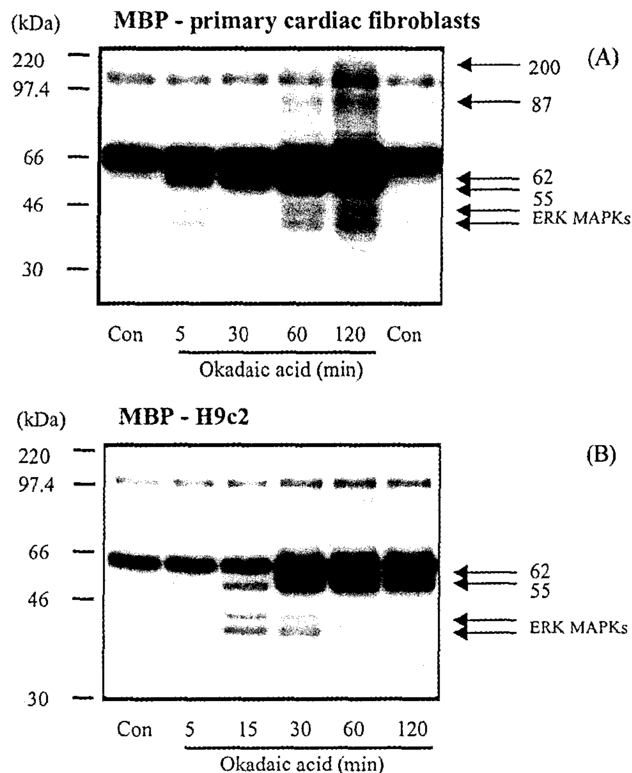


**Fig. 2.** Exposure to okadaic acid activates similar renaturable MBP kinases in other cardiac cell types. Cultured (A) cardiac fibroblasts, or (B) the cardiac myoblast cell line H9c2 were stimulated for the indicated times with 1 μM okadaic acid, or left untreated (Con). Protein kinase activity using MBP as a substrate was assayed by the in-gel method, as described in the Materials and Methods. The arrows to the right of the panel (A) indicate the positions of 55 kDa, 62 kDa, 87 kDa, and 200 kDa renaturable kinases, as well as the ERK MAPKs (42 and 44 kDa). In panel (B), only changes in the activity of the 55 and 62kDa protein kinases and the ERK MAPKs (42 and 44 kDa) were observed. These experiments were repeated twice with similar results.

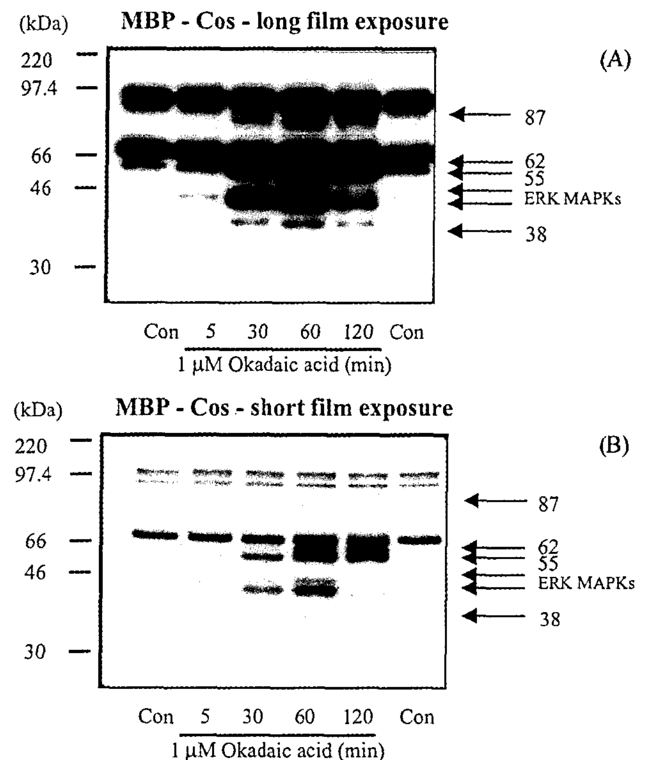


**Fig. 3.** Exposure to okadaic acid activates similar renaturable MBP kinases in the Cos cell line. Confluent cultures of Cos cells were stimulated for the indicated times with 1 μM okadaic acid, or left untreated (Con). Protein kinase activity using MBP as a substrate was assayed by the in-gel method, as described in the Materials and Methods. The arrows to the right of the panels indicate the positions of 38kDa, 55 kDa, 62 kDa, and 87 kDa renaturable kinases, as well as the ERK MAPKs (42 and 44 kDa). Panel (A) shows that a renaturable protein kinase of 200 kDa could not be observed, even with the prolonged exposure of the autoradiograph. Panel (B) shows typical results obtained following overnight exposure of the autoradiograph. These experiments were repeated twice with similar results.

protein kinase activation in HEK293 cells prompted a closer examination of the dose-response relationship between the OA concentration and protein kinase activation. As shown in Fig. 4A and Fig. 4B, we observed that protein kinase activation was not in the observed cultured cardiac myocytes that were exposed to 0.1 μM for either 1 or 2 h. Extending these studies in Cos cells, we observed that 0.5 μM was moderately effective in stimulating ERK MAPK activity, but failed to activate the 38, 55, 66, or 87 kDa protein kinases (Fig. 4C). From these data we conclude that very high external concentrations of okadaic acid are required to elicit the activation of these protein kinases in either myocytes or Cos cells.

We further confirmed that the activation of these protein kinases could be readily observed in Cos cells that had not been previously subjected to serum starvation overnight, prior to the exposure to OA (results not shown). The process of serum starvation, therefore, neither primes nor inhibits the actions of OA to activate these protein kinases.

**Treatment with other serine/threonine phosphatase inhibitors fails to activate MBPKs** It has been recognized that OA can be applied to intact cells at concentrations up to 1 μM, and leads to the inhibition of PP2A without significant inhibition of the PP1, PP2B, or PP2C, (see review, Millward *et al.*, 1999). However, we examined the possible role that other protein phosphatases may play in regulating the activation of the renaturable MBPKs. We exposed Cos cells to 1 μM cyclosporin A (to inhibit PP2B) for up to 2 h, and observed no significant activation of any protein kinases, judged by the in-gel MBP kinase assays (results not shown). Increasing the dose of cyclosporin A to 100 μM also was without effect (results not shown).

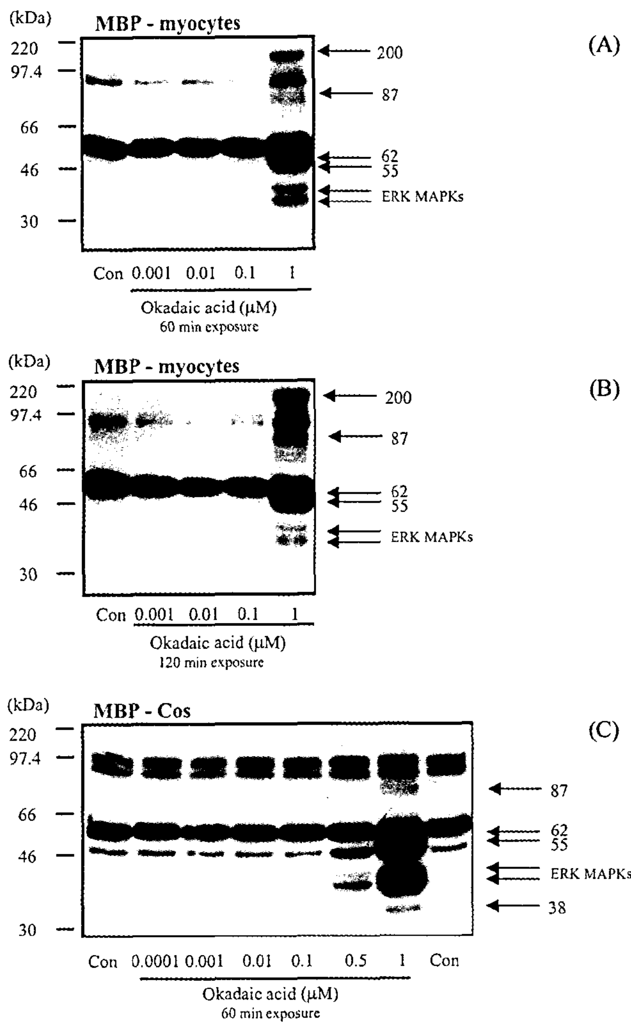


**Fig. 4.** The dose response relationship for okadaic acid activation of MBPKs. In Panels (A) and (B), cultured ventricular myocytes were stimulated for either (A) 60 or (B) 120 minutes with a control serum-free medium (Con), a range of concentrations (0.001 to 1 μM) okadaic acid. In Panel (C), cultured Cos cells were stimulated for 60 minutes with a control serum-free medium (Con), a range of concentrations (0.0001 to 1 μM) okadaic acid. In all Panels, the protein kinase activity using MBP as a substrate was assayed by the in-gel method, as described in the Materials and Methods. The arrows to the right of the panels indicate the positions of 38kDa, 55 kDa, 62 kDa, 87 kDa, and 200 kDa renaturable kinases, as well as the ERK MAPKs (42 and 44 kDa). These experiments were repeated with similar results.

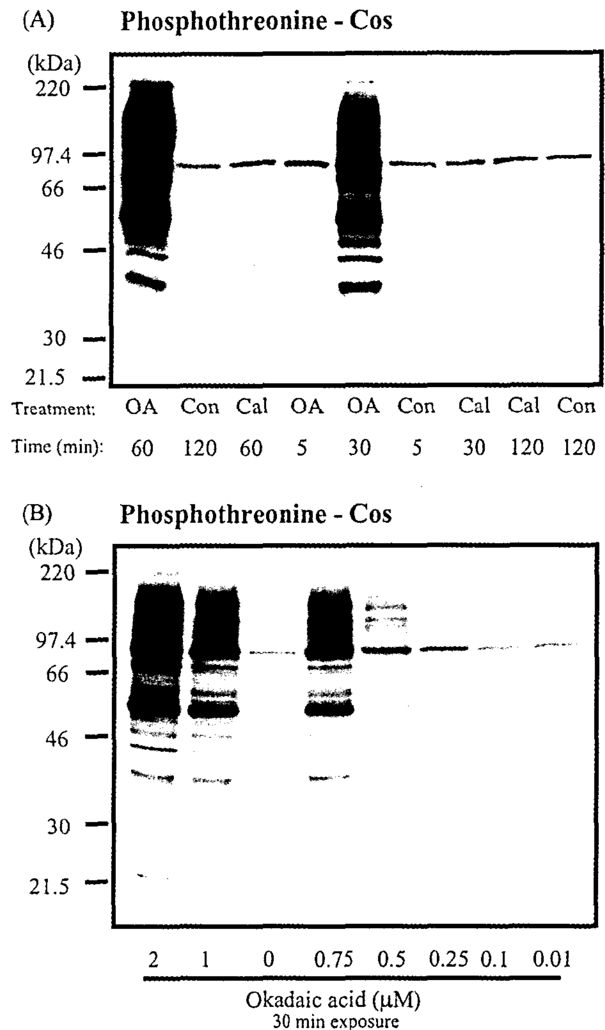


**Fig. 5.** The time-dependence and dose response relationship for okadaic acid enhancement of phosphorylated threonine proteins. In Panel (A), Cos cells were stimulated for the times indicated with either a control serum-free medium (CON), 1 μM okadaic acid (OA), or 0.01 μM calyculin A (Cal). Preliminary experiments indicated that higher doses of calyculin A could not be used due to problems with the toxicity of this compound. In Panel (B), cultured Cos cells were stimulated for 30 minutes with a range of concentrations (0 to 2 μM) okadaic acid. In all Panels, the cell lysates were then prepared and subjected to immunoblotting with antibodies for phosphothreonine, as described in the Materials and Methods. These experiments were repeated with similar results.

A similar protocol with 1 μM calyculin A (to inhibit both PP1 and PP2A with potentially equivalent potency) failed due to the toxicity of this compound. The maximum dose of calyculin A, tolerated for more than 30 min by Cos cells, was 0.01 μM. At this dose we observed no activation of renaturable MBPKs (results not shown).

We ensured the specificity of the OA-mediated effects in the control experiments using an inactive analog of OA, okadaic acid methyl ester. This compound at a final concentration of 1 μM failed to activate the renaturable MBPKs at time points up to 2 h (results not shown).

**Immunoblotting with phospho-threonine antibodies** As a control for the preceding experiments, we also evaluated the total profiles of phosphorylation events by immunoblotting. Specifically, commercially-available antibodies that were

directed at phosphorylated threonine residues showed that the threonine phosphorylation of a large number of proteins with molecular masses ranging from approximately 40 to 220 kDa could be stimulated by exposure of Cos cells to 1 μM OA (Fig. 5). Importantly, no changes could be observed following a brief 5-min exposure to OA (Fig. 5A). The low, non-toxic doses of calyculin A (0.01 μM) also failed to elicit significant increases in threonine phosphorylation, even during prolonged incubations up to 2 h. Furthermore, when we examined the dose response relationship of OA and threonine phosphorylation, it was clear that high doses of OA (i.e.≥ 0.75 μM OA) were required (Fig. 5B). These effects were therefore consistent with the requirement for prolonged exposure with high dose OA to in order enhance the activities of the renaturable MBPKs.

**Signal transduction inhibitors fail to inhibit okadaic acid-stimulated protein kinases** To evaluate the signal transduction mechanisms that were recruited following prolonged exposure to OA, we preincubated cells with a variety of different inhibitors prior to exposure of Cos cells to 1 μM OA. One possible mechanism to account for the delayed activation of the OA-sensitive protein kinases could be that their activation requires the synthesis of a positive regulator of the signal transduction pathway, such as the synthesis of autocrine growth factors or even a protein kinase intermediate itself. We eliminated this possibility when we failed to observe significant inhibition of the OA activation of these protein kinases following the exposure of the Cos cells to the protein synthesis inhibitor, cycloheximide (20 μM) (results not shown). We have previously shown that this dose of cycloheximide is sufficient to prevent synthesis of MAPK phosphatases (Andersson *et al.*, 1998). Furthermore, we eliminated the possibility of activation following the synthesis and autocrine release of growth factors (such as those interacting with the Epidermal Growth Factor-, Fibroblast Growth Factor- and Platelet Derived Growth Factor-Receptors (Baird *et al.*, 1988; Stein, 1993)) by showing that suramin (600 μM) also failed to inhibit the OA-activation of these protein kinases (results not shown). We have shown that this concentration of suramin is sufficient to inhibit the heat-shock-induced activation of protein kinases (Ng *et al.*, 2000). It should be further noted that suramin, an analog of heparin sulfate, has been implicated as an inhibitor of a heterogeneous population of growth factor and cytokine receptors. It has also been shown to inhibit ERK MAPK activation by serum, Epidermal Growth Factor, Platelet-Derived Growth Factor, Tumor Necrosis Factor-α, as well as ERK MAPK activation by oxidative stress that is elicited by hydrogen peroxide and arsenite (Grazioli *et al.*, 1992; Stein, 1993; Guyton *et al.*, 1996; Chen *et al.*, 1998). Suramin was previously reported to inhibit a number of other intracellular regulators in signal transduction, including protein-tyrosine phosphatases, protein kinase C, Cdc2, phosphatidylinositol 3'kinase (Zhang *et al.*, 1998 and the references therein). Thus, our inability to inhibit

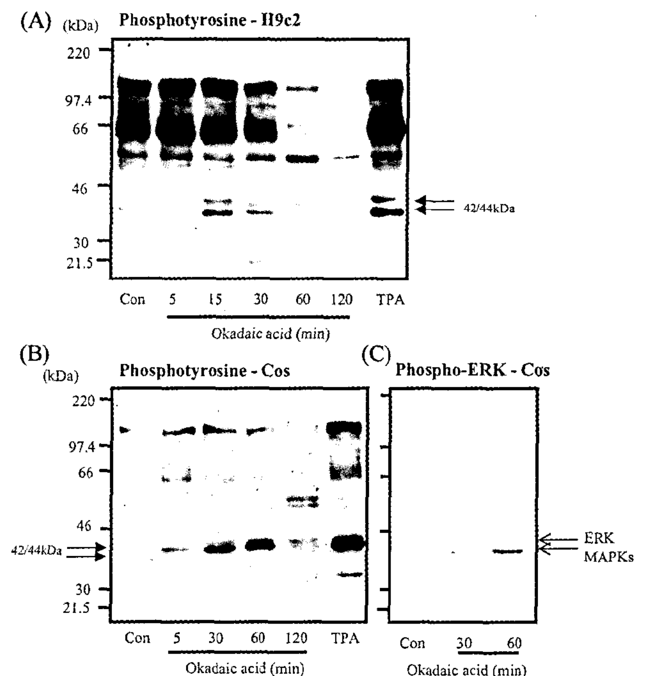


**Fig. 6.** Changes in phosphorylated tyrosine proteins upon okadaic acid treatment appear to coincide with changes in phosphorylated ERK MAPKs. The H9c2 cells (Panel (A)) or Cos cells (Panels (B) and (C)) were exposed to 1 μM okadaic acid for 5 to 120 min, or to 1 μM TPA for 5 min. In Panels (A) and (B) the cell lysates were immunoblotted for phosphotyrosine, and in Panel (C) for phospho-ERK MAPK, as described in the Materials and Methods. These experiments were repeated with similar results. The arrows in each panel indicate the positions of the ERK MAPKs (42 and 44 kDa).

the activities of MBPK-55, MBPK-62, MBPK-87, or MBPK-200 suggests that these events also do not lie upstream of kinase activation in our system.

To evaluate which protein kinases may regulate the activation of these OA-activated protein kinases, we then employed a range of different protein kinase inhibitors. We found that a general inhibitor of tyrosine kinases (genistein, 100 μM) failed to prevent the activation of the OA stimulated protein kinases. This is consistent with our failure to observe increases in tyrosine phosphorylation of proteins of approximately 55, 62, 87, or 200 kDa proteins, following the OA treatment of H9c2 cells (Fig. 6A) or Cos cells (Fig. 6B) for up to 2 h. Three important observations should also be noted. First, the basal level of tyrosine phosphorylation of the different cell types appears markedly different despite equivalent total protein loading, processing the immunoblots in parallel, and using equivalent lengths of exposure of these blots to film. Secondly, a significant decrease in tyrosine phosphorylation of many proteins appears to accompany prolonged (i.e. 2 h) stimulation with OA. This is especially prominent for the H9c2 cells, which begin with higher basal levels of tyrosine phosphorylation (Fig. 6A). Thirdly, there are

some tyrosine phosphorylated protein bands that appear following 15 or 30 min of either cell type to OA. These also appear to be observed following TPA exposure (Fig. 6A and 6B, denoted by arrows). The size of these proteins suggests that they may be the ERK MAPKs that are known to require phosphorylation on both the Thr and Tyr residues for activation. Indeed, when we probed the lysates from OA-treated Cos cells with the antibodies that were directed to phospho-ERK MAPK, we observed proteins of these sizes as expected (Fig. 6C).

We also found that an inhibitor of protein kinase C (Bisindolylmaleimide I, 1 μM) failed to prevent activation of the OA stimulated protein kinases (results not shown). When we examined the inhibitor of the ERK MAPK kinase (PD98059, 30 μM), we also found that it failed to prevent OA activation in any of the protein kinases, including the ERK MAPKs. This concentration of PD98059 is sufficient to inhibit ERK MAPK in response to a variety of growth factors, cytokine, or stress stimuli (Ng *et al.*, 2000; Ng *et al.*, 2001). This failure of PD98059 to inhibit the ERK MAPK activation in response to prolonged OA exposure suggests that the OA may be inhibiting the phosphatases that directly act on the ERK MAPKs. This is consistent with recent findings that protein phosphatases, such as PP2A, may indeed be tightly complexed with the protein kinases they regulate (Heriche *et al.*, 1997; Westphal *et al.*, 1998; and reviewed in Millward *et al.*, 1999; Westphal *et al.*, 1999).

## Discussion

Phosphorylation is a dynamic and reversible process that allows a cell to regulate the functions of its proteins. A protein's activity can be influenced by the net phosphorylation of critical amino acids. This is regulated by a balance of activities of the two classes of intracellular enzymes. Specifically, the protein kinases that catalyze the transfer of phosphate from ATP to specific serine, threonine, or tyrosine residues of that protein will lead to enhanced protein phosphorylation; whereas, the protein phosphatases that catalyze the removal of phosphate from the protein will lead to enhanced protein dephosphorylation. It is clear that there is likely to be approximately 500 different protein kinases that are encoded in the human genome (see www.kinase.com). Although there appears to be fewer phosphatases, the role of these phosphatases has generally been accepted as playing a major role in the regulation of signal transduction cascades (Charbonneau *et al.*, 1992; Shenolikar, 1994).

Of the protein phosphatases that dephosphorylate the serine or threonine residues of a protein, the overall importance of PP2A in regulating cellular functions is underscored by the observations that several organisms produce the PP2A-inhibiting toxins (Epand *et al.*, 1990). In this study we used one specific toxin, OA. This was originally isolated from marine sponges, and is now widely used in molecular cell biology to evaluate the contribution of PP2A to a variety of cellular functions (Epand *et al.*, 1990). We have previously shown that OA, when added at a final concentration of 1 μM, can lead to a delayed activation of a number of protein kinases in cultured heart ventricular myocytes (Andersson *et al.*, 1998). We have extended this study to show that this activation is also observable in a number of cell lines. Specifically, we have shown activation of 55 and 62 kDa protein kinases in H9c2 cells, and the activation of 38, 55, 62, and 87 kDa proteins in Cos cells (Fig. 2 and Fig. 3). In these cells, OA also activates the 42 and 44 kDa ERK MAPKs (Figs. 1-3 and Fig. 6). In contrast, we found that the human embryonic kidney 293 cell line was so sensitive to these high concentrations of OA that all 293 cells died before any activation of these protein kinases could be observed (results not shown). We have now also shown that activation of the kinases is only observed following exposure of Cos, or myocytes to 1 μM final concentrations of OA. The doses of OA of 0.1-0.5 μM are also poor activators of these kinases (Fig. 4). The use of this high concentration of OA has generally been accepted as required in order to affect a substantial inhibition of intracellular PP2A (Donaldson *et al.*, 1991; Casillas *et al.*, 1993; Rampal *et al.*, 1995; Favre *et al.*, 1997). However, we should point out that the lower doses of OA, including 10-500 nM, have also been used in previous studies (Morimoto *et al.*, 1997; Galigniana *et al.*, 1999). The marked difference in the concentration of OA that is required to elicit a biological or biochemical effect may very well depend on a number of variables, such as the level of expression and activity of PP2A in each cell type. Thus, when we examined the effects of OA to alter the total level of phosphothreonine-containing proteins in these cells, we confirmed that the high doses of OA are required (Fig. 5B). Also, long incubation times are required to see these effects (Fig. 5A).

One potential problem with this model that invokes the actions of PP2A could be the failure of calyculin A, a structurally different PP2A inhibitor, to lead to activation of the protein kinases of interest in this study (results not shown). However, this failure of calyculin A can be readily explained by the relative efficacy of inhibition in intact cells by calyculin A and OA, as shown in the study by Favre and colleagues (Favre *et al.*, 1997). Specifically, calyculin A at concentrations up to 100 nM can only affect up to 60% inhibition of PP2A activity in intact cells, such as the MCF7 breast cancer cell line (Favre *et al.*, 1997). In this same study, complete inhibition of PP2A activity could be readily demonstrated between a 30 and 60 min exposure to 1 μM OA (Favre *et al.*, 1997). We propose that the activation of the kinases, described in our study, may only be revealed when there is almost a complete inhibition of PP2A. Also, we can rule out a role for PP1, because 1 μM OA failed to inhibit PP1 in intact cells, but calyculin A inhibited PP1 by 80% (Favre *et al.*, 1997). Thus, the failure of calyculin A to permit activation of the protein kinases may reflect its relative inefficiency as a PP2A inhibitor in intact cells. Indeed, this suggestion appears to be

supported by the failure of non-toxic doses of calyculin A to alter the levels of phosphothreonine; whereas under the same conditions okadaic acid stimulated a range of proteins phosphorylated on threonine (Fig. 5A).

To further characterize the 55, 62, and 87 kDa protein kinases that are activated following exposure of cardiac myocytes to OA, we ensured that a structurally related OA derivative that is ineffective as a PP2A inhibitor failed to activate these kinases (results not shown). This helps to rule out the possibility that non-specific effects of OA contribute towards kinase activation in our study. Furthermore, we noted that the activation of the MBPKs precedes any visible sign of cell toxicity in response to OA, although cell death does eventually occur during exposure times of 2 or more hours. The differences in the time course of activation of the MBPKs (maximal at 30 to 60 min) and cell death (after 120 min) further suggest that cell death per se is not the initiator of MBPK activation.

We also evaluated the substrate specificity of these kinases by our in-gel assay protocol. We showed that the transcription factors c-Jun, Elk, ATF2, or c-Fos were all poor substrates for the 55, 62, and 87 kDa protein kinases. In contrast, c-Jun and ATF2 were efficient substrates for JNK MAPKs, and Elk was an efficient substrate for ERK MAPKs (Table 1). The protein kinase MAPKAPK2 could be efficiently phosphorylated by ERK MAPKs, but was not a substrate for the 55, 62, or 87 kDa protein kinases (Table 1). Instead we found that both the 55 and 62 kDa protein kinases could effectively phosphorylate the Histone IIIS substrate protein (Fig. 1B). Therefore, although the 55/62 kDa proteins and ERK MAPKs share the properties of renaturable kinase activity and the ability to utilize MBP as a substrate, they differ in their ability to utilize other substrates that have been commonly used to assess MAPK activation.

We also initiated studies to evaluate the signal transduction cascades that could culminate in the activation of the 55, 62, and 87 kDa renaturable protein kinases. We have shown that pretreatment with either cycloheximide (to inhibit protein synthesis) or suramin (to inhibit growth factor receptors) fails to attenuate the OA-elicited kinase activation (results not shown). This suggests that the delay in the activation of these kinases is not due to the OA-induced synthesis of a growth factor or other protein. Furthermore, Bisindolylmaleimide I (to inhibit protein kinase C) or genistein (to inhibit tyrosine kinases) were ineffective (results not shown). Of particular importance is the observation that the MEK inhibitor PD98059 is also without effect to attenuate the activation of either the 55, 62, or 87 kDa kinases, or indeed, the ERK MAPK activation following OA exposure. Taken together, we propose that our results support the recent data that PP2A will be found in a cell that is complexed with the proteins that it will regulate (Millward *et al.*, 1999). Our identification of the 55, 62, and 87 kDa protein kinases in the Cos cell line allows us to continue further purification from these readily cultured cells (Fig. 3) without the need for primary cultures of cardiac myocytes or fibroblasts.

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## References

Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M. and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr. Biol.* **5**, 283-295.

Andersson, M. B., Ketterman, A. J. and Bogoyevitch, M. A. (1998) Differential regulation of parallel mitogen-activated protein kinases in cardiac myocytes revealed by phosphatase inhibition. *Biochem. Biophys. Res. Commun.* **251**, 328-333.

Armstrong, S. C. and Ganote, C. E. (1992) Effects of the protein phosphatase inhibitors okadaic acid and calyculin A on metabolically inhibited and ischemic isolated myocytes. *J. Mol. Cell. Cardiol.* **24**, 869-884.

Baird, A., Schubert, D., Ling, N. and Guillemin, R. (1988) Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **85**, 2324-2328.

Bogoyevitch, M. A., Glennon, P. E., Andersson, M. B., Clerk, A., Lazou, A., Marshall, C. J., Parker, P. J. and Sugden, P. H. (1994) Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J. Biol. Chem.* **269**, 1110-1119.

Bogoyevitch, M. A., Clerk, A. and Sugden, P. H. (1995a) Activation of the mitogen-activated protein kinase cascade by pertussis toxin-sensitive and -insensitive pathways in cultured ventricular cardiomyocytes. *Biochem. J.* **309**, 437-443.

Bogoyevitch, M. A., Ketterman, A. J. and Sugden, P. H. (1995b) Cellular stresses differentially activate the c-Jun N-terminal protein kinases and the extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J. Biol. Chem.* **270**, 29710-29717.

Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J. and Sugden, P. H. (1996) Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal protein kinases are activated by ischemia/reperfusion. *Circ. Res.* **79**, 162-173.

Cano, E. and Mahadevan, L. C. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.* **20**, 117-122.

Casillas, A. M., Amaral, K., Chegini-Farahani, S. and Nel, A. E. (1993) Okadaic acid activates p42 mitogen-activated protein kinase (MAP kinase; ERK-2) in B-lymphocytes but inhibits rather than augments cellular proliferation: contrast with phorbol 12-myristate 13-acetate. *Biochem. J.* **290**, 545-550.

Charbonneau, H. and Tonks, N. K. (1992) 1002 protein phosphatases? *Ann. Rev. Cell Biol.* **8**, 463-493.

Chen, W., Martindale, J. L., Holbrook, N. J. and Liu, Y. (1998) Tumor promoter arsenite activates extracellular signal-regulated kinase through a signaling pathway mediated by epidermal growth factor receptor and Shc. *Mol. Cell. Biol.* **18**, 5178-5188.

Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990) Okadaic

acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**, 98-102.

Donaldson, R. W., Hagedorn, C. H. and Cohen, S. (1991) Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. *J. Biol. Chem.* **266**, 3162-3166.

Epand, R. M. and Lester, D. S. (1990) The role of membrane biophysical properties in the regulation of protein kinase C activity. *Trends Pharmacol. Sci.* **11**, 317-320.

Favre, B., Turowski, P. and Hemmings, B. A. (1997) Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin A, okadaic acid, and tautomycin. *J. Biol. Chem.* **272**, 13856-13863.

Galigniana, M. D., Housely, P. R., DeFranco, D. B. and Pratt, W. B. (1999) Inhibition of glucocorticoid receptor nucleocytoplasmic shuttling by okadaic acid requires intact cytoskeleton. *J. Biol. Chem.* **274**, 16222-16227.

Grazioli, L., Alzani, R., Ciomei, M., Mariani, M., Restivo, A., Cozzi, E. and Marcucci, F. (1992). *Int. J. Immunopharmacol.* **14**, 637-642.

Guyton, K. Z., Liu, Y. S., Gorospe, M., Xu, Q. B. and Holbrook, N. J. (1996) Activation of mitogen-activated protein kinase by $H_2O_2$-role in cell survival following oxidant injury. *J. Biol. Chem.* **271**, 4138-4142.

Heriche, J.-K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E. M. and Goldberg, Y. (1997) Regulation of protein phosphatase 2A by direct interaction with casein kinase 2a. *Science* **276**, 952-955.

Hunter, T. (1995) Protein kinases and phosphatases: The ying and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236.

Millward, T. A., Zolnierowicz, S. and Hemmings, B. A. (1999) Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* **24**, 186-191.

Morimoto, Y., Ohba, T., Kobayashi, S. and Haneji, T. (1997) The protein phosphatase inhibitors okadaic acid and calyculin A induce apoptosis in human osteoblastic cells. *Exp. Cell Res.* **230**, 181-186.

Ng, D. C. H. and Bogoyevitch, M. A. (2000) The mechanism of heat shock activation of ERK Mitogen-activated protein kinases in the interleukin 3-dependent ProB cell line BaF3. *J. Biol. Chem.* **275**, 40856-40866.

Ng, D. C. H., Long, C. S. and Bogoyevitch, M. A. (2001) A role for the extracellular signal-regulated kinase and p38 mitogen-activated protein kinases in Interleukin-1β-stimulated delayed signal tranducer and activator of transcription 3 activation, atrial natriuretic factor expression, and cardiac myocyte morphology. *J. Biol. Chem.* **276**, 29490-29498.

Rampal, A. L., Jhun, B. H., Kim, S., Liu, H., Manka, M., Lachaal, M., Spangler, R. A. and Jung, C. Y. (1995) Okadaic acid stimulates glucose transport in rat adipocytes by increasing the externalization rate constant of GLUT4 recycling. *J. Biol. Chem.* **270**, 3938-3943.

Shenolikar, S. (1994) Protein serine/threonine phosphatases-new avenues for cell regulation. *Annu. Rev. Cell Biol.* **10**, 55-86.

Stein, C. A. (1993) Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. *Cancer Res.* **53**, 2239-2248.

Weinbrenner, C., Baines, C. P., Liu, G,-S., Armstrong, S. C., Ganote, G. C. E., Walsh, A. H., Honkanen, R. E., Cohen, M. V. and Downey, J. M. (1998) Fostriecin, an inhibitor of protein phosphatase 2A, limits myocardial infarct size even when administered after onset of ischemia. *Circulation* **98**, 899-905.

Westphal, R. S., Anderson, K. A., Means, A. R. and Wadzinski, B. E. (1998) A signaling complex of $Ca^{2+}$-calmodulin-dependent protein kinase IV and protein phosphatase 2A. *Science* **280**, 1258-1261.

Westphal, R. S., Coffee, R. L., Marotta, A., Pelech, S. L. and Wadzinski, B. E. (1999) Identification of kinase-phosphatase signaling modules composed pf p70 S6kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J. Biol. Chem.* **274**, 687-692.

Wu, J., Lau, L. F. and Sturgill, T. W. (1994) Rapid deactivation of MAP kinase in PC12 cells occurs independently of induction of phosphatase MKP-1. *FEBS Lett.* **353**, 9-12.

Zhang, Y.-L., Keng, Y.-F., Zhao, Y., Wu, L. and Zhang, Z.-Y. (1998) Suramin is an active site-directed, reversible, and tight-binding inhibitor of protein-tyrosine phosphatases. *J. Biol. Chem.* **273**, 12281-12287.