Phosphorylation and activation of mitogen- and stress-activated protein kinase-1 in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires both extracellular-signal-regulated kinase and p38 mitogen-activated protein kinase

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G-protein-coupled receptor agonists are powerful stimulators of mitogen-activated protein kinase (MAPK) cascades in cardiac myocytes. However, little is known regarding the physiological activation of enzymes downstream of MAPKs. We examined the activation of mitogen- and stress-activated protein kinase-1 (MSK1), a downstream target of MAPKs, in adult rat cardiac myocytes by phenylephrine and endothelin-1. Both agonists induced the phosphorylation of MSK1 at Thr-581 and Ser-376 but not at Ser-360. Maximal phosphorylation was observed at 10–15 min after stimulation and it correlated with increased activity. Maximal activation of MSK1 in adult cardiomyocytes temporally coincided with maximal p38 MAPK activation while activation of the extracellular-signal-regulated kinase (ERK) cascade was more rapid. Phosphorylation and activation of MSK1 was completely inhibited by either PD98059 (ERK1/2 pathway inhibitor) or SB203580 (p38 MAPK inhibitor) alone. These data demonstrate that MSK1 activation in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires the simultaneous activation of both the ERK and p38 MAPK pathways. However, the lack of phosphorylation at Ser-360, an identified phosphorylation site targeted by MAPKs, may indicate that MSK1 is not a direct substrate of ERK1/2 and p38 MAPK in adult rat cardiomyocytes.

Key words: adrenergic agonist, endothelin-1.

INTRODUCTION

In cardiac myocytes, endothelin-1 (ET-1) and α_1-adrenergic agonists such as phenylephrine (PE) stimulate hypertrophic growth, inducing both the morphological and transcriptional changes associated with the response [1]. The signalling pathways utilized are probably manifold but mitogen-activated protein kinase (MAPK) pathways have been implicated in the regulation of this response [1,2]. MAPKs are a superfamily of proline-directed serine/threonine protein kinases that regulate the activities of transcription factors and other cell-signalling proteins [3–5]. These kinases are phosphorylated and activated by dual-specificity kinases that in turn are regulated by specific upstream kinases. Of the three best-characterized subfamilies, the extracellular-signal-regulated kinases (ERKs) are generally implicated in the regulation of growth responses of the cell, whereas the c-Jun N-terminal kinases (JNKs) and p38 MAPKs are more usually associated with cellular responses to stress [6,7]. The nature of these cascades allows considerable potential for signal integration and amplification as well as the possibility of cross-talk between pathways because of the apparent overlapping substrate specificities of some of the components and of the upstream signalling molecules.

All three MAPK subfamilies have been implicated in the regulation of cardiac myocyte hypertrophy but there is considerable debate as to which are physiologically relevant in this response [1,8]. Recent evidence also suggests that there may be additional effects on cell survival [9]. In neonatal rat ventricular myocytes, ET-1 and PE were shown to activate ERKs, JNKs and p38 MAPK [10–14]. In addition, PE activates all three MAPK subfamilies in the isolated perfused rat heart [15]. However, the regulation of MAPK cascades in adult rat cardiac cells has not been studied extensively. The extrapolation of the results from neonatal to adult cells is difficult, because there are marked phenotypic differences between neonatal and adult cardiac myocytes, including age-dependent changes in protein kinase expression [16] and α_1-adrenergic signalling [17]. In addition, alterations in ERK signalling between neonatal and adult cardiac myocytes in response to angiotensin II have been reported [18,19]. Therefore, identification of the cellular signalling events involved in MAPK activation and downstream targets in adult rat cardiac myocytes is important for understanding the *in vivo* changes in cardiac function.

Mitogen- and stress-activated protein kinase-1 (MSK1) is a previously identified enzyme that is present in various mammalian cell types [20]. MSK1 is activated *in vivo* by a vast variety of extracellular signals that include growth factors, phorbol esters, cell-damaging stimuli and pro-inflammatory cytokines. The reason for this is that MSK1 is activated *in vitro* and *in vivo* by a mechanism involving an interaction with either ERKs or p38 MAPK. MSK1 has been implicated in the regulation of transcriptional activation [20–22]. However, expression or activation of MSK1 has not yet been demonstrated in cardiac myocytes.

In the present study, we first characterized the activation of ERKs and p38 MAPK by hypertrophic G-protein-coupled receptor (GPCR) agonists, PE and ET-1, in adult rat cardiac myocytes. Furthermore, we investigated the effect of these agonists on MAPK signalling to MSK1. We show for the first

Abbreviations used: ERK, extracellular-signal-regulated kinase; ET-1, endothelin-1; GPCR, G-protein-coupled receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAPK1, MAPK-activated protein kinase-1; MSK1, mitogen- and stress-activated protein kinase-1; PE, phenylephrine; DTT, dithiothreitol; E64, trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane.

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time in cardiac myocytes that GPCR agonists activate MSK1 and that this effect requires activation of both the ERK and p38 MAPK pathways.

EXPERIMENTAL

Materials

PE, ET-1, D,L-propranolol, prazosin, DMSO, dithiothreitol (DTT), leupeptin, trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane (E64), PMSF and Protein G–agarose were obtained from Sigma (St. Louis, MO, U.S.A.). SB203580, PD98059, GF109203X and H89 were obtained from Calbiochem (La Jolla, CA, U.S.A.). [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (Glyfada, Greece). Bradford protein assay reagent was from Bio-Rad (Hercules, CA, U.S.A.). Nitrocellulose (0.45 μm) was obtained from Schleicher & Schuell (Keene, NH, U.S.A.). Rabbit polyclonal antibodies to total ERK1/2 and p38 MAPK, as well as antibodies specific for phosphorylated MSK1(Thr-581), MSK1(Ser-376) and MSK1(Ser-360), and the dually phosphorylated p38 MAPK were obtained from Cell Signalling (Beverly, MA, U.S.A.). A mouse monoclonal antibody to phosphorylated ERK1/2 was also from Cell Signalling. Antibody to total MSK1 and Crosstide peptide (Gly-Arg-Pro-Arg-Thr-Ser-Ser-Phe-Ala-Glu-Gly) were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Prestained molecular-mass markers were from New England Biolabs (Beverly, MA, U.S.A.). Western-blotting chemiluminencence reagent kit was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Secondary antibodies were from Dako (High Wycombe, Bucks., U.K.). X-OMAT AR film was purchased from Eastman Kodak Company (New York, NY, U.S.A.). General laboratory reagents were from Sigma or Merck (Darmstadt, Germany).

Isolation of cardiac myocytes

Ventricular cardiac myocytes were isolated from 200–250 g adult male Wistar rats by cardiac retrograde aortic perfusion as described previously [23], with minor modifications. Briefly, the hearts were perfused in a retrograde fashion for 5 min with Krebs–Henseleit medium (hereafter referred to as incubation medium) containing 25 mM $NaHCO_3$, 4.7 mM KCl, 118.5 mM NaCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 10 mM glucose and 5 μM added Ca^{2+} as $CaCl_2$. Perfusion continued for 40 min with incubation medium containing 0.5 mg/ml collagenase (CLS1; Biochrom KG) and 50 μM Ca^{2+}. The heart was gently dissociated through the bore of a large-tip pipette, followed by two decantations to separate dead cells. Cells were finally resuspended in incubation medium, in which added Ca^{2+} was gradually increased to 1 mM. Preparations were considered satisfactory only if the yield of rod-shaped cells was more than 70 %.

Cell treatment and lysis

Freshly isolated cardiomyocytes were incubated at 37 °C in incubation buffer containing agonists, inhibitors or vehicle for various times, lysed in ice-cold buffer containing 20 mM Hepes, pH 7.5, 20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4, 5 mM DTT, 10 mM benzamidine, 200 μM leupeptin, 10 μM E64, 300 μM PMSF and 1 % (v/v) Triton X-100, and extracted on ice for 30 min. Cell lysates were centrifuged (10000 ***g*** for 5 min at 4 °C) and the supernatants were boiled with 0.33 vol. of SDS sample buffer [0.33 M Tris/HCl, pH 6.8, 10 % (w/v) SDS, 13 % (v/v) glycerol, 133 mM DTT and 0.2 % (w/v) Bromophenol Blue]. Protein concentrations were determined using the Bio-Rad Bradford assay.

Immunoblot analysis

Proteins were separated by SDS/PAGE on 10 % (w/v; MAPKs) or 8 % (w/v; MSK1) acrylamide/0.275 % (w/v) bis-acrylamide slab gels and transferred electrophoretically on to nitrocellulose membranes (0.45 μm). Membranes were blocked with TBS-T [20 mM Tris/HCl, pH 7.5, 137 mM NaCl and 0.1 % (v/v) Tween 20] containing 5 % (w/v) non-fat milk powder for 30 min at room temperature, and then incubated with the appropriate antibody [1:1000 dilution in TBS-T containing 5 % (w/v) BSA] at 4 °C overnight. Proteins were detected with horseradish peroxidase-conjugated secondary antibody [1:5000 dilution in TBS-T containing 1 % (w/v) non-fat milk powder for 1 h at room temperature] and were visualized by enhanced chemiluminescence. Scanning densitometry was used for semi-quantitative analysis of the data.

Immunoprecipitation and assay of MSK1 activity

Myocytes were exposed to 100 μM PE with or without pretreatment with inhibitors and lysed in ice-cold buffer A [20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 10 % (v/v) glycerol, 100 mM KCl, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$, 0.05 % (v/v) mercaptoethanol, 10 mM β-glycerophosphate, 0.2 mM leupeptin, 0.01 mM E64, 5 mM DTT, 0.3 mM PMSF, 0.004 mM microcystin LR and 0.1 % (v/v) Triton X-100]. Extracts were centrifuged (10000 ***g*** for 5 min at 4 °C), supernatants were removed and protein content was determined using the Bio-Rad Bradford assay. Cell lysates (1 mg of protein) were incubated at 4 °C for 2 h on a shaking platform with 3 μg of MSK1 antibody (Upstate Biotechnology) coupled to 20 μl of Protein G–agarose. The immunoprecipitates were washed twice with 100 μl of buffer A and once with 100 μl of buffer B (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 10 mM $MgCl_2$, 3 mM DTT and 10 μM cAMP-dependent protein kinase inhibitor peptide). The immunoprecipitated MSK1 pellets were resuspended in 40 μl of buffer B containing 30 μM Crosstide peptide, 0.125 mM ATP and 2 μCi of [γ-^{32}P]ATP (5000 Ci/mmol). Assays were carried out for 15 min at 30 °C and reactions stopped by placing the tubes on ice. The samples were centrifuged (10000 ***g*** for 30 s at 4 °C) and 30 μl of supernatant was spotted on to P81 papers (Whatman International). The papers were washed three times in 0.75 mM orthophosphoric acid and the radioactivity incorparated into the peptide was measured by Cerenkov counting in a scintillation counter. One unit of activity was defined as the amount of enzyme that catalysed the phosphorylation of 1 nmol of peptide in 1 min.

Statistics

Data are presented as means $\pm$ S.E.M. from n independent experiments. Statistical analyses (ANOVA with Dunnet post test or two-tailed Student's t test where appropriate) were performed using Instat (Graph Pad Software, San Diego, CA, U.S.A.) with significance taken as being established at $P < 0.05$.

RESULTS

Activation of ERK1/2 and p38 MAPK by PE and ET-1

PE and ET-1 activate all three MAPK subfamilies in neonatal rat ventricular myocytes [10,13,14]. In addition, PE activates these kinases in the perfused adult rat heart [15], whereas the effect of ET-1 has not been tested in this setting. To examine the activation of ERKs and p38 MAPK by PE and ET-1 in adult rat cardiomyocytes, we determined the phosphorylation of these

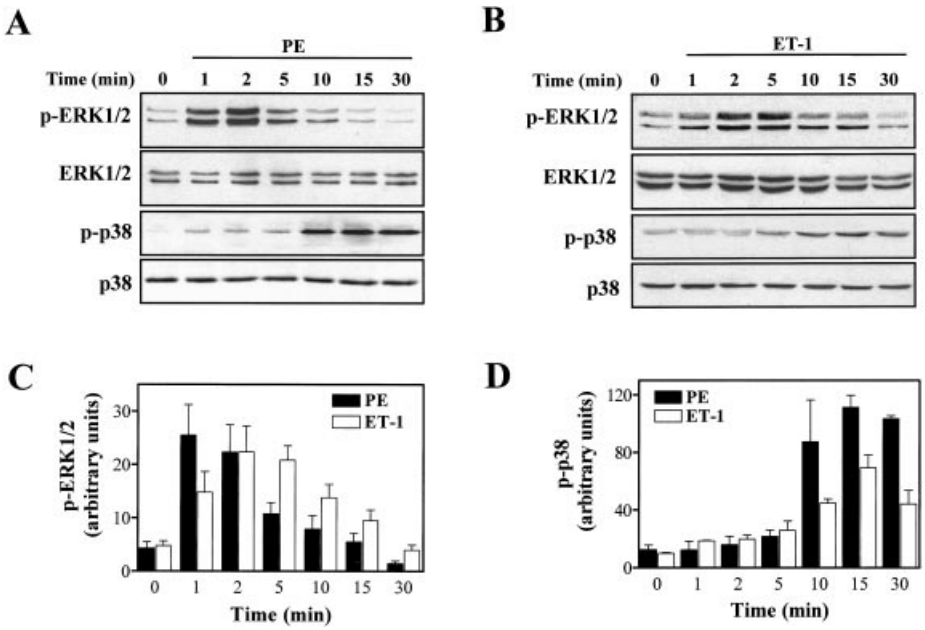

Figure 1 Phosphorylation of ERK1/2 and p38 MAPK by PE and ET-1 in adult rat cardiomyocytes

Cardiomyocytes were exposed to 100 μM PE (**A**) or 100 nM ET-1 (**B**) for the times indicated. Cell extracts (100 μg) were subjected to SDS/PAGE and immunoblotted with an antibody specific for phospho-ERK1/2 or phospho-p38 MAPK. Membranes were stripped and reprobed for total ERK1/2, or total p38 MAPK, as a control for equal protein loading. The experiments were repeated three times with comparable results. Immunoblotted phospho-ERK1/2 (**C**) and phospho-p38 MAPK (**D**) from cardiomyocytes exposed to PE (solid bars) or ET-1 (open bars) were quantified by laser scanning densitometry and plotted. The results are expressed as means $\pm$ S.E.M. from three independent experiments.

kinases by immunoblotting with antibodies specific for the dually phosphorylated forms of each of the kinases after exposure to agonists over a 1–30 min period. We confirmed the activation of ERK1/2 and p38 MAPK by PE. Similarly, ET-1 activated both kinases. Specifically, PE rapidly activated ERK1/2, stimulating a maximal response ($\approx$ 7.5-fold relative to controls) within 1 min, declining drastically after that and reaching basal levels by 15 min (Figures 1A and 1C). In comparison, ET-1-stimulated phosphorylation of ERK1/2 was also rapid, showing maximal stimulation at 2 min, and was equally robust ($\approx$ 7-fold relative to controls) but remained considerably elevated over 15 min (Figures 1B and 1C). p38 MAPK phosphorylation by PE ($\approx$ 9-fold compared with the controls) or ET-1 ($\approx$ 7-fold increase compared with the controls) was delayed compared with that of ERK1/2. A maximal increase in p38 MAPK phosphorylation was observed at 10–15 min, and this was sustained up to 30 min (Figures 1A, 1B and 1D). In all cases, equal protein loading was verified by reprobing the immunoblots with antibodies recognizing ERK1/2 or p38 MAPK independently of the phosphorylation state.

Further control experiments showed that, as expected, ERK1/2 phosphorylation by both agonists was inhibited by PD98059 and U0126 but not SB203580. PD98059 and U0126 had no effect on p38 MAPK phosphorylation (results not shown). In addition, we used GF109203X, which was originally developed as a protein kinase C inhibitor [24]. When cardiomyocytes were pre-exposed to 1 μM GF109203X, PE- or ET-1-stimulated ERK1/2 phosphorylation was abolished. However, at the same concentration GF109203X had no effect on p38 MAPK phosphorylation (Figure 2). In all cases, controls with the inhibitors alone were included. We confirmed equal loading of protein by reprobing the membranes with antibodies against total ERK1/2 or total p38 MAPK.

PE and ET-1 stimulate MSK1 phosphorylation

The MAPKs regulate a variety of different biological processes through their actions in the cell cytoplasm and nucleus. MSK1 has been identified previously as a downstream target of ERK and p38 MAPK pathways [20]. We therefore investigated whether the activation of MAPKs in response to GPCR agonists could also lead to the activation of MSK1. Exposure of cardiomyocytes to either PE or ET-1 resulted in increased phosphorylation of MSK1, as assessed by immunoblotting with an antibody specific for the kinase when phosphorylated at Thr-581. Maximal levels were attained at 10–15 min after exposure to agonists; thus enhanced phosphorylation of MSK1 coincides temporally with the activation of p38 MAPK (Figures 3A and 3C). MSK1 was activated to a somewhat lesser extent by ET-1 ($\approx$ 5.5-fold) compared with the activation induced by PE ($\approx$ 7.5-fold; Figures 3B and 3C). Equal protein loading was confirmed using an antibody against total MSK1. Similar results were obtained in additional experiments in which an antibody specific for MSK1 phosphorylated at Ser-376 was used (results not shown). However, we did not observe any phosphorylation when we used an antibody to the Ser-360 phosphorylated kinase. The PE-stimulated MSK1 phosphorylation was reversed by the α_1-adrenergic specific antagonist prazosin, whereas it was not affected by D,L-propranolol, a β-adrenergic-specific antagonist (Figure 3D).

PE- and ET-1-stimulated MSK1 phosphorylation is regulated by ERK and p38 MAPK

In other mammalian cells tested, MSK1 can be activated by either ERKs or p38 MAPK. We therefore used the ERK pathway inhibitor PD98059 and the p38 MAPK inhibitor SB203580 to assess the contribution of these MAPKs in the phosphorylation

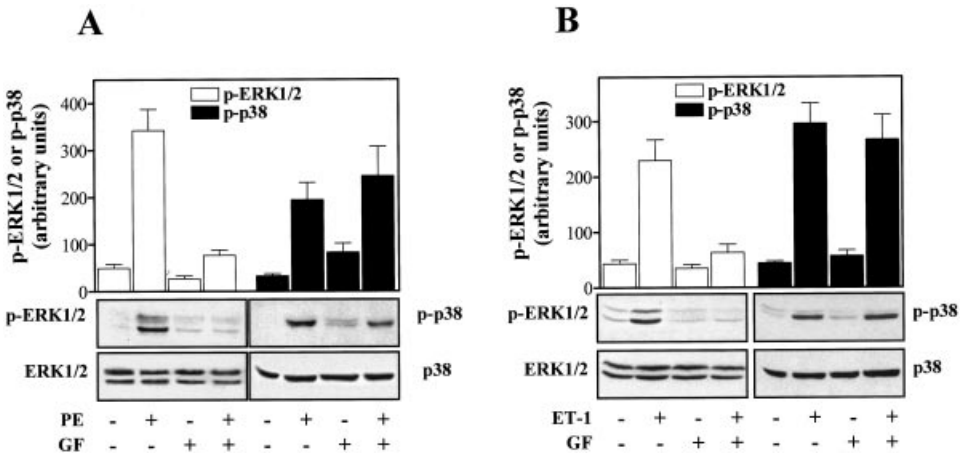

Figure 2 Effect of GF109203X on PE- and ET-1-induced phosphorylation of ERK1/2 and p38 MAPK

Cardiomyocytes were either not exposed to inhibitors (−) or pretreated for 10 min (+) with GF109203X (GF; 1 μM). Then they were incubated in the absence of agonists, or in the presence of 100 μM PE (**A**) or 100 nM ET-1 (**B**) for 15 min (p38 MAPK) or 2 min (ERK1/2). Cell extracts (100 μg) were immunoblotted for phosphorylated ERK1/2 or phosphorylated p38 MAPK. Membranes were stripped and reprobed for total ERK1/2, or total p38 MAPK, as a control for equal protein loading. These experiments were repeated three times with comparable results. ERK1/2 and p38 MAPK phosphorylation were analysed by laser scanning densitometry and plotted (**A** and **B**, top panels). The results are presented as means ± S.E.M. from three independent experiments.

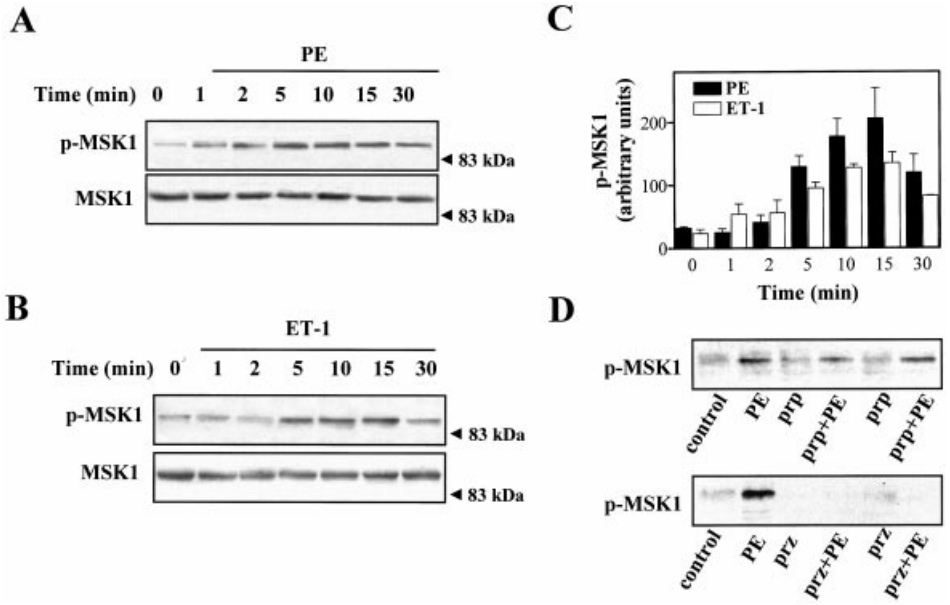

Figure 3 PE and ET-1 stimulate MSK1 phosphorylation

Cardiomyocytes were exposed to 100 μM PE (**A**) or 100 nM ET-1 (**B**) for the times indicated. Cell extracts (100 μg) were subjected to SDS/PAGE and immunoblotted with an antibody that detects MSK1 when phosphorylated at Thr-581 (**A** and **B**, top panels). An antibody against total MSK1 was used to verify equal protein loading (**A** and **B**, bottom panels). The molecular-mass markers are shown on the right. The data shown are representative of three independent experiments. (**C**) Phosphorylation of MSK1 by PE (solid bars) or ET-1 (open bars) was quantified by laser scanning densitometry and plotted. The results are expressed as means ± S.E.M. from three independent experiments. (**D**) Phosphorylation of MSK1 by PE (10 min) was completely inhibited by pretreatment with the α_1-adrenoceptor-selective antagonist prazosin (1 μM, 10 min; bottom panel) but not affected by D,L-propranolol (2 μM, 10 min; top panel). The data shown are representative of three independent experiments. prz, prazosin; prp, propranolol.

of MSK1 in adult rat ventricular myocytes. We also used the GF109203X, which effectively blocks ERK1/2 activation by PE or ET-1 but has no effect on p38 MAPK in our cell system (Figure 2). Phosphorylation of MSK1 by ET-1 and PE was completely prevented by PD98059 or SB203580 alone (Figures 4A and 4B). Similarly, prior treatment of cardiomyocytes with GF109203X resulted in the complete inhibition of MSK1 phosphorylation. Similar results were obtained when either the MSK1(Thr-581) or MSK1(Ser-376) antibody was used. These results imply a role for both ERK1/2 and p38 MAPK in regulating MSK1 phosphorylation by PE or ET-1. To test the possibility that the above effects were not due to a non-specific inhibitory effect, H89, which was originally developed as an inhibitor of cAMP-dependent protein kinase but which has been also shown to inhibit several other protein kinases [25], was added prior to exposure to ET-1 or PE. Incubation of cardio-

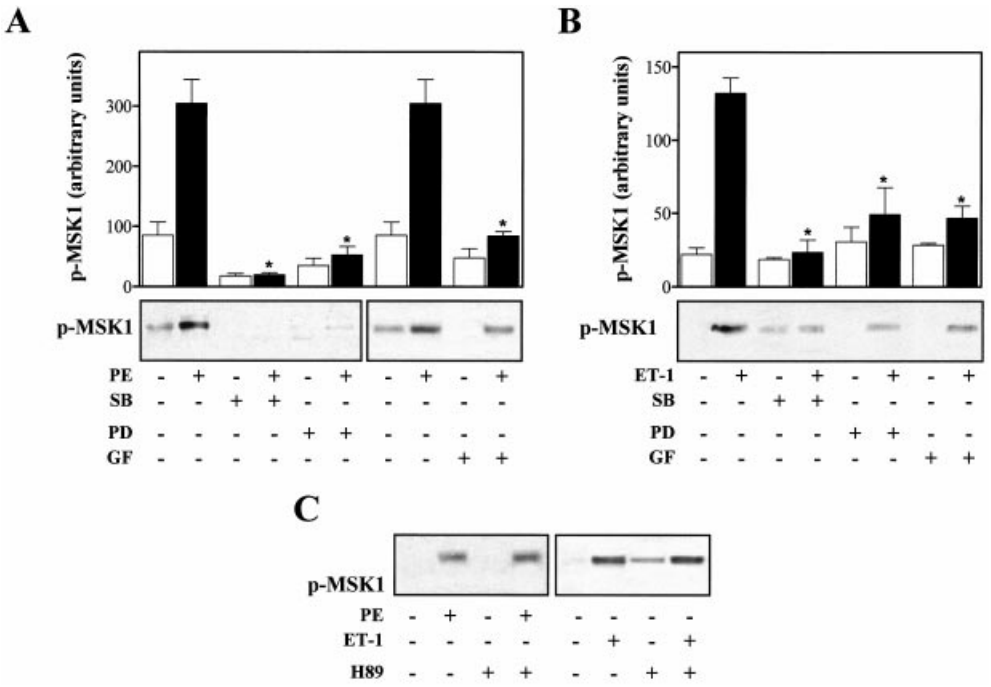

Figure 4 Inhibition of ERK1/2 or p38 MAPK attenuates MSK1 phosphorylation by PE or ET-1

Cardiomyocytes were either left untreated or preincubated with SB203580 (SB; 10 μM), PD98059 (PD; 10 μM) or GF109203X (GF; 1 μM). Then they were incubated in the absence of agonists (**A** and **B**, open bars) or in the presence of 100 μM PE (**A**, solid bars) or 100 nM ET-1 (**B**, solid bars) for 10 min. Proteins (100 μg) were separated by SDS/PAGE and phospho-MSK1-(-Thr-581) was detected by immunoblotting (**A** and **B**, bottom panels). This experiment was repeated three times with comparable results. Blots were quantified by laser scanning densitometry (**A** and **B**, top panels). The results are expressed as means $\pm$ S.E.M. from three independent experiments. Asterisks indicate significant inhibition of MSK1 phosphorylation ($P < 0.05$, ANOVA with Dunnet post test) compared with identically treated cells in the absence of inhibitors. (**C**) MSK1 phosphorylation by PE or ET-1 was not affected by pretreatment of cardiomyocytes with H89 (10 μM; 10 min). The data shown are representative of three independent experiments.

myocytes with 10 μM H89 had no effect on MSK1 phosphorylation (Figure 4C).

PE increases MSK1 activity through ERK and p38 MAPK pathways

To test whether the increased phosphorylation of MSK1 at Thr-581 and Ser-376 observed in adult cardiomyocytes in response to PE and ET-1 resulted in the activation of the kinase, MSK1 was immunoprecipitated and the activity was measured using Crosstide as a substrate. As shown in Figure 5(A), activation of the kinase followed a time course that paralleled that of the increase in phosphorylation, showing a maximum at 10–15 min after stimulation with PE. The activation of MSK1 was completely suppressed when the cardiomyocytes were incubated with PE in the presence of either PD98059 or SB203580 (Figure 5B). The addition of both inhibitors did not reduce the activity further.

DISCUSSION

This study elucidates further the signal-transduction pathways involved in GPCR-induced activation of MAPKs in cardiac myocytes by focusing on a target downstream of ERK1/2 and p38 MAPK. We show that MSK1 is present in adult rat cardiomyocytes and it is activated in response to hypertrophic GPCR agonists. The phosphorylation and activation of MSK1 is inhibited by drugs that prevent activation of ERK1/2 (PD98059, GF109203X) or p38 MAPK (SB203580). These observations suggest that both pathways are required for the activation of MSK1.

The regulation of MAPK pathways by GPCR agonists such as ET-1 and PE has been studied extensively and characterized in primary cultures of neonatal rat ventricular myocytes [10,13,14]. These studies have reported that GPCR agonists activate all three of the well-characterized MAPK subfamilies (ERK1/2, p38 MAPK and JNKs). These kinases have been implicated in the transcriptional changes associated with the hypertrophic response, although evidence also suggests that they may have a role in cell survival [1,2]. Although less extensively studied, the activation of ERK1/2, p38 MAPK and JNKs by the α_1-adrenergic agonist PE has been also shown in intact perfused rat heart [15]. Here we confirmed that the GPCR agonists PE and ET-1 stimulate both ERK1/2 and p38 MAPK in adult rat cardiac myocytes (Figure 1). The temporal pattern of activation of the two kinases was different. It is of interest that the phosphorylation of ERK1/2 by PE was very rapid and transient. The ET-1-induced ERK1/2 phosphorylation was more sustained, showing a maximum at 2–5 min, and it followed more closely the typical time course of ERK activation by agonists and growth factors observed in neonatal cardiomyocytes [13,26]. However, in primary cultures of adult rat cardiomyocytes, noradrenaline-stimulated ERK1/2 activation persisted for at least 48 h [27]. The discrepancy between our results and the latter study may reflect differences related to the different cell models. On the other hand, p38 MAPK phosphorylation was slow, requiring 10–15 min to reach maximal levels, and it remained elevated for at least 30 min (Figures 1A, 1B and 1D). These

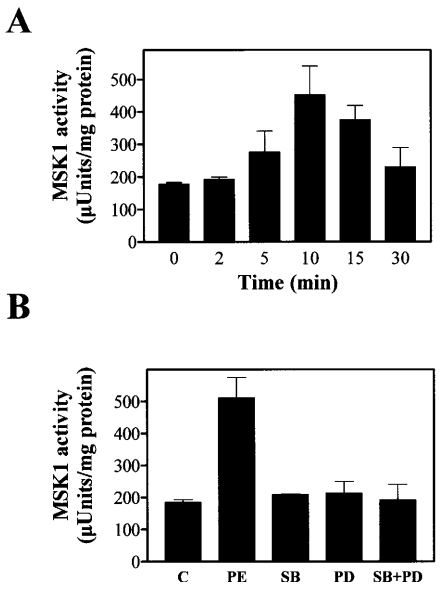

Figure 5 PE increases MSK1 activity

(**A**) Cardiomyocytes were exposed to 100 μM PE for the times indicated. After cell lysis, MSK1 was immunoprecipitated and assayed as described in the Experimental section. The data are expressed as means $\pm$ S.E.M. from three independent experiments. (**B**) Cardiomyocytes were pretreated for 10 min with SB203580 (SB; 10 μM), PD98059 (PD; 50 μM) or both together. They were then stimulated with 100 μM PE for 10 min, in the continued presence of inhibitors. MSK1 was immunoprecipitated and assayed. The data are presented as means $\pm$ S.E.M. from three independent experiments.

results are consistent with the more sustained p38 MAPK activation (maximal at 10 min) reported in the neonatal ventricular myocytes and perfused rat heart [14,15]. It is of note that PE strongly activates ERK1/2 and p38 MAPK in adult cardiomyocytes, comparable with ET-1 (Figure 1), whereas PE is considered to be a less powerful activator of these pathways in neonatal cardiomyocytes [1,2]. This may be due to developmentally related changes in α_1-adrenoceptor physiology in rat cardiac myocytes. The use of GF109203X inhibitor revealed that, in adult cardiomyocytes, phosphorylation of p38 MAPK was not affected at concentrations that completely abolished ERK1/2 phosphorylation (Figure 2). This is in contrast to the situation in neonatal cardiac myocytes where activation of p38 MAPK by ET-1 was inhibited by GF109203X [14]. GF109203X was originally developed as a specific inhibitor of protein kinase C [24] but this has been argued against in recent studies [25]. However, in the context of the present study, it should be noted that GF109203X has a differential effect on ERK1/2 and p38 MAPK activation.

To date, little is known of the regulation of the downstream molecular targets of ERK1/2 and p38 MAPK in the adult heart. A new MAPK target protein MSK1 has been identified [20]. In HEK-293 cells, MSK1 is activated in an ERK-dependent manner by stimulation with phorbol esters or epidermal growth factor, whereas activation induced by UV irradiation, oxidative stress and other cell-damaging stimuli is mediated through p38 MAPK [20]. These results suggest that either ERK or p38 MAPK activity are sufficient for activation of MSK1. Here we provide evidence that GPCR agonists phosphorylate and activate MSK1 in adult rat cardiomyocytes (Figures 3 and 5). MSK1, like the closely related MAPK-activated protein kinase-1 (MAPKAPK1) isoforms (also known as RSK), is unusual in that it contains two distinct protein kinase domains within a single polypeptide. The four regulatory phosphorylation sites identified in MAPKAPK1 are conserved in MSK1, suggesting a similar mechanism of activation where the C-terminal domain is essential for the activation of the N-terminal domain, which phosphorylates exogenous substrates [20,28]. By analogy to MAPKAPK1, two of the four phosphorylation sites, Thr-581 in the C-terminal domain and Ser-360, which is located between the two kinase domains, are phosphorylated by MAPKs. The phosphorylation of Thr-581 activates the C-terminal kinase domain, allowing it to phosphorylate Ser-376. The combined phosphorylation of Ser-360 and Ser-376 appears to be critical for the activation of the N-terminal domain, which may then enhance phosphorylation of Ser-212 [20,28]. Using antibodies against MSK1 phosphorylated at Thr-581, Ser-376 or Ser-360, we observed increased phosphorylation in response to stimulation of cardiomyocytes with PE or ET-1 at Thr-581 and Ser-376 but not at Ser-360. Given the above-described mechanism of activation, both Ser-360 and Ser-376 should be critical for the activation of the N-terminal kinase of MSK1. However, increased phosphorylation of only Thr-581 and Ser-376, which was observed in response to PE in cardiomyocytes, resulted in the activation of the kinase (Figures 3 and 5). Nevertheless, the observed activation may not represent full activity. It cannot be excluded that another site, which we have not detected, is also required for full activation of the MSK1 isoform in cardiomyocytes.

Maximal activation of MSK1 in adult cardiomyocytes temporally coincides with maximal p38 MAPK activation, whereas activation of the ERK cascade is more rapid. Thus it would be expected that p38 MAPK is more likely to be the upstream kinase responsible for MSK1 phosphorylation. However, at 10 min, when the maximal activation of MSK1 is observed, the agonist-stimulated ERK1/2 phosphorylation is still increased over 2-fold compared with the unstimulated cells, and this could still contribute to MSK1 phosphorylation. It should be noted that, considering only the temporal profiles of the phosphorylation of the three kinases, it is difficult to assess the contribution of each of the MAPKs to the phosphorylation and activation of MSK1 because it is unclear how the kinetics of ERK1/2 and p38 MAPK phosphorylation interact and coordinate MSK1 activation. One factor that should be taken into account is the potentially differing compartmentalization of the three kinases.

PD98059 and SB203580 independently and completely inhibited PE- and ET-1-induced MSK1 phosphorylation and activation (Figures 4A, 4B and 5B), implying that MSK1 is a substrate of both ERK1/2 and p38 MAPK. Furthermore, these results suggest that both ERK1/2 and p38 MAPK are required for MSK1 activation in adult cardiomyocytes in response to GPCR agonists and contradict published data in other cell systems where simultaneous inhibition of ERK1/2 and p38 MAPK activity is required to fully inhibit MSK1 activation in response to growth factors [20,22]. Further support for the requirement of both ERK and p38 MAPK pathways for MSK1 activation can be obtained from the use of GF109203X. GF109203X, which inhibits ERK1/2 but not p38 MAPK in adult cardiomyocytes (Figure 2), abolished phosphorylation of MSK1. However, these results should be interpreted with caution since GF109203X may have inhibited other kinases required for MSK1 phosphorylation in cardiomyocytes. Considering the inhibition data together, with the fact that MSK1 is not phosphorylated at Ser-360, we cannot exclude the possibility that MSK1 is not a direct substrate of ERK1/2 and p38 MAPK in adult rat cardiac myocytes.

In conclusion, we provide evidence demonstrating for the first time in cardiac myocytes that MSK1 is phosphorylated at Thr-581 and Ser-376, activated by hypertrophic GPCR agonists and that this activation requires the simultaneous activation of both the ERK and p38 MAPK pathways. The physiological role of activated MSK1 in cardiac myocytes is unknown. In other cell types, activated MSK1 has been shown to phosphorylate nucleosomal components, such as histone H3/HMG-14 [29], as well as transcription factors, such as cAMP-response-element-binding protein and activating transcription factor 1 [20–22], indicating that it may play a role in the regulation of gene expression. This issue has not been addressed in the present study and will require further investigation. In this regard, several studies have suggested cAMP-response-element-binding protein as an important regulator of gene expression in cardiomyocytes, which has possible relevance to the pathophysiology of the heart [30,31].

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