Regulation of the phosphorylation of elongation factor 2 by MEK-dependent signalling in adult rat cardiomyocytes

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Abstract The Gq-coupled agonists phenylephrine and endothelin-1 each activate protein synthesis in cardiomyocytes as part of the programme that leads to cardiac hypertrophy. Here we show that they each induce the dephosphorylation of elongation factor (eEF) 2, a protein that in its dephosphorylated state mediates the translocation step of elongation. The ability of both agonists to induce dephosphorylation of eEF2 requires signalling via the mTOR and MEK/Erk signalling pathways, but is independent of phosphoinositide 3-kinase. Expression of an activated form of MEK leads to dephosphorylation of eEF2, in an mTOR independent manner, indicating that signalling via MEK/Erk suffices to cause dephosphorylation of eEF2.

Key words: Translation; Elongation; eEF2; MEK; Cardiomyocyte; Hypertrophy

1. Introduction

Eukaryotic elongation factor 2 (eEF2) mediates the translocation step of peptide-chain elongation in the cytoplasm of mammalian cells. This step involves the co-ordinated movement of the ribosome relative to the mRNA by three nucleotides and occurs every time a new amino acid has been added to the nascent polypeptide chain. eEF2 is a phosphoprotein [1,2]: phosphorylation at threonine 56 prevents eEF2 from binding to ribosomes and thus inhibits its activity [3–5]. The kinase that phosphorylates eEF2 is an unusual and highly specific one. It belongs to a small group of protein kinases whose primary sequences are unrelated to main serine/threonine/tyrosine kinase superfamily [6,7]. Its only known substrate is eEF2 and it is therefore known as eEF2 kinase. This is a calcium/calmodulin (CaM)-dependent enzyme [2,8] and was formerly called Ca/CaM-kinase III [1,2].

Insulin brings about the dephosphorylation of eEF2 in several cell types [9–12] and this effect can be blocked by rapamycin, a compound that inhibits the function of the mammalian target of rapamycin, mTOR. Recent work has shown that p70 S6 kinase 1 (S6K1), a target of the mTOR pathway, phosphorylates eEF2 kinase (at Ser365, rat), resulting in decreased activity of eEF2 kinase [11]. S6K1 is activated by insulin in a rapamycin-sensitive manner (for review see [13]). It thus provides a link between insulin, mTOR signalling and the inactivation of eEF2 kinase leading to the stimulation of eEF2 and of peptide-chain elongation.

Ser365 in eEF2 kinase is also phosphorylated by 90-kDa ribosomal protein S6 kinase ($p90^{RSK}$), a protein kinase that lies downstream of the classical Erk (MAP kinase) pathway [11]. This could allow stimuli that activate Erk signalling to turn on peptide-chain elongation. However, very little work has been carried out to establish whether MEK/Erk signalling does regulate eEF2 phosphorylation in response to physiological agonists and it is also unclear whether MEK/Erk/$p90^{RSK}$ is sufficient to cause dephosphorylation of eEF2 in response to such stimuli. Here we have studied the effects of Gq-protein coupled receptor agonists (phenylephrine, PE, and endothelin-1, ET-1) on the phosphorylation of eEF2 in adult rat cardiomyocytes. These agonists are of particular interest because they stimulate protein synthesis and induce hypertrophy of ventricular cardiomyocytes (see [14,15]). This clinically important condition is primarily characterised by inappropriate stimulation of protein accumulation, leading to increased cell size which underlies cardiac hypertrophy. Another agent that causes cardiac hypertrophy, angiotensin II, has been reported to induce dephosphorylation of eEF2 through mechanisms that require signalling via MEK and phosphoinositide (PI) 3-kinase [16]. However, PE and ET-1 stimulate cardiac protein synthesis without activating signalling through PI 3-kinase [17], although they do activate the MEK/Erk pathway [18]. Increasing evidence points to a key role for MEK/Erk signalling in the induction of hypertrophy by PE and ET-1 [19–21]. Two key questions that we have addressed here is whether PE and ET-1 regulate eEF2 phosphorylation via MEK-dependent mechanisms and, if so, whether activation of this pathway is sufficient for dephosphorylation of eEF2.

2. Materials and methods

2.1. Chemicals, biochemicals and other reagents

These were obtained as described earlier [18] with the following additions. The anti-phospho (Thr56)eEF2 and anti-eEF2 antibodies were described earlier [22,23]. Antisera for S6K1 and $p90^{RSK}$ were kindly provided by the Division of Signal Transduction Therapy, University of Dundee. Wortmannin was from Calbiochem (Nottingham, UK).

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Abbreviations: ARVC, adult rat ventricular cardiomyocyte; eEF2, eukaryotic elongation factor 2; Erk, extracellular ligand-regulated kinase; ET-1, endothelin-1; MEK, mitogen-activated protein-ERK kinase; $p90^{RSK}$, 90-kDa ribosomal protein S6 kinase; PE, phenylephrine; S6K1, ribosomal protein S6 kinase 1

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2.2. Isolation, virus infection, culture, treatment and extraction of cardiac myocytes

Ventricular myocytes were isolated by collagenase digestion from adult rat hearts as described previously [10]. Cells were plated in dishes pretreated with laminin and cultured overnight in 199 medium [10]. Virus infection using adenovirus encoding an activated form of MEK (MEK^{CA}; from Dr S. Tanaka, Tokyo, Japan [24], in which key regulatory serine residues in the catalytic domain of MEK are mutated to glutamates) was performed as described earlier [18]. The control adenovirus expressing β-galactosidase was kindly provided by Professor T. Kuroki (Tokyo, Japan [25]). Following virus infection, cells were given fresh medium and incubated for a further 36 h before treatment. Cells were lysed (50 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_3, 50 mM NaF, 5 mM sodium pyrophosphate, 270 mM sucrose, 1% (v/v) Triton X-100, 1 mM DTT, 1 μM microcystin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, and 0.2 mM phenylmethylsulphonyl fluoride) and extracts were prepared as described earlier [10]. Protein concentrations were determined by the method of Bradford [26].

2.3. Gel electrophoresis and western blotting

These procedures were performed as described earlier [18,27] using Immobilon® membrane. Blots were developed by enhanced chemiluminescence (ECL).

2.4. Other procedures

S6K1 was assayed after immunoprecipitation using a 32-residue peptide substrate as described earlier [28]. $p90^{RSK}$ activity was assayed after immunoprecipitating the enzyme from 150 μg of adult rat ventricular cardiomyocyte (ARVC) cell lysates with anti-$p90^{RSK}$ antibody (from DSTT, Dundee). Immunoprecipitates were washed once with cell lysis buffer containing 0.5M NaCl, once with cell lysis buffer, once with kinase buffer (50 mM MOPS, pH7.5, 0.1 mM EDTA, 0.03% (w/v) Brij 35, 0.1% (v/v) β-mercaptoethanol), and were assayed in this kinase buffer containing 5 μM PKI, 10 mM $MgCl_2$/100 μM ATP and 2 μCi [γ-^{32}P]ATP at 30°C for 20 min, using Crosstide (GRPRTSSFAEG, 50 μM, from Dr. D.R. Alessi, Dundee) as substrate.

3. Results and discussion

3.1. PE and ET-1 elicit the dephosphorylation of eEF2 in ARVC

As shown in Fig. 1, treatment of ARVC with either PE or ET-1 resulted in the dephosphorylation of eEF2 as determined using a phosphospecific antibody that only detects eEF2 when it is phosphorylated at Thr56 [22]. Separate immunoblots with an antibody that detects eEF2 irrespective of its state of phosphorylation demonstrated equal loading of all lanes. In each case, dephosphorylation was already evident after 5 min of treatment and further dephosphorylation was seen up to 30–60 min following addition of agonist, after which phosphorylation increased. In the case of ET-1 treatment, phosphorylation had returned to initial levels by 120 min. In the case of PE, phosphorylation was higher at 120 min than at 30/60 but was still below the control levels seen at time zero.

Given that the effects of PE were greater than those of ET-1, most subsequent work focused on the effects of PE. To assess the signalling pathways that might mediate the effects of this agent on eEF2 phosphorylation, we studied the activities of S6K1 and $p90^{RSK}$ in cells treated with PE. Both enzymes can phosphorylate and inactivate eEF2 kinase, at least in vitro [11], and are therefore candidates for mediating the effect of PE on the phosphorylation of eEF2.

3.2. PE activates $p90^{RSK}$ and S6K1 with distinct kinetics

As shown in Fig. 2A, PE elicited a rapid and marked activation of $p90^{RSK}$. Activity peaked by 5 min and then fell gradually, although it was still sustained well above basal levels by 120 min. In contrast, activation of S6K1 was slower (reaching a maximum only after 60 min; Fig. 2B), as reported earlier by us for the activation of this enzyme [17] and of S6K2 [18] by PE in these cells. As shown in Fig. 1A, eEF2 phosphorylation had already decreased by 5 min, a time at which there is no significant activation of S6K1 (or S6K2 [18]). It is therefore most unlikely that phosphorylation of eEF2 kinase by S6K1/2 is responsible for this early fall in eEF2 phosphorylation. Activation of S6K1 by ET-1 in ARVC also occurs after a similar lag period to that seen in response to PE, but reaches a peak earlier at around 30 min [17]. This may, at least in part, explain the different kinetics observed here for the effects of ET-1 and PE on the phosphorylation of eEF2.

3.3. Dephosphorylation of eEF2 is blocked by inhibitors of MEK or mTOR

To study the upstream signalling pathways that mediate the dephosphorylation of eEF2 in response to PE or ET-1, we selected a time point at which dephosphorylation was maximal. This was 1 h for PE and 30 min for ET-1. Fig. 3A,B shows that the dephosphorylation of eEF2 induced by PE or ET-1 was blocked by treatment of cells with either rapamycin or PD098059, indicating that signalling via both mTOR and MEK is required for this effect. The dephosphorylation of eEF2 in response to PE or ET-1 was also blocked by two other structurally unrelated inhibitors of MEK, U0126 [29] and PD184352 [30] (data not shown), strongly supporting the idea that signalling through MEK is essential for this effect. These observations are not consistent with an ability of $p90^{RSK}$ alone to bring about the dephosphorylation of eEF2, via phosphorylation and inactivation of eEF2 kinase [11]. If this were the case, one would not expect rapamycin to block the effect (as it does not impair activation of the Erk pathway). In fact, we have shown earlier that MEK signalling is required for activation of S6K1 [17] or S6K2 [18] by PE or ET-1. This likely explains why the MEK inhibitors block the dephosphorylation of eEF2, since they will prevent the activation of both $p90^{RSK}$ and the S6 kinases by PE/ET-1.

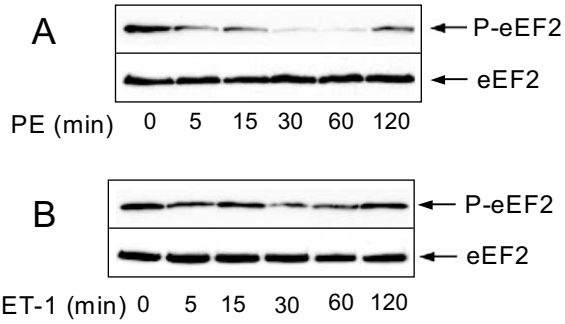

Fig. 1. PE and ET-1 induce dephosphorylation of eEF2 in adult cardiomyocytes. A,B: ARVC were treated with PE (10 μM) or ET-1 (0.1 μM) for the times indicated and cells were then lysed and equal amounts of cell extract protein were analysed by SDS–PAGE and Western blotting, using antisera that recognise eEF2 only when it is phosphorylated at Thr56 (upper part of each panel, indicated P-eEF2) or eEF2, irrespective of its state of phosphorylation (lower part of each panel, eEF2). '0' indicates untreated cells. All experiments were performed at least three times with similar results.

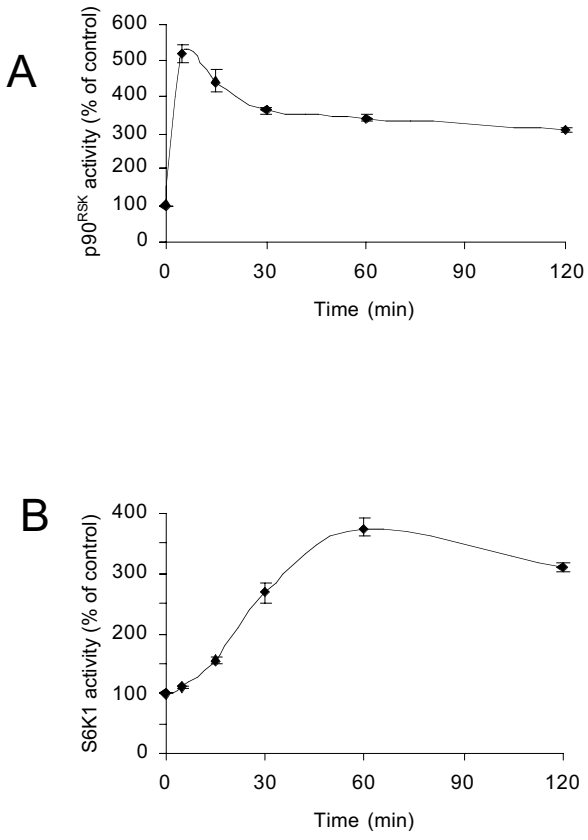

Fig. 2. Time courses of activation of $p90^{RSK}$ and S6K1 by PE in ARVC. ARVC were treated with PE (10 μM) for the times indicated and cells were then lysed and equal amounts of cell extract protein were subjected to immunoprecipitation with anti-$p90^{RSK}$ (A) or anti-S6K1 (B). Immunoprecipitates were assayed for kinase activity as described in the methods. Data are mean ± S.D. ($n = 6$; i.e. data are from six experiments, each using a different batch of cardiomyocytes).

It remained possible that $p90^{RSK}$ might contribute to the dephosphorylation of eEF2, especially at earlier times where its activation is maximal (Fig. 2A). To test this idea, we tested the effects of PD098059 and rapamycin on the ability of PE to induce the dephosphorylation of eEF2 at an early time point, where $p90^{RSK}$ activation is maximal and S6K1 activation has not yet occurred (5 min). At this time, the decrease in eEF2 phosphorylation is of course smaller, but is significant and reproducible. As shown in Fig. 3C, PD098059 blocked the dephosphorylation of eEF2 at this time without affecting its levels in control cells. This indicates that MEK/Erk signalling contributes to dephosphorylation of eEF2 even at an early time prior to activation of S6K1: this could indicate that activation of $p90^{RSK}$ is enough to induce the dephosphorylation of eEF2 and is studied further below. Surprisingly, rapamycin also blocked the PE-induced dephosphorylation of eEF2 at 5 min, a time when S6K1 (Fig. 2B) and S6K2 [18] are not activated.

One possible explanation for this is that the effect of rapamycin on the phosphorylation of eEF2 also involves an input from protein phosphatase activity. The major protein phosphatase acting on mammalian eEF2 is thought to be PP2A [31,32]. PP2A can interact with the regulatory protein α4 [33], which is related to the protein Tap42p, which is implicated in the control of translation by TOR in yeast [34]. Indeed, there is evidence that α4 modulates the phosphorylation of eEF2 via its interaction with PP2A [35]. It is clearly possible that the effect of rapamycin of eEF2 phosphorylation involves inputs from PP2A/α4 as well as from the regulation of eEF2 kinase by S6K. Further analysis of this lies beyond the scope of this study.

3.4. Dephosphorylation of eEF2 in response to ET-1 or PE does not involve signalling via PI 3-kinase

Earlier data showed that the dephosphorylation of eEF2 caused by insulin or angiotensin was blocked by inhibition of PI 3-kinase [9,16]. To study whether PI 3-kinase is involved in the effects of PE and ET-1 on the phosphorylation of eEF2, we treated ARVC with wortmannin prior to challenging them with PE or ET-1. We previously showed that the doses of wortmannin used here block PI 3-kinase signalling (it prevents the activation of protein kinase B induced by insulin [17]). Over a 100-fold range of concentrations, wortmannin had no effect on the ability of PE or ET-1 to elicit the dephosphorylation of eEF2 (Fig. 4A,B). Thus, PE and ET-1 regulate eEF2 phosphorylation by mechanisms that do not require PI 3-kinase. This is consistent with a role of S6K1 in these effects as wortmannin does not block its activation by PE [17] and ET-1 (data not shown).

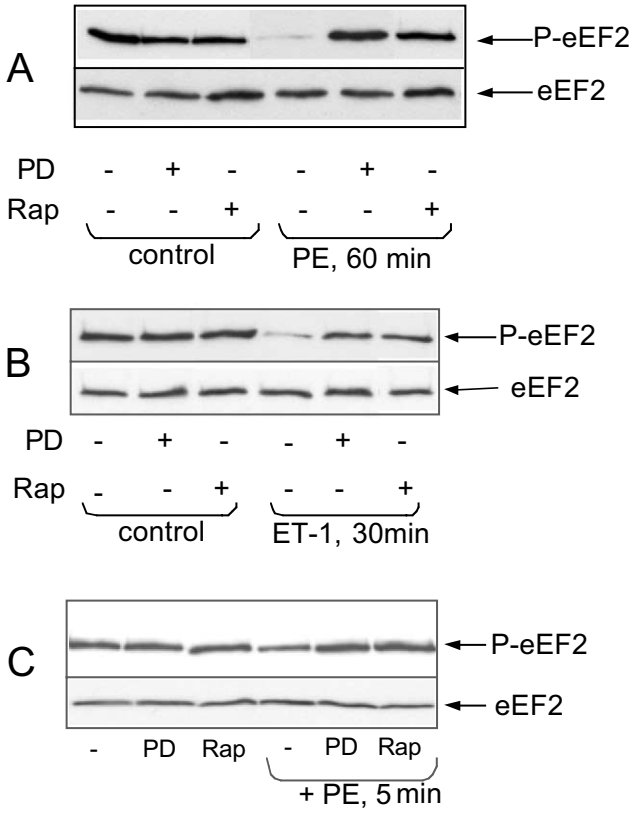

Fig. 3. Dephosphorylation of eEF2 in response to PE or ET-1 is blocked by rapamycin or PD098059. ARVC were treated with PE (10 μM; panels A,C), ET-1 (0.1 μM; panel B) or vehicle (MeOH) for the indicated times in the presence or absence of PD098059 (50 μM, added 45 min before agonist) or rapamycin (100 nM, added 30 min before agonist). Samples were then analysed as described for Fig. 1. These experiments were performed at least three times with similar results.

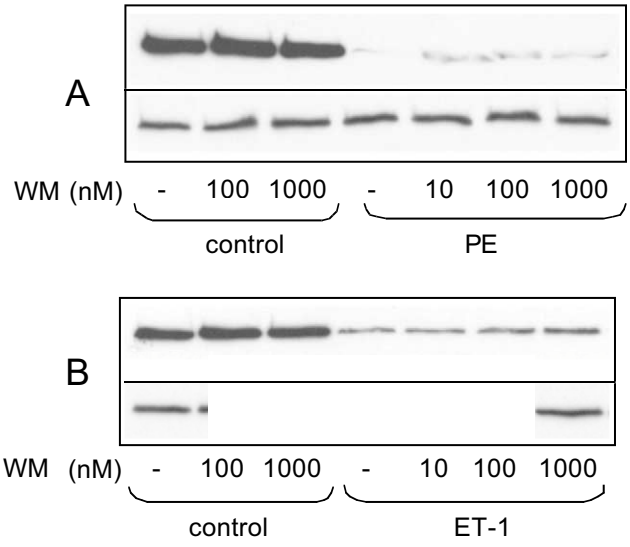

Fig. 4. Wortmannin does not block the dephosphorylation of eEF2 by PE or ET-1. ARVC were treated with PE (10 μM; 60 min; panel A) or ET-1 (0.1 μM; 30 min; panel B) in the presence or absence of wortmannin (WM; concentrations indicated; added 15 min before agonist and again 15 min after agonist as this compound is unstable in aqueous solution). Samples were then analysed as described for Fig. 1. These experiments were performed at least three times with similar results.

3.5. Activated MEK drives the dephosphorylation of eEF2 in an mTOR-independent manner

The above data are consistent with a role for S6K1 (and probably S6K2) in regulating the phosphorylation state of eEF2 in response to PE or ET-1. It remained to be established whether activation of $p90^{RSK}$ itself could suffice to bring about the dephosphorylation of eEF2, as one might expect from in vitro experiments [11]. Analysis of its role in the effects of PE or ET-1 is complicated by the fact that, although the time courses of activation of S6K1/2 and $p90^{RSK}$ differ, they do overlap (Fig. 2; [17,18]). As an alternative approach to studying this, we therefore explored the effects of prolonged activation of $p90^{RSK}$ on eEF2 phosphorylation. This was achieved by expressing an activated form of MEK (MEK^{CA}) using an adenoviral vector [24]. As negative control, we used virus encoding *LacZ*. Infection of ARVC with this virus had no effect on the activity of $p90^{RSK}$ or the phosphorylation of S6K1 [17] or of eEF2 (Fig. 5). Expression of MEK^{CA} caused a marked increase in $p90^{RSK}$ activity which was, as expected, reduced to basal levels by application of the MEK inhibitor PD098059 (Fig. 5A). In contrast, rapamycin had no effect on the activity of $p90^{RSK}$. MEK^{CA} expression also caused an increase in the phosphorylation of S6K1, as manifested by a retardation of its mobility on SDS–PAGE (Fig. 5B). Phosphorylation of S6K1 leads to its activation [13] and these data are consistent with our earlier studies showing that expression of MEK^{CA} causes activation of S6K1 (and S6K2) in ARVC [17,18], effects which are blocked by rapamycin and by PD098059. Expression of MEK^{CA} led to dephosphorylation of eEF2 and as expected this effect was blocked by the MEK inhibitor (Fig. 5C). However, the MEK^{CA}-induced dephosphorylation of eEF2 was not affected by rapamycin, indicating that activation of ERK signalling can induce dephosphorylation of eEF2 without a requirement for activation of S6K1. The simplest explanation for this, consistent with the present data and those published earlier [11,17], is that $p90^{RSK}$ can mediate the inactivation of eEF2 kinase in vivo, leading to dephosphorylation and activation of eEF2.

3.6. Concluding remarks

The data presented here show that the Gq-coupled receptor agonists PE and ET-1 each induce the dephosphorylation of eEF2 in cardiac myocytes. These agents activate protein synthesis and induce hypertrophy, and it is likely that the activation of eEF2 contributes to these end effects. The present data also show that signalling via MEK and mTOR is required for the modulation of eEF2 phosphorylation under these conditions. This is likely to involve phosphorylation and inactivation of eEF2 kinase by $p90^{RSK}$/S6K1, which are downstream components of these signalling pathways [11]. The effect of rapamycin may also involve inputs from PP2A to the regulation of eEF2 phosphorylation [35]. The data showing that

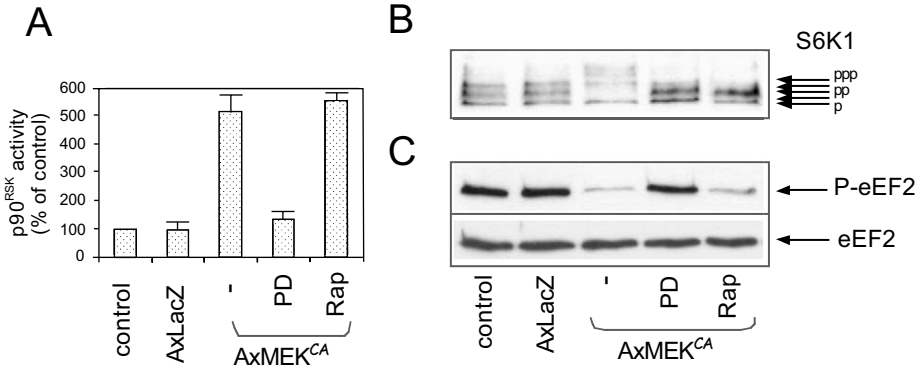

Fig. 5. Expression of activated MEK induces dephosphorylation of eEF2 in ARVC. ARVC were left uninfected ('control'), or infected with adenovirus (multiplicity of infection (m.o.i.) 10) encoding lacZ (AxLacZ, as control for effects of adenoviral infection) or activated MEK ($AxMEK^{CA}$, 10 m.o.i.). 36 h later, cells were treated (for 4 h) with PD098059 (50 μM) or rapamycin (rap; 100 nM). Cells were then lysed and samples of extracts were used for analysis of $p90^{RSK}$ activity (A) or subjected to SDS–PAGE and Western blotting (B,C). B: The blot was developed with anti-S6K1 antiserum. The labelled arrows indicate the differently migrating species of S6K1, which differ in their states of phosphorylation, so that the most highly phosphorylated (ppp; active) forms of S6K1 migrate slowest. C: Blots were developed with anti-eEF2 (Thr56[P]) or (as loading control) anti-eEF2 that recognises eEF2, whether or not it is phosphorylated. In A, data are derived from four separate experiments; in B and C, experiments were performed four times with similar results.

MEK signalling is critical in regulating eEF2 phosphorylation in adult rat cardiomyocytes is similar to the finding that blocking MEK inhibited the ability of a quite distinct G-protein coupled receptor agonist, angiotensin II, to induce dephosphorylation of eEF2 in neonatal cardiomyocytes [16]. However, this agonist works through additional pathways to PE/ET-1 since, in addition to Gq, it activates Gi and signalling via PI 3-kinase and the dephosphorylation of eEF2 induced by angiotensin II required PI 3-kinase signalling [16], in contrast to the data reported here. These data add to the growing number of examples where MEK/Erk signalling regulates the phosphorylation or activity of components of the translational machinery in mammalian cells [16,17,36–38].

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